Selective loss of cortical endothelial tight junction proteins during Alzheimer's disease progression

Yu Yamazaki,¹ Mitsuru Shinohara,¹ Motoko Shinohara,¹ Akari Yamazaki,¹ Melissa E. Murray,¹ Amanda M. Liesinger,¹ Michael G. Heckman,² Elizabeth R. Lesser,² Joseph E. Parisi,^{3,4} Ronald C. Petersen,⁴ Dennis W. Dickson,¹ Takahisa Kanekiyo¹ and Guojun Bu¹

While the accumulation and aggregation of amyloid- β and tau are central events in the pathogenesis of Alzheimer's disease, there is increasing evidence that cerebrovascular pathology is also abundant in Alzheimer's disease brains. In brain capillaries, endothelial cells are connected closely with one another through transmembrane tight junction proteins forming the blood–brain barrier. Because the blood–brain barrier tightly regulates the exchange of molecules between brain and blood and maintains brain homeostasis, its impairment is increasingly recognized as a critical factor contributing to Alzheimer's disease pathogenesis. However, the pathological relationship between blood–brain barrier properties and Alzheimer's disease progression in the human brain is not fully understood. In this study, we show that the loss of cortical tight junction proteins is a common event in Alzheimer's disease, and is correlated with synaptic degeneration. By quantifying the amounts of major tight junction proteins, claudin-5 and occludin, in 12 brain regions dissected from post-mortem brains of normal ageing ($n = 10$), pathological ageing ($n = 14$) and Alzheimer's disease patients ($n = 19$), we found that they were selectively decreased in cortical areas in Alzheimer's disease. Cortical tight junction proteins were decreased in association with the Braak neurofibrillary tangle stage. There was also a negative correlation between the amount of tight junction proteins and the amounts of insoluble Alzheimer's disease-related proteins, in particular amyloid- β_{40} , in cortical areas. In addition, the amount of tight junction proteins in these areas correlated positively with those of synaptic markers. Thus, loss of cortical tight junction proteins in Alzheimer's disease is associated with insoluble amyloid- β_{40} and loss of synaptic markers. Importantly, the positive correlation between claudin-5 and synaptic markers, in particular synaptophysin, was present independent of insoluble amyloid- β_{40} , amyloid- β_{42} and tau values, suggesting that loss of cortical tight junction proteins and synaptic degeneration is present, at least in part, independent of insoluble Alzheimer's disease-related proteins. Collectively, these results indicate that loss of tight junction proteins occurs predominantly in the neocortex during Alzheimer's disease progression. Further, our findings provide a neuropathological clue as to how endothelial tight junction pathology may contribute to Alzheimer's disease pathogenesis in both synergistic and additive manners to typical amyloid- β and tau pathologies.

1 Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA

2 Division of Biomedical Statistics and Informatics, Mayo Clinic, Jacksonville, FL, USA

3 Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA

4 Department of Neurology, Mayo Clinic, Rochester, MN, USA

Correspondence to: Guojun Bu

Department of Neuroscience, Mayo Clinic Jacksonville, 4500 San Pablo Road, Jacksonville FL 32224, USA

E-mail: bu.guojun@mayo.edu

Correspondence may also be addressed to: Takahisa Kanekiyo
E-mail kanekiyo.takahisa@mayo.edu

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Abbreviations: CAA = cerebral amyloid angiopathy; PMI = post-mortem interval; PSD-95 = postsynaptic density protein 95

Introduction

Alzheimer's disease is the most common type of dementia in the elderly. Alzheimer's disease pathologically is defined by the aggregation and accumulation of amyloid- β in the brain parenchyma as senile plaques and in the vasculature as cerebral amyloid angiopathy (CAA), as well as abnormally phosphorylated tau in neurons as neurofibrillary tangles (Serrano-Pozo *et al.*, 2011; Nelson *et al.*, 2012). In addition to these central pathological hallmarks, significant vascular pathologies such as circle of Willis atherosclerosis, cerebral infarcts, microbleeds, and white matter changes, frequently are detected in Alzheimer's disease brains (Yarchoan *et al.*, 2012; Kapasi and Schneider, 2016), suggesting a link between the cerebrovascular dysfunctions and the pathogenesis of Alzheimer's disease.

In the CNS, capillary endothelial cells have distinct barrier properties referred to as the blood–brain barrier (Deeken and Loscher, 2007; Daneman and Prat, 2015). Since the blood–brain barrier controls entry of blood components into the brain and elimination of toxic molecules, it maintains brain homeostasis (Zlokovic, 2008; Daneman, 2012; Daneman and Prat, 2015; Zhao *et al.*, 2015). It is increasingly recognized that blood–brain barrier impairment may contribute to the pathogenesis of various neurological diseases including late-onset Alzheimer's disease (Zlokovic, 2005; Erickson and Banks, 2013; Montagne *et al.*, 2017; Yamazaki and Kanekiyo, 2017; Zenaro *et al.*, 2017). Dysfunction in the blood–brain barrier has been shown to contribute to amyloid- β accumulation and neuronal loss in Alzheimer's disease mouse models of amyloidosis (Deane *et al.*, 2004; Cirrito *et al.*, 2005; Winkler *et al.*, 2015; Storck *et al.*, 2016). Conversely, ageing and amyloid- β accumulation cause blood–brain barrier dysfunction by affecting multiple properties of endothelial cells (Kook *et al.*, 2012; Montagne *et al.*, 2015; Yamazaki *et al.*, 2016). Thus, current evidence from animal studies suggests that blood–brain barrier impairment might contribute to Alzheimer's disease pathogenesis in a synergism to amyloid- β accumulation (Erickson and Banks, 2013; Yamazaki and Kanekiyo, 2017). However, since Alzheimer's disease is a complex multifactorial disease that is difficult to recapitulate in mouse models (Drummond and Wisniewski, 2017; Jankowsky and Zheng, 2017), clarification is needed regarding the association of markers of blood–brain barrier integrity and Alzheimer's disease-related pathology in humans.

Region-specific appearances of Alzheimer's disease pathologies, including amyloid- β accumulation and

neurofibrillary tangles, have provided important insights into disease pathogenesis (Klunk *et al.*, 2004; Buckner *et al.*, 2005; Jack *et al.*, 2008; Vlassenko *et al.*, 2010; Wang *et al.*, 2016). Indeed, by analysing biochemical markers in multiple brain regions dissected from post-mortem brains of normal ageing, pathological ageing and Alzheimer's disease, we have found novel evidence regarding pathomechanisms of amyloid- β accumulation and disease development (Shinohara *et al.*, 2013, 2014, 2017). In this study, we quantified the amounts of tight junction proteins, which uniquely constitute the para-cellular barrier in the blood–brain barrier, in 12 brain areas, compared these among three disease groups, and assessed their associations with the Braak neurofibrillary tangle stage, CAA severity, vascular pathology severity, and other Alzheimer's disease-related features. Our results indicate that the amounts of tight junction proteins selectively are decreased in the cortex of Alzheimer's disease brains in association with insoluble amyloid- β accumulation and loss of synaptic markers.

