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Untangling the association of amyloid-β and tau with synaptic and axonal loss in Alzheimer's disease

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It is currently unclear how amyloid-β and tau deposition are linked to changes in synaptic function and axonal structure over the course of Alzheimer's disease. Here, we assessed these relationships by measuring presynaptic (synaptosomal-associated protein 25, SNAP25; growth-associated protein 43, GAP43), postsynaptic (neurogranin, NRGN) and axonal (neurofilament light chain) markers in the CSF of individuals with varying levels of amyloid-β and tau pathology based on ^{18}F-flutemetamol PET and ^{18}F-flortaucipir PET. In addition, we explored the relationships between synaptic and axonal markers with cognition as well as functional and anatomical brain connectivity markers derived from resting-state functional MRI and diffusion tensor imaging. We found that the presynaptic and postsynaptic markers SNAP25, GAP43 and NRGN are elevated in early Alzheimer's disease i.e. in amyloid-β-positive individuals without evidence of tau pathology. These markers were associated with greater amyloid-β pathology, worse memory and functional changes in the default mode network. In contrast, neurofilament light chain was abnormal in later disease stages, i.e. in individuals with both amyloid-β and tau pathology, and correlated with more tau and worse global cognition. Altogether, these findings support the hypothesis that amyloid-β and tau might have differential downstream effects on synaptic and axonal function in a stage-dependent manner, with amyloid-related synaptic changes occurring first, followed by tau-related axonal degeneration.

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Introduction

Alzheimer's disease is a slowly progressing disorder in which pathophysiological changes precede clinical symptoms by many years (Jack *et al.*, 2010). There is growing evidence suggesting that increases in amyloid-β may be one of the triggers that initiates Alzheimer's disease by setting off a chain of events that include the accumulation of toxic forms of tau, which eventually cause downstream neurodegeneration and dementia (Jack *et al.*, 2010, 2013*a*, 2014). However, it is currently unclear how rising amyloid-β levels lead to tau deposition and why there is such a long delay between them.

Recently, a hypothesis has been proposed that could explain how these two events are temporally related (Edwards, 2019). This hypothesis suggests that, in early stages of Alzheimer's disease, as amyloid-β levels start to rise, plaques begin to be deposited and progressively increase in size, reducing glutamatergic transmission and damaging nearby synapses (Wu *et al.*, 2010; Burgold *et al.*, 2011). This initial damage to synapses close to amyloid-β plaques produces a local network dysfunction that evokes a response from microglia, which remove damaged synapses to prevent further damage or major network disruption (Cummings *et al.*, 2017). However, as more amyloid-β plaques continue to accumulate, particularly across multiple locations, the synaptic damage becomes more pronounced and spreads, resulting in phosphorylation of tau (Howlett *et al.*, 2008; Hong *et al.*, 2016), dissociation of tau from microtubules and the formation of tau tangles, which in turn triggers axon loss and neurodegeneration (Jack *et al.*, 2018).

Although this proposed sequence of toxic effects is supported by several animal studies of Alzheimer's disease (Zempel *et al.*, 2010, 2013; Rozkalne *et al.*, 2011; Cohen *et al.*, 2013; Spires-Jones and Hyman, 2014; Koffie *et al.*, 2019), to our knowledge it has yet to be extensively tested in human subjects. This is particularly timely because of the recent development of tau tracers with PET and the novel assays for measuring synaptic and axonal proteins in the CSF. Thus, the aim of this study was to understand the differential impact of amyloid-β and tau on synaptic loss and axonal integrity during earlier Alzheimer's disease stages, i.e. in amyloid-positive and amyloid-negative individuals who do not yet have tau pathology, as well as later Alzheimer's disease stages, i.e. in tau-positive and tau-negative individuals with established amyloid-β pathology. To this end, we measured the concentrations of presynaptic (synaptosomal-associated protein 25, SNAP25; growth-associated protein 43, GAP43), postsynaptic (neurogranin, NRGN) and axonal (neurofilament light chain, NfL) markers in the CSF. We then tested which of these CSF markers was associated with regional amyloid-β and tau deposition measured with amyloid-PET and tau-PET, and we assessed their relationship with memory and global cognition. Finally, using resting-state functional MRI and diffusion tensor imaging, we identified which of the available CSF and PET markers was more closely associated with changes in the default-mode network and degeneration of the parahippocampal tract, which are well-established connectivity measures of Alzheimer's disease (Pievani *et al.*, 2011). Our underlying hypothesis was that global amyloid-β deposition would be associated with changes in synaptic markers, memory dysfunction and default-mode network activity, whereas elevated tau PET would be accompanied by more pronounced axonal degeneration, global cognitive decline and parahippocampal white matter damage. The unique combination of imaging and CSF measures places this study in an ideal position to test the multimodal biomarker relationships in the course of Alzheimer's disease.