Materials and methods

Subjects

Post-mortem tissues were obtained through the Mayo Clinic Brain Bank for neurodegenerative diseases, whose operating procedures are approved by the Mayo Institutional Review Board. In addition to 19 patients with sporadic Alzheimer's disease, we evaluated 10 neurologically normal elderly subjects without amyloid- β accumulation (normal ageing) and 14 neurologically normal elderly subjects with extensive cortical amyloid- β deposits (pathological ageing) (Dickson *et al.*, 1992; Murray and Dickson, 2014). All normal ageing, pathological ageing and sporadic Alzheimer's disease patients were subjects in the Mayo Clinic Alzheimer Disease Research Center (ADRC; P50 AG016574) or Mayo Clinic Study of Aging (MCSA; U01 AG006786) and had standardized neurological and neuropsychological assessments. Neuropathological findings were reported using recommendations from the Consortium to Establish a Registry for Alzheimer's disease (CERAD) (Mirra *et al.*, 1991). A modified Bielschowsky stain was used to determine the Braak neurofibrillary tangle stages (Braak and Braak, 1991). The cohort and enzyme-linked immunosorbent assay (ELISA) measurements of Alzheimer's disease-related molecules other than tight junction proteins overlapped with that of our previous studies (Shinohara *et al.*, 2013, 2014, 2017).

Vascular pathology scores

Haematoxylin and eosin stained sections were used to assess vascular pathology. Cerebrovascular pathology was assessed in each case using a modified 'Kalaria vascular score' as described by Craggs *et al.* (2013). A maximum score of 10 was calculated from six derived from all cortical regions and four from the basal ganglia. The modified 'Kalaria vascular score' (Craggs *et al.*, 2013) provides an incremental scoring system based on the presence of the following vascular pathology: cortical region, vessel wall modifications (score 1–2), degree of perivascular and white matter modifications (score 3–4), microinfarcts (score 5), large infarcts (score 6); and basal ganglia region, vessel wall modifications (score 1), degree of perivascular space dilation (score 2), microinfarcts (score 3), and large infarcts (score 4). Vascular pathology scores were available for 32 patients in our cohort (six subjects with normal ageing, 10 subjects with pathological ageing and 16 patients with sporadic Alzheimer's disease).

CAA scores

The amyloid- β immunostained section was used to assign a semi-quantitative CAA score in both the leptomeninges and brain parenchyma in cortical sections. The CAA severity was semi-quantitatively assessed on a 4-point scale in both the leptomeninges and the brain parenchyma: 0, no evidence of CAA; 1, mild involvement of vessels; 2, some circumferential amyloid deposition; and 3, severe, diffuse involvement (Love *et al.*, 2014; Shinohara *et al.*, 2016).

Sample preparation

Samples were prepared as described previously (Shinohara *et al.*, 2013, 2014, 2017). In brief, grey matter from seven cortical areas (dorsolateral prefrontal, orbitofrontal, inferior temporal, inferior parietal, primary visual cortex, posterior cingulate, entorhinal) and five subcortical areas (amygdala, striatum, thalamus, hypothalamus, cerebellum) was dissected and kept frozen until protein extraction. Brain lysates were prepared by the three-step extraction method, based upon differential solubility in buffer alone, detergent (TritonTM X-100) and chaotropic agent [guanidine hydrochloride (GuHCl)]. After removal of meninges and blood vessels, 100–200 mg of frozen brain tissue were homogenized in ice-cold Tris-buffered saline (TBS) containing a protease inhibitor cocktail (Roche Diagnostics) by Polytron homogenization (KINEMATICA). After centrifugation at 100 000 g for 60 min at 4°C, the supernatant was aliquoted and stored at –80°C (referred to as 'TBS fraction' or 'TBS'). The residual pellet was rehomogenized in TBS plus 1% TritonTM X-100 with protease inhibitor cocktail, incubated with mild agitation for 1 h at 4°C and centrifuged as above. The resultant supernatant was aliquoted and stored at –80°C (referred to as TX fraction or TX). The residual pellet was rehomogenized in TBS plus 5 M GuHCl, pH 7.6, and incubated with mild agitation for 6–12 h at 22°C. After centrifugation as above, the resultant supernatant (referred to as the GuHCl fraction) was diluted with nine volumes of TBS, aliquoted and stored at –80°C.

Quantification of tight junction proteins and the endothelial cell marker CD31

The amount of claudin-5 was determined by a mouse monoclonal capture antibody (Invitrogen) and a rabbit polyclonal detection antibody (Abcam), as described (Kazmierski *et al.*, 2012). Goat anti-rabbit IgG (H+L) antibody (Invitrogen) was used as the secondary antibody for the claudin-5 ELISA. The amount of occludin was determined by using a rabbit polyclonal capture antibody (Invitrogen) and horseradish peroxidase (HRP)-conjugated mouse monoclonal capture antibody (Invitrogen). Recombinant claudin-5 and occludin proteins (Novus Biologicals) were used as standards. Colorimetric quantification was performed using a Synergy HT plate reader (BioTek) using HRP-linked streptavidin (Vector) or Poly-HRP 40 streptavidin (Fitzgerald) and 3,3',5,5'-tetramethylbenzidine substrate (Sigma). The amount of endothelial cell marker, CD31 (also known as PECAM-1) was determined by a commercial ELISA kit according to the manufacturer's instruction (R&D). The TX fraction was used to measure the amounts of these membrane proteins. To minimize the possibility that any observed associations with tight junction protein simply reflect associations with number of endothelial cells, the amounts of tight junction proteins in each brain-region were normalized (i.e. divided) by the CD31 amount in the given brain region; these normalized tight junction protein measures (hereinafter referred to as CLDN5 and OCLN, respectively) were used in all analyses. Of note, CLDN5 and OCLN were moderately to highly correlated, with Spearman's correlation coefficients for the 12 different brain regions ranging from 0.32 to 0.71 (median = 0.61). The regional distribution of CLDN5 and OCLN in normal ageing brains is shown in Supplementary Fig. 1.