Materials and methods

Participants

The present study included 128 individuals from the Swedish BioFINDER cohort (http://biofinder.se/), an ongoing longitudinal study designed to identify and develop new markers for neurodegenerative disorders, particularly Alzheimer's disease. For this study, only subjects with ^{18}F-flutemetamol PET, ^{18}F-flortaucipir PET and CSF measures were included. Cognitively normal subjects were required to have a Clinical Dementia Rating score of 0, 27–30 points on the Mini-Mental State Examination, not fulfil criteria for mild cognitive impairment or dementia, have no history of cognitive change over time, and be fluent in Swedish. Cognitively impaired subjects were required to fulfil the DSM-5 criteria for mild neurocognitive disorder or major neurocognitive disorder due to Alzheimer's disease (American Psychiatric Association, 2013), and exhibit abnormal amyloid-β accumulation based on ^{18}F-flutemetamol PET. All subjects underwent the Mini-Mental State Examination (Folstein *et al.*, 1975) and delayed word list recall test from the ADAS-Cog (Alzheimer's Disease Assessment Scale – Cognitive Subscale) (Rosen *et al.*, 1984) to assess global cognition and episodic memory, respectively. There were not global ADAS-Cog scores available for this sample.

The Regional Ethical Review Board of Lund University, the Swedish Medicines and Products Agency, and the Radiation Safety Committee of Skåne University Hospital in Sweden approved the study and written, informed consent was obtained from all participants according to the Declaration of Helsinki.

Image acquisition

All subjects underwent structural MRI on a Siemens Tim Trio 3 T scanner, ^{18}F-flutemetamol PET on a Philips Gemini TF 16 scanner, and ^{18}F-flortaucipir PET on a General Electrics Discovery 690 scanner. ^{18}F-flutemetamol PET images were acquired 90 to 110 min after injection of 185 MBq ^{18}F-flutemetamol and reconstructed into 4 × 5 frames using the line-of-response row-action maximum-likelihood algorithm (Palmqvist *et al.*, 2016). ^{18}F-flortaucipir PET images were acquired 80 to 100 min after injection of 370 MBq ^{18}F-flortaucipir, reconstructed into 4 × 5 frames using an iterative Vue Point HD algorithm with six subsets, 18 iterations with 3 mm filter and no time-of-flight correction (Hahn *et al.*, 2016). The structural T_1-weighted images were acquired using a magnetization-prepared rapid gradient echo sequence using the following parameters: 176 slices, repetition time: 1950 ms, echo time: 3.4 ms, inversion time: 900 ms, flip angle: 9°, 1 mm isotropic voxels.

In addition, a subsample also underwent resting-state functional MRI and diffusion tensor imaging on a Siemens Tim Trio 3 T scanner. Resting-state functional MRI images were acquired using a gradient-echo planar imaging pulse sequence with the following parameters: 180 volumes, 33 slices, repetition time: 2000 ms, echo time: 30 ms, 3 mm isotropic voxels. Diffusion tensor imaging scans were acquired with 64 diffusion-weighted directions at a b-value of 1000 s/mm^2 using an echo-planar imaging sequence with the following parameters: 65 axial slices; repetition time: 8200 ms; echo time: 86 ms, no inter-slice gap, 2 mm isotropic voxels.

Image preprocessing

All PET images were motion-corrected, time-averaged and coregistered to their skull stripped T_1-weighted scans. Amyloid-β positivity was established using a composite cortical region normalized by the whole cerebellum, brainstem and eroded subcortical white matter (Landau *et al.*, 2015) on ^{18}F-flutemetamol data with a cut-off of >0.693. This cut-off was obtained from linear mixed models in a large group of subjects of BioFINDER (n = 406) using a composite cortical region of interest in addition to the whole cerebellum, the pons/brainstem and eroded cortical white matter as a reference region (Palmqvist *et al.*, 2016). We excluded borderline cases and used cut-offs that were ±5% from the original cut-offs to avoid drawing conclusions based on cases that could be easily misclassified because of variability in the measurements or subthreshold amyloid-β positivity. Tau positivity was calculated using a composite set of brain regions corresponding to a temporal meta-region of interest normalized by the inferior cerebellum grey matter on ^{18}F-flortaucipir data with a previously established cut-off of >1.34 (Ossenkoppele *et al.*, 2018).