Quantification of Alzheimer's disease-related proteins

The amounts of total amyloid- β_{40} ($A\beta_{x-40}$), total amyloid- β_{42} ($A\beta_{x-42}$), full-length amyloid- β_{40} ($A\beta_{1-40}$), full-length amyloid- β_{42} ($A\beta_{1-42}$), tau, postsynaptic density protein 95 (PSD-95, also known as DLG4), synaptophysin (SYP), and glial fibrillary acidic protein (GFAP) were determined by ELISAs as described previously (Shinohara *et al.*, 2013, 2014, 2017). The amounts of N-terminally truncated amyloid- β_{40} ($A\beta_{t-40}$) and amyloid- β_{42} ($A\beta_{t-42}$) were calculated by subtracting values of $A\beta_{1-40}$ and $A\beta_{1-42}$ from $A\beta_{x-40}$ and $A\beta_{x-42}$ values, respectively (Shinohara *et al.*, 2017). The TBS fraction was used to measure the amounts of cytosolic or secreted proteins, and molecules (GFAP, and PSD-95), while the TX fraction was used to measure the amounts of membrane proteins (i.e. SYP). The GuHCl fraction was used to measure the amounts of pathological aggregated proteins for $A\beta_{x-40}$, $A\beta_{1-40}$, $A\beta_{x-42}$, $A\beta_{1-42}$ and tau as described previously (Shinohara *et al.*, 2013, 2014, 2017). Alzheimer's disease-related proteins are summarized for each brain region in Supplementary Table 1 separately for the normal ageing, pathological ageing, and sporadic Alzheimer's disease groups.

Outcome measures and calculation of average protein levels in cortical and subcortical regions

The outcome measures of the study were the values of two tight junction proteins (CLDN5 and OCLN), each of which were measured in seven cortical and five subcortical brain regions. In addition to these region-specific tight junction protein amounts, the average (i.e. mean) CLDN5 and OCLN levels across cortical regions and also across subcortical regions were also calculated. Similarly, averages of Alzheimer's disease-related proteins across cortical and subcortical regions were calculated. Although brain-region-specific values were analysed independently, to present our results more succinctly given the large number of region-specific tight junction and Alzheimer's disease-related protein values that were measured, the average values of cortical and subcortical regions were sometimes emphasized.

There was a small amount of missing data regarding CLDN5 and OCLN levels (0.6%), which has potential to bias average CLDN5 and OCLN levels across cortical and subcortical regions. Therefore, in calculating averages of cortical and subcortical tight junction protein values, if a patient did not have a given tight junction protein measure for a given brain region, we imputed the missing data using the mean of the values of patients who did have this information available in the given disease group (normal ageing, pathological ageing, sporadic Alzheimer's disease) for that specific brain region. Any patient with missing data for >50% of the cortical or subcortical brain regions for a given tight junction protein measure was not included in any analysis, involving the given cortical or subcortical region. (Only one patient was missing data for CLDN5 for all 12 brain regions.) Missing data were handled in the same fashion when calculating cortical and subcortical averages of Alzheimer's disease-related proteins (0.5% of Alzheimer's disease-related protein data were missing).

Statistical analysis

Continuous variables were summarized with the sample median and range. Comparisons of characteristics [age at death, sex, post-mortem interval (PMI), Braak stage, CAA score, vascular pathology score, Alzheimer's disease-related proteins] between normal ageing, pathological ageing, and sporadic Alzheimer's disease groups were made using a Kruskal-Wallis rank sum test or a chi-square test.

Comparisons of CLDN5 and OCLN values among the three groups were made using linear regression models that were adjusted for age at death, sex, and PMI. In particular, we adjusted all of the analyses for PMI as we observed several nominally significant ($P < 0.05$) associations with PMI (where increased PMI was associated with a lower OCLN level) prior to correction by multiple testing. Specifically, increased PMI was associated with a lower OCLN level in the dorsolateral prefrontal region ($P = 0.025$), inferior temporal region ($P = 0.028$), striatum ($P = 0.041$), and also when examining the average of all cortical areas ($P = 0.011$) (Supplementary Table 2). It should also be noted that none of these associations remained significant [all false discovery rate (FDR)-

corrected $P \geq 0.13$] when correcting for multiple testing using a FDR correction as we did in our other analyses.

An overall test of difference of each tight junction protein measure between the three disease groups was first performed. To account for the fact that overall tests of difference were made for each of the 12 different brain regions and for averages of cortical and subcortical areas, an FDR correction for multiple testing was used. Assuming an FDR of 5%, FDR-corrected P -values were calculated and denoted as P_c . When a statistically significant overall difference between groups was observed (i.e. $P_c \leq 0.05$), pair-wise comparisons were made, and FDR-corrected P -values were subsequently calculated to correct for the three pair-wise comparisons made between the three disease groups. Comparisons of un-normalized claudin-5, un-normalized occludin, and CD31 values between the three disease groups were made in a similar manner.

Linear regression models that were adjusted for age at death, sex, and PMI were used to examine associations of CLDN5 and OCLN values with Braak stage, CAA score, and vascular pathology score. Regression coefficients and 95% confidence intervals (CIs) were estimated and are interpreted as the increase in the mean tight junction protein level corresponding to a 1-unit increase in the given measure. Separately, for associations with Braak stage, CAA, and vascular pathology scores, FDR-corrected P -values (P_c) were calculated to correct for the 12 different brain regions that were examined, as well as the cortical and subcortical averages, assuming an FDR of 5%.

Associations between CLDN5 and OCLN values with Alzheimer's disease-related proteins were evaluated using linear regression models that were adjusted for age at death, sex, and PMI. For Alzheimer's disease-related proteins, because of the large number of measures that were considered (each measured in the 12 different brain regions), distributions varied widely and many were skewed or contained outlying/influential points. With this in mind, these Alzheimer's disease-related proteins were divided into four-level categorical variables based on the sample quartiles (i.e. 1 = ≤ 25 th percentile, 2 = 26th to 50th percentile, 3 = 51st to 75th percentile, 4 = >75th percentile) for use in linear regression analysis. Regression coefficients and 95% CIs were estimated and interpreted as the increase in the mean tight junction protein level corresponding to an increase of one quartile of the given Alzheimer's disease-related protein. For associations of tight junction proteins with Alzheimer's disease-related proteins, analysis of the cortical and subcortical averages was considered to be primary (with region-specific analysis considered to be secondary), and FDR-corrected P -values (P_c) were calculated to correct for the 10 Alzheimer's disease-related proteins that were examined in the primary analysis, assuming an FDR of 5%. In the secondary region-specific analysis, no adjustment for multiple testing was made, and therefore uncorrected P -values are presented (denoted as P) and $P \leq 0.05$ is considered as significant.

All statistical tests were two-sided. Statistical analyses were performed using SAS (version 9.4; SAS Institute, Inc. Cary, North Carolina) and R Statistical Software (version 3.2.3; R Foundation for Statistical Computing, Vienna, Austria).