The resting-state functional MRI scans were analysed using multivariate exploratory linear decomposition into independent components (MELODIC), as implemented in FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl). This is a data-driven method that extracts temporally related signals, which represent functionally relevant networks. Using different modules of FSL, the following preprocessing was applied to the functional MRI scans: removal of the first five volumes, motion correction, slice-timing correction, brain extraction, spatial smoothing using a Gaussian kernel of 8 mm and high-pass temporal filtering of 100 s. After preprocessing, all images were registered to the MNI (Montreal Neurological Institute) space using a mean echo-planar image (EPI) generated from all subjects. The subject's time series were then temporally concatenated into a single 4D time series and separated in 20 independent components. Of these 20 components, two quantitatively overlapped with the anterior and posterior default mode networks provided by Biswal *et al.* (2010). We binarized these network maps and extracted the mean temporal time series of each map from all subjects.

Diffusion tensor images were corrected for distortions caused by eddy currents and head motion, and skull-striped using the eddy_correct tool of FSL (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki). A diffusion tensor model was then fitted at each voxel to calculate the fractional anisotropy maps for each subject using the Diffusion Toolbox (Behrens *et al.*, 2007). All images were visually inspected to ensure whole brain coverage, absence of artefacts (venetian blind, checkers and stripe blurring artefacts) or spatial distortions in the temporal poles caused by field inhomogeneities. To extract structural connectivity values from a relevant Alzheimer's disease connectivity marker we used the hippocampal cingulum mask from the John Hopkins University (JHU) white matter tractography atlas (Hua *et al.*, 2008), corresponding to the parahippocampal tract. We binarized this mask, coregistered it to the native fractional anisotropy images of all subjects and extracted their mean fractional anisotropy values.

CSF biomarker measurements

NRGN and NfL were measured at the Clinical Neurochemistry Laboratory at University of Gothenburg, Mölndal, Sweden, using an in-house immunoassay for NRGN (Kvartsberg *et al.* 2019) and GAP43 (Sandelius *et al.* 2019) and a commercial ELISA for NfL (NF-light® ELISA, Uman Diagnostics) (Zetterberg *et al.*, 2016). For SNAP25 measurements, we used an assay that consists of enrichment with immunoprecipitation (KingFisher™ Flex System) followed by digestion, addition of heavy isotope-labelled standards, and quantitation with liquid chromatography/selected reaction monitoring mass spectrometry (LC-SRM/MS) (Agilent 6490 QQQ MS).

Statistical analyses

Statistical analyses were carried out using SPSS 25.0 (IBM Corp., Armonk, NY, USA) and R (version 3.5.1). Square root, logarithmic or reciprocal transformations were applied to CSF biomarkers that were not normally distributed. Then, a set of pairwise t-tests was used to compare these biomarkers between amyloid-positive and amyloid-negative individuals without evidence of tau pathology (earlier disease stages) and between tau-positive and tau-negative individuals with amyloid-β pathology (later disease stages). These analyses were adjusted for age in addition to sex for the NfL comparisons since this marker was significantly higher in males compared to females ($P < 0.05$). Sixteen subjects had missing SNAP25 levels due to technical problems. For all the analyses, we report the degrees of freedom or number of subjects.

To assess the ability of synaptic and axonal markers in predicting amyloid-β and tau pathology, we built linear regression models with the markers as dependent variables and amyloid-β and tau as the outcomes, using the forward selection method and adjusting for age and sex. For these analyses, we verified that the residuals were normally distributed, there was no

heteroscedasticity, and no multicollinearity between the variables, which was determined using a variance inflation factor below five.

Spearman partial correlation analyses were then used to assess the relationship between memory or global cognition with synaptic and axonal markers, while adjusting for the previous covariates in addition to education. These analyses were carried out in earlier and later Alzheimer's disease stages.

We then used linear regression models to assess which of the PET (amyloid-β, tau), synaptic (SNAP25, GAP43, NRGN) and axonal (NfL) markers was the best predictor of functional activity in the anterior and posterior default-mode networks as well as fractional anisotropy in the parahippocampal tract. Again, here we verified that all linear regression model assumptions were met.

Finally, we conducted mediation analyses to test different models based on the expression 'X → Y, mediated by M', while controlling for covariates. The significance of the mediation was assessed by calculating bias-corrected 95% confidence intervals (CIs) using bootstrapping (500 resamples).

All analyses were adjusted for multiple comparisons using false discovery rate (FDR) corrections ($q < 0.05$, two-tailed).

Data availability

Anonymized data will be shared by request from a qualified academic investigator for the sole purpose of replicating procedures and results presented in the article and as long as data transfer is in agreement with EU legislation on the general data protection regulation and decisions by the Ethical Review Board of Sweden and Region Skåne, which should be regulated in a material transfer agreement.

Results

Study participants

In this cross-sectional and multimodal study of 128 participants, 62 were cognitively normal and 66 were cognitively impaired, the latter being diagnosed with mild cognitive impairment or Alzheimer's disease dementia. Since previous evidence shows that amyloid and tau accumulation have different non-linear trajectories over the course of Alzheimer's disease, with amyloid initially accelerating and then later decelerating or even decreasing, whereas tau is initially low and then later accelerates (Kadir *et al.*, 2012; Jack *et al.*, 2010, 2013*a*, *b*, 2014, 2018), we divided the participants into three groups using a global amyloid-β composite cortical region and a temporal tau-PET meta-region of interest. Thus, using PET measures, in the current study 37 subjects were amyloid-negative and tau-negative (amyloid-β–Tau–), 35 were amyloid-positive and tau-negative (amyloid-β + Tau–) and 53 were both amyloid-positive and tau-positive (amyloid-β + Tau +) (Table 1). Only three subjects were amyloid-β–Tau + , who were excluded from the analyses because of the small number of cases in the group. To motivate our approach of dividing the sample into groups, we explored the non-linear relationships between the biomarkers using spline models across the entire cohort (Supplementary material). These models showed that the relationships between amyloid PET and synaptic markers are quadratic: they are positively correlated in initial stages, then reach a stable point, and afterwards they become slightly negatively correlated. This suggests that amyloid seems to have a detrimental effect on synaptic damage only in early stages and that this effect cannot be captured by assessing these relationships across the entire sample. In contrast, the spline models also show that tau PET only has an effect on NfL in later disease stages, with Tau– subjects being concentrated on one side of the plot, whereas Tau + subjects spread out on the other side, driving the correlations.

A subsample of our cohort also underwent resting state functional MRI (66%, 43 cognitively unimpaired, 41 cognitively impaired) and diffusion tensor imaging (49%, 25 cognitively unimpaired, 38 cognitively impaired) to assess the relationship between synaptic, axonal and PET maskers with brain connectivity measures of Alzheimer's disease.

All the analyses performed in this study were repeated using a less stringent cut-off of 1.22 to define tau positivity and divide subjects into Tau + and Tau– groups. This cut-off was calculated following the recommendations by Jack *et al.* (2017) to calculate a sensitivity-based tau PET cut-off. The results of these analyses can be found in the Supplementary material.

Synaptic and axonal biomarkers change in earlier and later Alzheimer's disease stages

Our findings showed significant increases in the synaptic markers SNAP25 [$F(2,63) = 9.08$, $P = 0.004$], GAP43 [$F(2,70) = 6.12$, $P = 0.016$] and NRGN [$F(2,70) = 7.75$, $P = 0.007$] but not in the axonal marker NfL [$F(2,70) = 0.73$, $P = 0.395$] (Fig. 1A) in earlier Alzheimer's disease stages, i.e. in amyloid-β + Tau– compared to amyloid-β– Tau– cases. Similar results were found after excluding subjects who did not have values for all CSF markers [SNAP25: $F(2,61) = 10.63$, $P = 0.002$; GAP43: $F(2,63) = 4.35$, $P = 0.041$; NRGN: $F(2,61) = 10.30$, $P = 0.002$; NfL: $F(2,61) = 2.75$, $P = 0.103$].