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Results

Characteristics of normal and pathological ageing, and sporadic Alzheimer's disease patient groups

Table 1 details characteristics of each of the three disease groups. Median age was significantly higher ($P = 0.003$) for pathological ageing patients (median = 94 years) compared to the normal ageing (median = 83 years) and Alzheimer's disease (median = 86 years) groups, while sex ($P = 0.30$) and PMI ($P = 0.77$) were relatively similar between groups. As expected, both Braak stage and CAA score were lowest for normal ageing patients, followed by pathological ageing, and then by Alzheimer's disease (both $P < 0.001$). Vascular pathology scores did not differ noticeably between the three groups ($P = 0.77$).

Tight junction proteins are selectively decreased in the cortical areas of patients with Alzheimer's disease

To determine whether the expression of tight junction proteins is altered in Alzheimer's disease, the levels of two major tight junction proteins, CLDN5 and OCLN, were quantified by ELISA in TX fractions of each of the 12 brain regions in

each of the three groups. The average CLDN5 level across cortical areas was significantly lower in Alzheimer's disease compared to normal ageing and pathological ageing (both $P_c = 0.002$; Fig. 1A and Supplementary Table 3). Loss of cortical CLDN5 in Alzheimer's disease was confirmed by brain region-wise analysis; CLDN5 was significantly lower in multiple cortical areas in Alzheimer's disease compared to those with normal ageing and/or pathological ageing (Fig. 1C and Supplementary Table 3). Specifically, when adjusting for age at death, sex, and PMI, CLDN5 was significantly lower for Alzheimer's disease compared to both normal and pathological ageing in the dorsolateral prefrontal, orbitofrontal, and inferior temporal regions (all $P_c \leq 0.023$). Additionally, CLDN5 in the inferior parietal region was significantly lower for Alzheimer's disease compared to pathological ageing ($P_c = 0.027$), whereas in the primary visual cortex it was lower for Alzheimer's disease compared to normal ageing ($P_c = 0.033$). In contrast, there were no significant differences in the level of CLDN5 in normal and pathological ageing in the cortical areas (Supplementary Table 3), and no differences in CLDN5 in the three groups for subcortical areas (Fig. 1A, C and Supplementary Table 3).

Specific loss of cortical tight junction protein in Alzheimer's disease also was evident when the level of OCLN was examined. When considering the average OCLN across cortical areas, this was significantly lower

Table 1 Comparison of characteristics between normal aging, pathological ageing, and sporadic Alzheimer's disease patients

Variable	Normal ageing (n = 10)	Pathological ageing (n = 14)	Alzheimer's disease (n = 19)	P-value
Age at death, years	83 (57, 95)	94 (82, 101)	86 (62, 95)	0.003
Sex, male (%)	3 (30.0)	1 (7.1)	5 (26.3)	0.30
PMI, h	12 (5, 18)	12 (2, 23)	11 (2, 24)	0.77
Braak stage (%)				<0.001
0	2 (20.0)	0 (0.0)	0 (0.0)	
I	1 (10.0)	1 (7.1)	0 (0.0)	
II	4 (40.0)	2 (14.3)	0 (0.0)	
III	2 (20.0)	5 (35.7)	0 (0.0)	
IV	1 (10.0)	6 (42.9)	0 (0.0)	
V	0 (0.0)	0 (0.0)	4 (21.1)	
VI	0 (0.0)	0 (0.0)	15 (78.9)	
CAA score (%)				<0.001
0	8 (80.0)	6 (42.9)	0 (0.0)	
0.5	2 (20.0)	3 (21.4)	3 (15.8)	
I	0 (0.0)	3 (21.4)	5 (26.3)	
1.5	0 (0.0)	0 (0.0)	4 (21.1)	
2	0 (0.0)	1 (7.1)	4 (21.1)	
2.5	0 (0.0)	0 (0.0)	1 (5.3)	
3	0 (0.0)	1 (7.1)	2 (10.5)	
VP score (%)				0.77
2–3	3 (50.0)	4 (40.0)	8 (50.0)	
4–5	1 (16.7)	3 (30.0)	5 (31.3)	
6–10	2 (33.3)	3 (30.0)	3 (18.8)	

The sample median (minimum, maximum) is given for age at death and PMI. P-values result from a Kruskal-Wallis rank sum test or Fisher's exact test. Information was unavailable regarding vascular pathology (VP) score for 11 subjects.

Figure 1 Widespread decreases of tight junction proteins in cortical areas of Alzheimer's disease brains. **(A)** Boxplot of average claudin-5/CD31 across cortical and subcortical areas for normal ageing (NA), pathological ageing (PA), and sporadic Alzheimer's disease (AD) patients. Average claudin-5/CD31 across cortical and subcortical areas was not available for one pathological ageing patient. **(B)** Boxplot of average occludin/CD31 across cortical and subcortical areas for normal ageing, pathological ageing, and sporadic Alzheimer's disease patients. **(C)** Heat map summarizing statistically significant differences in tight junction proteins between normal ageing, pathological ageing and sporadic Alzheimer's disease patients in each brain region. FDR corrected P -values (P_c) were calculated. P -values from each cell in **C** are available in Supplementary Table 3. * $P_c < 0.05$, ** $P_c < 0.01$, N.S. = not significant after FDR correction. Cortical areas: dorsolateral prefrontal, orbitofrontal, inferior temporal, inferior parietal, primary visual cortex, posterior cingulate and entorhinal cortex. Subcortical areas: amygdala, striatum, thalamus, hypothalamus and cerebellum. AD = Alzheimer's disease; NA = normal ageing; PA = pathological ageing.

for Alzheimer's disease compared to the other groups (both $P_c \leq 0.011$; Fig. 1B and Supplementary Table 3). Similar to the results for CLDN5, brain region-wise analysis showed that OCLN was significantly lower in Alzheimer's disease in several cortical areas. As shown in Fig. 1C and Supplementary Table 3, for cortical areas, OCLN was significantly lower in Alzheimer's disease compared to both normal and pathological ageing for the inferior temporal, posterior cingulate, and entorhinal regions (all $P_c \leq 0.030$). OCLN level did not differ noticeably between normal and pathological ageing in cortical areas (Supplementary Table 3), and also did not differ significantly between the three disease groups for subcortical areas (Fig. 1B, C and Supplementary Table 3). Collectively, these results indicate

that the amounts of tight junction proteins are predominantly decreased in the cortical, but not in subcortical areas in Alzheimer's disease.

Results from comparisons of un-normalized claudin-5, un-normalized occludin, and CD31 between normal ageing, pathological ageing, and sporadic Alzheimer's disease patients are presented in Supplementary Tables 4 and 5.