In contrast, significant increases in NfL [$F(2,86) = 18.70$, $P < 0.001$] but not SNAP25 [$F(2,75) = 1.83$, $P = 0.180$], GAP43 [$F(2,86) = 1.99$, $P = 0.162$] or NRGN [$F(2,86) = 0.66$, $P = 0.421$] were found in later Alzheimer's disease stages, i.e. in amyloid-β + Tau + versus amyloid-β + Tau– cases (Fig. 1B). Again, these results were similar after excluding subjects without all markers [SNAP25: $F(2,73) = 0.76$, $P = 0.385$; GAP43: $F(2,75) = 3.78$, $P = 0.100$; NRGN: $F(2,73) = 0.944$, $P = 0.334$; NfL: $F(2,73) = 17.79$, $P < 0.001$]. To examine whether the later results were influenced by the presence of cognitive impairment, we performed an additional analysis that compared the same panel of markers between amyloid-β + Tau + and amyloid-β + Tau– groups but only in subjects who were cognitively

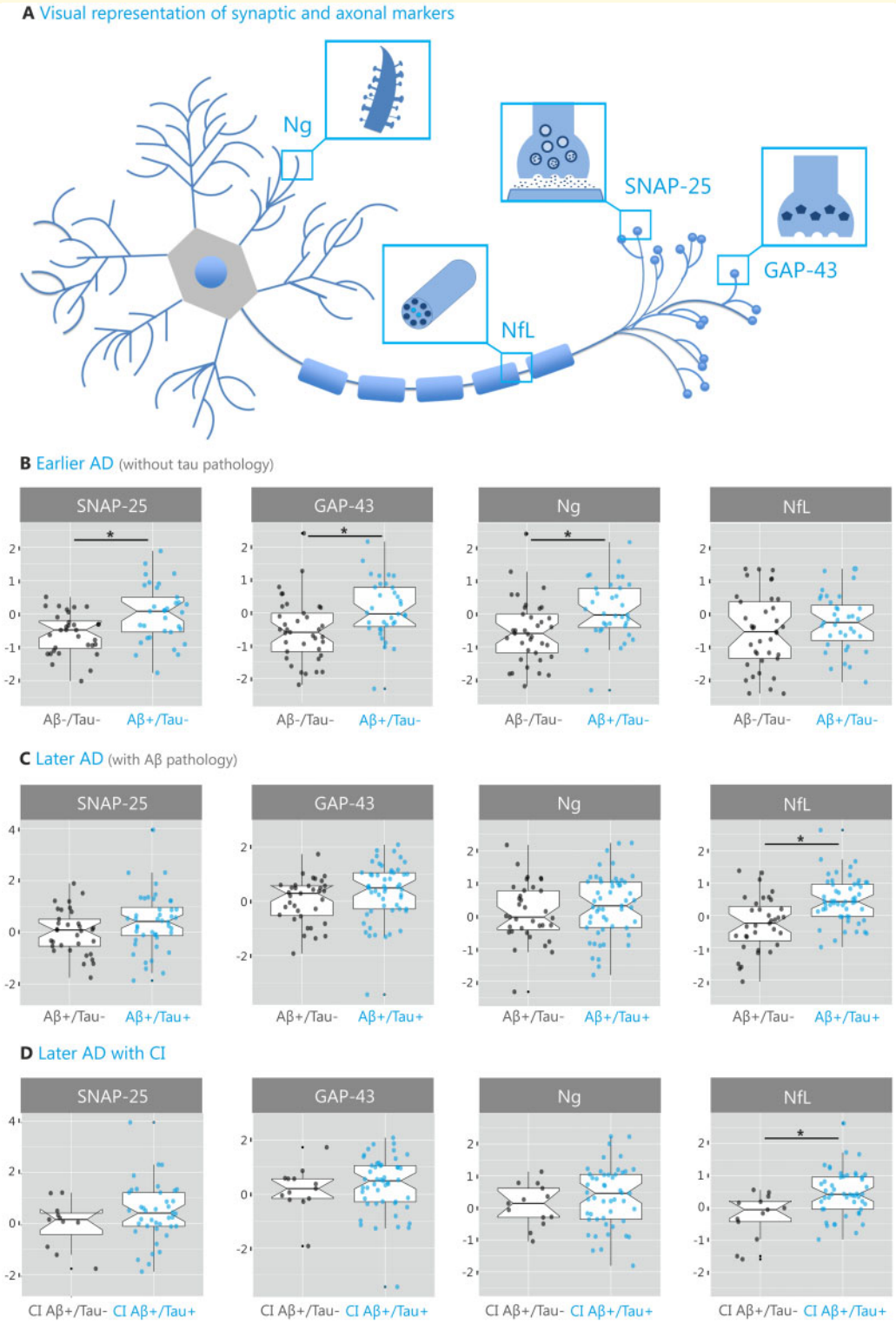

Figure 1 Synaptic and axonal markers change in earlier and later Alzheimer's disease stages. (**A**) Representation of the neuronal aspects measured by the postsynaptic [NRGN (Ng)], axonal (NfL) and presynaptic markers (SNAP25; GAP43). (**B**) In early Alzheimer's disease stages, SNAP25, GAP43 and NRGN show significant increases in amyloid-β+ (Aβ+) compared to amyloid-β– (Aβ–) individuals without evidence of tau PET retention (Tau–). (**C**) In later Alzheimer's disease stages, NfL shows significant increases in Tau+ compared to Tau– individuals who are all amyloid-β+. (**D**) The increases in NfL in the previous groups can still be observed when only cognitively impaired (CI) individuals are considered. These analyses were performed using *t*-tests, while controlling for covariates. The values presented in the plots are the residuals of SNAP25, GAP43, NRGN and NfL after regressing out the effects of age (all CSF markers) in addition to sex (NfL).

impaired. This analysis showed that NfL was still significantly increased in cognitively impaired tau-positive compared to tau-negative individuals [$F(2,60) = 8.23$, $P = 0.006$], whereas synaptic proteins did not show changes between these groups [SNAP25: $F(2,49) = 1.21$, $P = 0.277$; GAP43: $F(2,60) = 0.17$, $P = 0.678$; NRGN: $F(2,60) = 0.61$, $P = 0.437$] (Fig. 1C). Altogether, these results are in line with the hypothesis that amyloid-related synaptic changes occur early in Alzheimer's disease, even when widespread tau pathology is not yet present, whereas axonal degeneration is more closely associated with the development of tau pathology in cases that are amyloid-positive. We note that these results do not indicate there is no synaptic damage in later stages of Alzheimer's disease. In fact, if we compare amyloid-β + Tau + subjects to the amyloid-β–Tau– group, there are significant differences in all synaptic markers [NRGN: $F(2,88) = 16.79$, $P < 0.001$; GAP43: $F(2,88) = 17.043$, $P < 0.001$; SNAP25: $F(2,74) = 19.76$, $P < 0.001$]. Our results suggest that synaptic damage is not significantly different between amyloid-β + Tau– and amyloid-β + Tau + subjects, in contrast to axonal damage, which is significantly different, indicating that synaptic changes emerge in earlier disease stages, whereas axonal damage emerges in later disease stages.

Synaptic degeneration and axonal damage correlate with high amyloid-β and tau

To assess whether synaptic and axonal proteins are associated with the severity of amyloid-β and tau deposition in earlier and later Alzheimer's disease stages, we performed linear regression models using global amyloid-PET and temporal tau-PET standard uptake value ratio (SUVR) values as the outcome and SNAP25, GAP43, NRGN and NfL as the predictors. The goal of these analyses is to select the CSF marker that is the best predictor of amyloid and tau deposition, while excluding the other markers that were also associated with amyloid and tau but did not significantly improve the fit of the model when added to it. We found that increasing NRGN levels were associated with elevated global amyloid-PET SUVR ($r^2 = 0.11$, $t = 2.51$, $P = 0.015$) in cases with normal tau-PET (i.e. in amyloid-β–Tau– and amyloid-β + Tau– cases) (Fig. 2A), explaining 11% of the variance in amyloid-β deposition, in contrast to SNAP25 ($t = 0.933$, $P = 0.355$), GAP43 ($t = -0.573$, $P = 0.569$) or NfL ($t = 0.858$, $P = 0.394$), which did not reach significance. These findings indicate that postsynaptic changes are associated with the amount of amyloid-β pathology even before development of evident tau pathology.

On the other hand, increasing NfL levels correlated best with tau-PET SUVR values ($r^2 = 0.39$, $t = 5.42$, $P < 0.001$), explaining 39% of the variance in tau deposition in cases with amyloid-β pathology (i.e. in amyloid-β + Tau– and amyloid-β + Tau +) (Fig. 2B), in contrast to SNAP25 ($t = 0.35$, $P = 0.728$), GAP43 ($t = 1.66$, $P = 0.102$) and NRGN ($t = 1.48$, $P = 0.145$), indicating that axonal degeneration is more closely related to the levels of tau pathology in Alzheimer's disease.