Cortical tight junction proteins are decreased as Braak stage increases

To determine whether the amounts of tight junction proteins are decreased in association with severity of

Alzheimer's disease pathology, the associations between tight junction proteins and Braak stage were examined across all three groups. After adjusting for age at death, sex, and PMI, the average level of CLDN5 across cortical areas decreased significantly as Braak stage increased (both $P_c \leq 0.008$; Fig. 2A and Supplementary Table 6). The association between tight junction protein amount and Braak stage in cortical areas was further confirmed by brain region-wise analysis. As shown in Fig. 2C and Supplementary Table 6, as Braak stage increased, CLDN5 level in the dorsolateral prefrontal, orbitofrontal, inferior temporal, and inferior parietal cortical regions significantly decreased (all $P_c \leq 0.025$).

Similarly, a significant association between cortical tight junction protein and Braak stage was evident for OCLN. The average level of OCLN across cortical areas decreased significantly as Braak stage increased ($P_c \leq 0.008$; Fig. 2B and Supplementary Table 6). In addition, brain region-wise analysis showed that as Braak stage increased, OCLN decreased in dorsolateral prefrontal, inferior temporal, and entorhinal cortical regions (all $P_c \leq 0.048$; Fig. 2C and Supplementary Table 6). All of the aforementioned associations for Braak stage appear to be driven by the tight junction protein values in high Braak stage subjects (all Alzheimer's disease patients), while tight junction protein values in Braak 0–II and III–IV patients were relatively

Figure 2 Brain region-wise association between Braak stages and tight junction proteins across normal, pathological ageing and Alzheimer's disease. (A) Boxplot of average claudin-5/CD31 across cortical and subcortical areas according to Braak stage (0–II, III–IV, or V–VI) when considering the combined series of normal ageing, pathological ageing, and patients with Alzheimer's disease. Average claudin-5/CD31 across cortical and subcortical areas was not available for one Braak stage 0–II patient. (B) Boxplot of average occludin/CD31 across cortical and subcortical areas according to Braak stage (0–II, III–IV, or V–VI) when considering the combined series of normal ageing, pathological ageing, and patients with Alzheimer's disease. (C) Heat map summarizing statistically significant correlations between tight junction proteins and Braak stage in each brain region when considering the combined series of normal ageing, pathological ageing, and patients with Alzheimer's disease. Regression coefficients are interpreted as the increase in the mean tight junction protein measure corresponding to each 1-unit increase in Braak stage. FDR-corrected P -values (P_c) were calculated. P -values from each cell in C are available in Supplementary Table 6. ** $P_c < 0.01$, N.S. = not significant after FDR correction. Cortical areas: dorsolateral prefrontal, orbitofrontal, inferior temporal, inferior parietal, primary visual cortex, posterior cingulate and entorhinal cortex. Subcortical areas: amygdala, striatum, thalamus, hypothalamus and cerebellum.

similar (Supplementary Table 6). With the exception of an association between increased Braak stage and higher OCLN in the cerebellum ($P_c = 0.020$), no associations with Braak stage were evident for CLDN5 or OCLN in subcortical areas (Fig. 2A–C and Supplementary Table 6). Together, these results indicate that the amounts of cortical tight junction proteins are decreased as Braak stage increases, suggesting that the loss of cortical tight junction proteins is region-specific and correlates with progression of Alzheimer's disease pathology.

Cortical tight junction proteins are decreased, in part, in association with higher CAA scores

To determine the relationship between decreases in tight junction proteins in Alzheimer's disease and the degree of cerebrovascular pathology or CAA, associations of CLDN5 and OCLN with CAA and vascular pathology scores were examined. In the combined cohort of normal, pathological ageing, and Alzheimer's disease, vascular pathology scores were not significantly correlated with CLDN5 or OCLN in specific brain regions or when considering cortical and subcortical averages in analysis adjusted for age at death, sex, and PMI (Supplementary Fig. 2 and Supplementary Table 7). In contrast, while CAA scores were not significantly correlated with CLDN5 when considering cortical averages (Fig. 3A and Supplementary Table 8), as CAA score increased, CLDN5 in the dorsolateral prefrontal cortical region significantly decreased ($P_c = 0.031$; Fig. 3C and Supplementary Table 8). The average level of OCLN across cortical areas decreased significantly as CAA score increased ($P_c = 0.048$; Fig. 3B and Supplementary Table 8). In addition, brain region-wise analysis showed that as CAA score increased, OCLN decreased in inferior temporal cortical region ($P_c = 0.006$; Fig. 3C and Supplementary Table 8). CAA score was not correlated with CLDN5 and OCLN in subcortical areas (Fig. 3A–C and Supplementary Table 8). Together, these results indicate that cortical tight junction proteins are decreased, at least in part, as CAA score increases, but are not associated with vascular pathology score. Thus, loss of tight junction proteins occurs independently of the overall structural abnormalities in cerebrovasculature, whereas loss of tight junction proteins and overall severity of CAA might be related in cortical areas.

Cortical tight junction proteins decrease in association with higher insoluble amyloid- β and lower synaptic marker levels

The observed negative correlations between the amount of cortical tight junction proteins and Braak stage or CAA score (see above) suggest that tight junction pathology may occur synergistically with the development of Alzheimer's

disease pathology. To determine potential molecular mechanisms underlying the loss of tight junction proteins in Alzheimer's disease, correlations between the amounts of tight junction proteins and Alzheimer's disease-related molecules were measured in normal ageing, pathological ageing, and Alzheimer's disease. After adjusting for age at death, sex, and PMI, significant negative correlations with cortical CLDN5 and OCLN levels for insoluble A β x–40 and A β x–42 in the GuHCl fraction (all $P_c \leq 0.046$; Table 2) were found. In addition, significant positive correlations were observed for average cortical CLDN5 and OCLN with PSD-95 and SYP (all $P_c \leq 0.019$). There were no significant correlations between averaged tight junction protein amounts and those of insoluble tau in the GuHCl fraction or GFAP in cortical areas. Thus, the inverse relationship observed between tight junction protein levels and Braak stage (Fig. 2A–C and Supplementary Table 6) might be confounded by the presence of amyloid- β pathology. For illustration, the strongest five of these associations ($P_c < 0.01$) are shown in Fig. 4A–E. Together, the results indicate that there are quantitative correlations between the amount of tight junction proteins and both insoluble amyloid- β and synaptic markers in cortical areas.

Quantitative correlations in cortical areas were investigated by a brain region-wise analysis. Noticeably, as shown in Fig. 5 and Supplementary Table 9, significant correlations between the amount of tight junction proteins and insoluble A β x–40 and synaptic markers were almost exclusively present in cortical areas harbouring loss of tight junction proteins (Fig. 1C). On the other hand, while quantitative correlations between the amount of tight junction proteins and insoluble A β x–42 and tau were also detected in multiple cortical regions, they were less likely to be present in the areas harbouring loss of tight junction proteins (Fig. 5 and Supplementary Table 9). Collectively, these results indicate that loss of cortical tight junction proteins in Alzheimer's disease is characterized by associations with insoluble total amyloid- β_{40} accumulation (when compared to total amyloid- β_{42}) and loss of synaptic markers.