Memory and global cognition correlate with synaptic and axonal markers in a stage-dependent manner

The temporal course of cognitive decline in Alzheimer's disease is characterized by early changes in memory, which are then followed by other deficits and global cognitive decline. To test the hypothesis that synaptic and axonal proteins would be differentially associated with cognitive functions that are affected in earlier or later disease stages, we conducted correlation analyses between delayed memory (word list recall from ADAS-Cog), global cognition (Mini-Mental State Examination), SNAP25, GAP43, NRGN and NfL.

When the analyses were restricted to individuals without evident tau pathology (i.e. in amyloid-β–Tau– and amyloid-β + Tau– cases), we found that the only correlations that remained significant were between memory and both presynaptic (GAP43; $\rho = 0.30$, $P = 0.011$) (Fig. 3A) and postsynaptic (NRGN: $\rho = 0.28$, $P = 0.017$) (Fig. 3B) markers (there were also a trend for SNAP25: $\rho = 0.26$, $P = 0.039$, uncorrected for multiple comparisons). In contrast, when the analyses were restricted to individuals with evident amyloid-β pathology (i.e. amyloid-β + Tau– and amyloid-β + Tau + cases), the only remaining significant correlation was between NfL and global cognition ($\rho = -0.25$, $P = 0.020$) (Fig. 3C). This correlation was no longer significant when it was tested in amyloid-β + Tau– and amyloid-β + Tau + cases separately, possibly due to a lower number of subjects in the separate groups.

Functional and structural connectivity changes are associated with synaptic loss and tau pathology

For individuals who underwent resting state functional MRI and diffusion tensor imaging, we extracted connectivity measures that are known to be sensitive to Alzheimer's disease (Pievani *et al.*, 2011) and tested their relationship with our panel of CSF and PET markers. These connectivity measures consisted of brain activation signals from the anterior and posterior default mode networks as well as the fractional anisotropy values of the parahippocampal tract.

Our models showed that NRGN was associated with both reduced connectivity in the posterior default-mode network ($r^2 = 0.33$, $t = -3.27$, $P = 0.002$) (Fig. 4A) and increased connectivity in the anterior default-mode network ($r^2 = 0.37$, $t = 2.278$, $P = 0.020$) (Fig. 4B) in early Alzheimer's disease. When the signals of the anterior and posterior default-mode networks were combined, no significant associations with any marker were