There were no significant associations of CLDN5 and OCLN and Alzheimer's disease-related proteins when examining subcortical region (Table 2). This lack of association was also observed for the vast majority of individual brain regions in brain region-wise analysis (Fig. 5 and Supplementary Table 9).

Association between tight junction proteins and amyloid- β species

Total amyloid- β values (values in A β x–40 and A β x–42) in our measurements include those from the full-length amyloid- β (A β 1–40 and A β 1–42) and N-terminally truncated amyloid- β (A β t–40 and A β t–42), which may play different roles in Alzheimer's disease pathogenesis (Bayer and Wirths, 2014; Shinohara *et al.*, 2017). To determine the relationship between decreases in tight junction proteins

Figure 3 Association between tight junction proteins and CAA scores across normal, pathological ageing and Alzheimer's disease. (A) Boxplot of average claudin-5/CD31 across cortical and subcortical areas according to CAA score (0–0.5, 1–1.5, 2–3) when considering the combined series of normal ageing, pathological ageing, and Alzheimer's disease patients. Average claudin-5/CD31 across cortical and subcortical areas was not available for one CAA score 0–0.5 patient. (B) Boxplot of average occludin/CD31 across cortical and subcortical areas according to CAA score (0–0.5, 1–1.5, 2–3) when considering the combined series of normal ageing, pathological ageing, and Alzheimer's disease patients. (C) Heat map summarizing statistically significant correlations between tight junction proteins and CAA score in each brain region when considering the combined series of normal ageing, pathological ageing, and patients with Alzheimer's disease. Regression coefficients are interpreted as the increase in the mean tight junction protein measure corresponding to each 1-unit increase in CAA score. FDR-corrected *P*-values (*P_c*) were calculated. *P*-values from each cell in C are available in Supplementary Table 8. **P_c* < 0.05, N.S. = not significant after FDR correction. Cortical areas: dorsolateral prefrontal, orbitofrontal, inferior temporal, inferior parietal, primary visual cortex, posterior cingulate and entorhinal cortex. Subcortical areas: amygdala, striatum, thalamus, hypothalamus and cerebellum.

and the accumulation of each of these amyloid- β species, correlations between the amounts of tight junction proteins and amyloid- β species measured in a combined analysis of normal ageing, pathological ageing, and Alzheimer's disease were made. After adjusting for age at death, sex, and PMI, significant negative correlations with average cortical CLDN5 and OCLN for insoluble A β 1–40, A β t–40 and A β t–42 in the GuHCl fraction (all *P_c* $\leq$ 0.035, Supplementary Table 10) were found. In contrast, there were no significant correlations between average tight junction protein and insoluble A β 1–42 in GuHCl fractions in cortical areas. Thus, these results indicate that tight junction proteins correlate with insoluble full-length amyloid- β ₄₀ and

N-terminally truncated amyloid- β ₄₀ and amyloid- β ₄₂ species in cortical areas.

The amyloid- β species-dependent difference in its association with tight junction proteins was confirmed further by a brain region-wise analysis. As shown in Fig. 6 and Supplementary Table 9, significant correlations were found between tight junction proteins and insoluble A β t–40, A β t–42 and A β 1–40 across most cortical areas that had loss of tight junction proteins (Fig. 1C). On the other hand, there were no significant associations of tight junction protein with A β 1–42 in cortical areas (Fig. 6 and Supplementary Table 9). Collectively, these results indicate that loss of cortical tight junction proteins in Alzheimer's

Table 2 Association between tight junction proteins and Alzheimer's disease-related proteins when examining averages across cortical and subcortical regions

Alzheimer's disease-related protein	Analysis of average values of cortical areas			Analysis of averages of subcortical areas		
	Association with claudin-5/CD31		P-value _c	Association with occludin/CD31		P-value _c
	Regression coefficient (95% CI)	P-value _c		Regression coefficient (95% CI)	P-value _c	
Aβx-40	-0.17 (-0.28, -0.06)	0.006	0.002	-0.13 (-0.28, 0.01)	0.47	0.52
Aβx-42	-0.13 (-0.24, -0.01)	0.046	0.019	0.01 (-0.14, 0.16)	0.92	0.48
Tau	-0.07 (-0.19, 0.05)	0.27	0.068	-0.10 (-0.25, 0.04)	0.23	0.62
PSD-95	0.19 (0.08, 0.29)	0.005	0.007	0.16 (0.01, 0.31)	0.098	0.48
SYP	0.18 (0.08, 0.29)	0.005	0.019	0.18 (0.03, 0.32)	0.097	0.62
GFAP	-0.07 (-0.18, 0.05)	0.27	0.58	-0.16 (-0.30, -0.02)	0.097	0.79

Regression coefficients, 95% CI, and P-values result from linear regression models that were adjusted for age at death, sex, and PMI. Alzheimer's disease-related proteins were divided into four-level categorical variables based on the sample quartiles (i.e. 1 = ≤25th percentile, 2 = 26–50th percentile, 3 = 51–75th percentile, 4 = >75th percentile). Regression coefficients are interpreted as the increase in the mean tight junction protein measure corresponding to an increase in the given Alzheimer's disease-related protein from one quartile to the next highest quartile (i.e. from the first quartile to the second quartile). All P-values have been adjusted for multiple testing using FDR correction, assuming an FDR of 5%. Statistically significant P-values are given in bold. Aβx-40 = total amyloid-β₄₀; Aβx-42 = total amyloid-β₄₂.

disease is characterized by predominant associations with full-length amyloid-β₄₀ and N-terminally truncated amyloid-β₄₀ and amyloid-β₄₂, but not with full-length amyloid-β₄₂.

No associations were found for amyloid-β with CLDN5 or OCLN in subcortical areas (Supplementary Table 10). The lack of association in subcortical areas was also observed for most brain regions in brain region-wise analysis (Fig. 6 and Supplementary Table 9).

Positive correlation of CLDN5 and synaptic markers, in particular SYP, exists independently of insoluble amyloid-β and tau accumulation

The correlations of tight junction proteins with PSD-95 and SYP (when considering cortical averages) were evaluated by additionally adjusting for insoluble Aβx-40, Aβx-42 and tau to address any potential confounding influence of these three variables on synaptic loss. These additional model adjustments weakened associations of PSD-95 and SYP with CLDN5, but were still either significant or borderline-significant ($P_c = 0.055$ and $P_c = 0.032$, respectively). On the other hand, associations of PSD-95 and SYP with OCLN weakened and were no longer significant ($P_c = 0.12$ and $P_c = 0.17$, respectively). Thus, the correlation of CLDN5 and synaptic markers, in particular SYP, exists independent of insoluble amyloid-β and tau. This suggests that loss of cortical tight junction proteins is associated with synaptic degeneration, at least in part, independent of insoluble Alzheimer's disease-related protein pathology.

Discussion

In this study, we analysed the amounts of endothelial tight junction proteins in multiple brain regions from neocortical, limbic and subcortical areas in post-mortem brains of normal ageing, pathological ageing and Alzheimer's disease. Our results demonstrated that the amounts of two major tight junction proteins, CLDN5 and OCLN, are predominantly decreased in cortical areas of Alzheimer's disease compared to normal ageing and pathological ageing, but not in subcortical areas. Supporting the interrelationship between loss of cortical tight junction proteins and Alzheimer's disease, there were significant correlations of cortical tight junction protein reductions with accumulation of insoluble amyloid-β and synaptic loss in the combined series of normal ageing, pathological ageing, and Alzheimer's disease patients. While the potential alternation of tight junction proteins in post-mortem brains from Alzheimer's disease have been studied previously, only one or two brain regions were usually investigated in these prior studies (Romanitan *et al.*, 2007, 2010; Carrano *et al.*, 2011, 2012; Keaney *et al.*, 2015). Moreover, the immunohistochemical approaches frequently

Figure 4 Cortical tight junction proteins are decreased for patients with higher insoluble amyloid- β and lower synaptic marker levels. (A) Boxplot of average claudin-5/CD31 across cortical areas according to average total amyloid- β_{40} (A β x-40) across cortical areas. Average A β x-40 across cortical areas was divided into four groups based on sample quartiles (i.e. 25th percentile, 50th percentile, 75th percentile). (B) Boxplot of average occludin/CD31 across cortical areas according to A β x-40 across cortical areas. Average A β x-40 across cortical areas was divided into four groups based on sample quartiles (i.e. 25th percentile, 50th percentile, 75th percentile). (C) Boxplot of average claudin-5/CD31 across cortical areas according to average PSD-95 across cortical areas. Average PSD-95 across cortical areas was divided into four groups based on sample quartiles (i.e. 25th percentile, 50th percentile, 75th percentile). (D) Boxplot of average occludin/CD31 across cortical areas according to average PSD-95 across cortical areas. Average PSD-95 across cortical areas was divided into four groups based on sample quartiles (i.e. 25th percentile, 50th percentile, 75th percentile). (E) Boxplot of average claudin-5/CD31 across cortical areas according to average SYP across cortical areas. Average SYP across cortical areas was divided into four groups based on sample quartiles (i.e. 25th percentile, 50th percentile, 75th percentile). Regression coefficients are interpreted as the increase in the mean tight junction protein measure corresponding to an increase in the given Alzheimer's disease-related protein from one quartile to the next highest quartile [i.e. from the first quartile (Q1) to the second quartile (Q2)]. FDR-corrected P -values (P_c) were calculated. ** $P_c < 0.01$, N.S. = not significant after FDR correction. Cortical areas: dorsolateral prefrontal, orbitofrontal, inferior temporal, inferior parietal, primary visual cortex, posterior cingulate and entorhinal cortex. A β x-40 = total amyloid- β_{40} .

used have limitations in determining quantitative endothelial pathology (Harik, 1992; Horwood and Davies, 1994; Mooradian *et al.*, 1997; Deane *et al.*, 2004; Romanitan *et al.*, 2007; Miller *et al.*, 2008; Carrano *et al.*, 2011, 2012; Keaney *et al.*, 2015). Therefore, to fill these gaps, we took advantage of established biochemical methods (Shinohara *et al.*, 2013, 2014, 2017) that allow us to

quantify tight junction protein in multiple brain regions and that were also studied for other Alzheimer's disease-related molecules. Our results not only support previous reports showing an intimate relationship between cerebrovascular pathology and Alzheimer's disease (Yarchoan *et al.*, 2012; Toledo *et al.*, 2013), but also extend these results by demonstrating how the changes in membrane-

Figure 5 Heat map summarizing statistically significant correlations between tight junction proteins and Alzheimer's disease-related molecules. Correlations in each brain region were calculated when considering the combined series of normal ageing, pathological ageing, and Alzheimer's disease patients. *P*-values from each cell are available in Supplementary Table 9. Aβx-40 = total amyloid-β₄₀; Aβx-42 = total amyloid-β₄₂; TJ = tight junction.

Figure 6 Heat map summarizing statistically significant correlations between tight junction proteins and amyloid-β species. Correlations in each brain region were calculated when considering the combined series of normal ageing, pathological ageing, and Alzheimer's disease patients. *P*-values from each cell are available in Supplementary Table 9. Aβx-40 = total amyloid-β₄₀; Aβx-42 = total amyloid-β₄₂; Aβ1-40 = full-length amyloid-β₄₀; Aβ1-42 = full-length amyloid-β₄₂; Aβt-40 = N-terminally truncated amyloid-β₄₀; Aβt-42 = N-terminally truncated amyloid-β₄₂; TJ = tight junction.

associated tight junction proteins, a critical measure of blood–brain barrier integrity, occurs during Alzheimer's disease progression in a brain region-dependent manner.

One major finding of this study is that loss of cortical tight junction proteins occurs in association with accumulation of Alzheimer's disease-related insoluble proteins, in particular amyloid- β . The associations between total amyloid- β_{40} and tight junction proteins were stronger than that between total amyloid- β_{42} and tight junction proteins in cortical areas, suggesting that the relationship of endothelial pathology to amyloid- β may differ between amyloid- β_{40} and amyloid- β_{42} . Since higher amyloid- β_{40} levels are correlated with CAA severity (Shinohara *et al.*, 2016), decreases in tight junction proteins in Alzheimer's disease may reflect a change in endothelial cell properties associated with CAA pathology. Indeed, cortical tight junction proteins are decreased, at least in part, as CAA score increases, suggesting that loss of tight junction proteins and overall severity of CAA might be related in cortical areas. More specifically, since leptomeningeal vessels were removed from those brain samples, parenchymal rather than leptomeningeal CAA is reflected in these results (Carrano *et al.*, 2011). Alternatively, it is also possible that endothelial cell damage during Alzheimer's disease progression could predominantly interfere with the clearance of amyloid- β_{40} , causing a significant regional association between decreases of tight junction proteins and amyloid- β_{40} accumulation in brain parenchyma. In either scenario, our results suggest a synergistic mechanism that promotes both endothelial and amyloid- β pathology during progression of Alzheimer's disease pathology.

Of note, by focusing on amyloid- β isoforms, we also found significant associations of tight junction proteins for both N-terminally truncated amyloid- β_{40} and amyloid- β_{42} . Thus, relationships between N-terminally truncated amyloid- β and endothelial pathology may be different from that of full-length amyloid- β with endothelial pathology, which is in line with the concept that truncated amyloid- β species may play distinct roles in Alzheimer's disease pathogenesis (Bayer and Wirths, 2014; Shinohara *et al.*, 2017). How these species-specific interactions of amyloid- β with endothelial pathology occur and contribute to Alzheimer's disease pathogenesis require further clarification.

Since loss of synapses strongly correlates with a cognitive decline in Alzheimer's disease (Terry *et al.*, 1991; DeKosky *et al.*, 1996; Coleman and Yao, 2003; Koffie *et al.*, 2011), identifying biological processes related to a change in synaptic molecules in Alzheimer's disease adds to our understanding of Alzheimer's disease pathogenesis. In this context, it is noteworthy that loss of tight junction proteins was associated with loss of synaptic markers in cortical areas. Although further studies are needed, blood–brain barrier disruption due to tight junction protein reduction may exacerbate neuroinflammation, oxidative stress and structural changes in the neurovascular unit, resulting in synaptic loss (Zlokovic, 2011; Marques *et al.*, 2013;

Zenaro *et al.*, 2017). Conversely, since neuronal function likely mediates angiogenesis and blood–brain barrier development in endothelial cells through co-patterning and mutual crosstalk (Walchli *et al.*, 2015), neuronal damage during Alzheimer's disease progression may reduce the tight junction protein expression through a loss of physiological crosstalk between these two cell types. Importantly, previous studies suggested that circle of Willis or intracranial atherosclerosis is significantly associated with cognitive decline regardless of Alzheimer's disease pathology (Dolan *et al.*, 2010; Arvanitakis *et al.*, 2016). Moreover, recent human brain imaging studies suggested that cerebrovascular pathology affects cognitive impairment independent of amyloid- β deposition in both normal and cognitively impaired patients (Vemuri *et al.*, 2015; Ye *et al.*, 2015). Since the correlation of CLDN5 and synaptic markers, in particular SYP, was independent of insoluble A β _{x-40}, A β _{x-42} and tau, it is reasonable to postulate that tight junction pathology may also contribute to cognitive decline in Alzheimer's disease above and beyond amyloid- β and tau pathologies.

In this study, normalized tight junction protein measures (the amount of tight junction protein in each brain-region normalized against that of CD31 in the given brain region) were used as surrogates to represent tight junction protein expression in endothelial cells. Accordingly, decreases in CLDN5 or OCLN can be explained by the relative increase in CD31, the relative decrease in claudin-5 and occludin, or both. In fact, in terms of the severity and number of regions involved, the differences in claudin-5 and occludin measures (without normalization by CD31) between the three groups were much more prominent than that of CD31 (Supplementary Table 4 and 5); this suggests that decreases in CLDN5 and OCLN were indeed primarily driven by the relative decreases in claudin-5 and occludin rather than changes in CD31 in Alzheimer's disease brains. Thus, with the assumption that the amount of CD31 expression represents the number of endothelial cells, it is reasonable to argue that loss of CLDN5 and OCLN represents tight junction pathology in endothelial cells. Alternatively, considering CD31 is classified as an adhesion molecule in endothelial cells, loss of CLDN5 and OCLN might also represent the selective reduction of tight junction protein expression among other molecules vital for endothelial cell functions. Measurements of additional endothelial cell-specific molecules such as Tie2 and glucose transporter 1 (GLUT1) might help to distinguish these possibilities. In either scenario, however, our results highlight the presence of prominent endothelial tight junction pathology in Alzheimer's disease.

Several limitations of our study need to be acknowledged. First, all subjects were Caucasian, and therefore our findings may not be directly extrapolated to other populations. Second, information regarding other possible covariates such as APOE genotype, hypertension, hyperlipidaemia, diabetes and medications as statins or anti-hypertensives was not assessed. Thus, possible contributions of

these factors to endothelial pathology need to be determined in future studies. Third, considering the complexity and diversity of vascular pathobiology in Alzheimer's disease (Hawkes *et al.*, 2013; Ramirez *et al.*, 2016; Banerjee *et al.*, 2017), inclusion of subcortical white matter regions in the analysis might provide more comprehensive insight regarding how tight junction pathology in subareas of the brain contributes to Alzheimer's disease. In addition, molecular profiling studies using human brain tissues have provided critical insight into the mechanism by which vascular pathology occurs in association with Alzheimer's disease (Thomas *et al.*, 2015; Miners *et al.*, 2016, 2018). Thus, pericyte and astrocyte markers, plasma-derived surrogate markers for blood–brain barrier disruption, and cytokines and signalling molecules vital for maintaining vascular homeostasis represent key biomarkers to be analysed in parallel with endothelial tight junction pathology in future studies. Finally, because of the relatively small sample size, power to detect associations with tight junction protein is limited after adjustment for multiple testing. Therefore, the possibility of a type II error (i.e. a false-negative finding) needs to be considered when interpreting our results. For example, brain region-wise negative association between Braak stages (Fig. 2) or insoluble tau (Fig. 5) and tight junction proteins might represent the association between tau accumulation and tight junction pathology. On the other hand, there were no significant correlations between averaged tight junction protein amounts and those of insoluble tau. These seemingly inconsistent findings might be attributed to the relatively small sample sizes in our study. Considering that tau pathological changes could influence endothelial cell biology *in vivo* (Bennett *et al.*, 2018), further studies where several forms of pathological tau are investigated in a larger number of samples would be warranted. Nonetheless, our study overall indicates that tight junction pathology increases with the severity of Alzheimer's disease pathology.

In conclusion, we have shown that the tight junction proteins are selectively decreased in cortical areas in Alzheimer's disease. Biochemical analysis in 12 brain regions revealed that loss of cortical tight junction proteins in Alzheimer's disease is associated with insoluble amyloid- β_{40} accumulation and loss of synaptic markers. Furthermore, quantitative correlation between CLDN5 and synaptic markers, in particular SYP, was at least partially independent of insoluble amyloid- β and tau. Taken together, these results suggest that tight junction pathology may contribute to Alzheimer's disease pathogenesis in both synergistic and additive manners to amyloid- β and tau.

As loss of tight junction proteins is not a complete measure of blood–brain barrier disruption, and correlation of readouts in post-mortem brain does not necessarily represent causality, the results of our study should serve as a premise for future prospective studies that aim at determining whether blood–brain barrier impairment *per se* causatively contributes to Alzheimer's disease pathogenesis. It is also critical to test above mentioned mechanistic hypothesis

generated from the neuropathological clue (i.e. interactions of amyloid- β and synaptic loss with tight junction pathology) in future studies perhaps using experimental model systems.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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