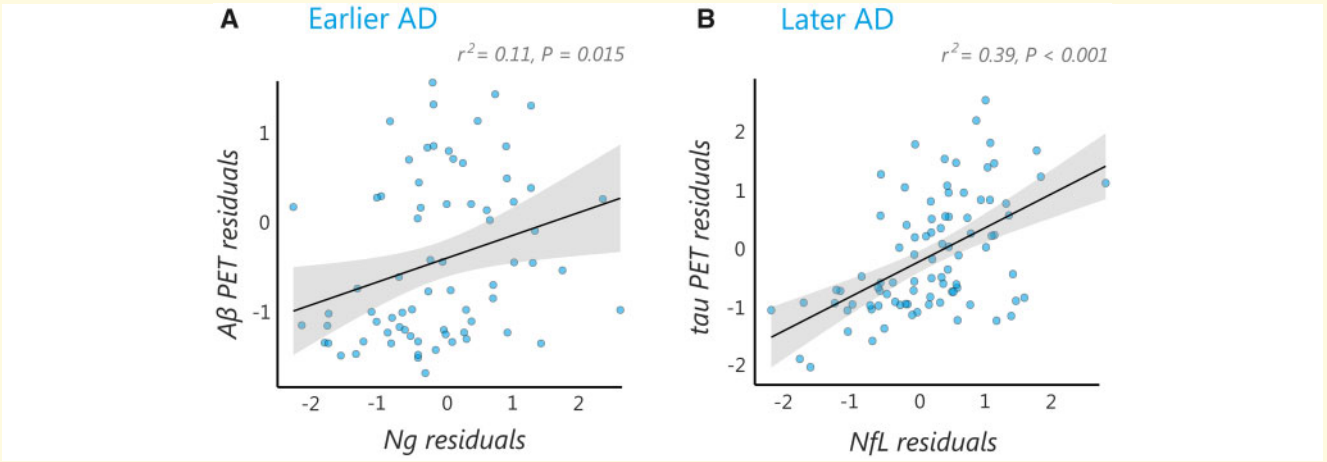

Figure 2 Synaptic degeneration and axonal damage correlate with high amyloid-β and tau. (**A**) Among the synaptic and axonal markers, increasing NRGN (Ng) correlates best with greater amyloid-β PET retention, i.e. in individuals who are amyloid– but do not show evidence of tau pathology. (**B**) In contrast, higher NfL correlates best with greater tau PET retention in later Alzheimer's disease stages, i.e. in individuals who are Tau– and Tau + with amyloid-β pathology. The analyses were carried out using linear regression models. The values presented in the plots are the residuals of NRGN and NfL after regressing out the effects of age (all markers) in addition to sex (NfL). AD = Alzheimer's disease.

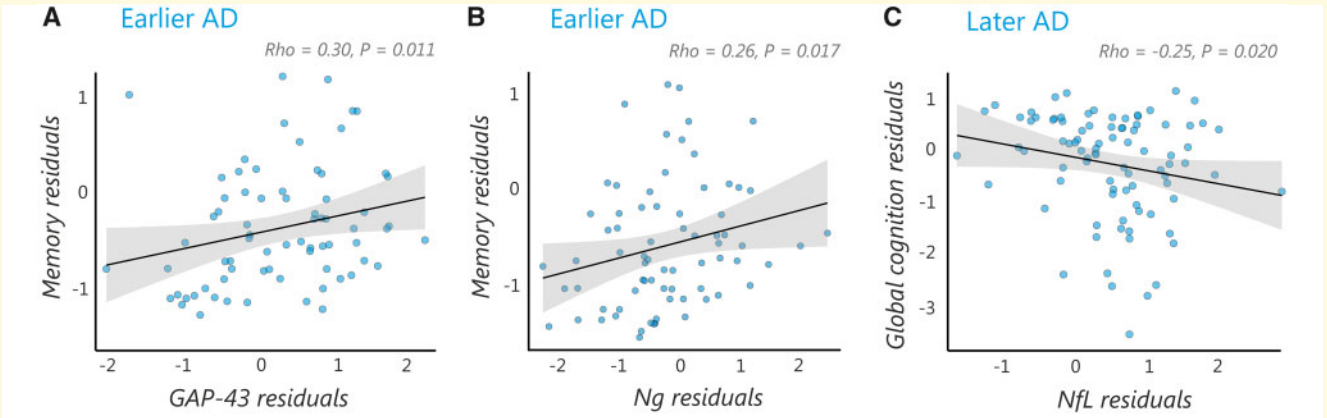

Figure 3 Memory and global cognition are associated with synaptic and axonal markers in a stage-dependent manner. (**A**) Memory measured by the word list recall test (ADAS-Cog) correlates with GAP43 and NRGN levels in early Alzheimer's disease stages (amyloid-β + Tau– and amyloid-β–Tau–), whereas (**B**) global cognition measured by the Mini-Mental State Examination correlates with NfL in later Alzheimer's disease stages (amyloid-β + Tau + and amyloid-β + Tau–). The correlation analyses were performed using Spearman's ρ. These results are in line with findings showing memory deficits in early Alzheimer's disease and global cognitive impairment in later disease stages. The values presented in the plots are the residuals of GAP43, NRGN and NfL after regressing out the effects of age, sex and education. AD = Alzheimer's disease.

found. On the other hand, contrary to what we expected, tau PET SUVR correlated with reduced parahippocampal white matter integrity in later disease stages (r^2 = 0.16, t = –2.91, P = 0.006) (Fig. 5), instead of NfL (t = 0.90, P = 0.374).

Synaptic loss mediates the effect of amyloid-β on tau, whereas tau mediates the effect of NfL on cognition

To test the hypothesis that amyloid-β deposition is followed by synaptic damage, tau aggregation, axonal degeneration and cognitive impairment, we conducted three mediation analyses. The first analysis tested whether the relationship between amyloid-β and tau was mediated by synaptic loss in earlier Alzheimer's disease stages. Since NRGN was the synaptic marker that was most strongly associated with amyloid-β in our previous analyses, we selected it as the synaptic mediator. The results of this analysis showed that NRGN mediates the effect between amyloid-β and tau pathologies in early Alzheimer's disease (0.118, 95% CI: 0.03 to 0.24, P = 0.008).

The second analysis assessed whether, in later Alzheimer's disease stages, the effects of tau on global cognition were mediated by axonal damage measured with NfL. The results

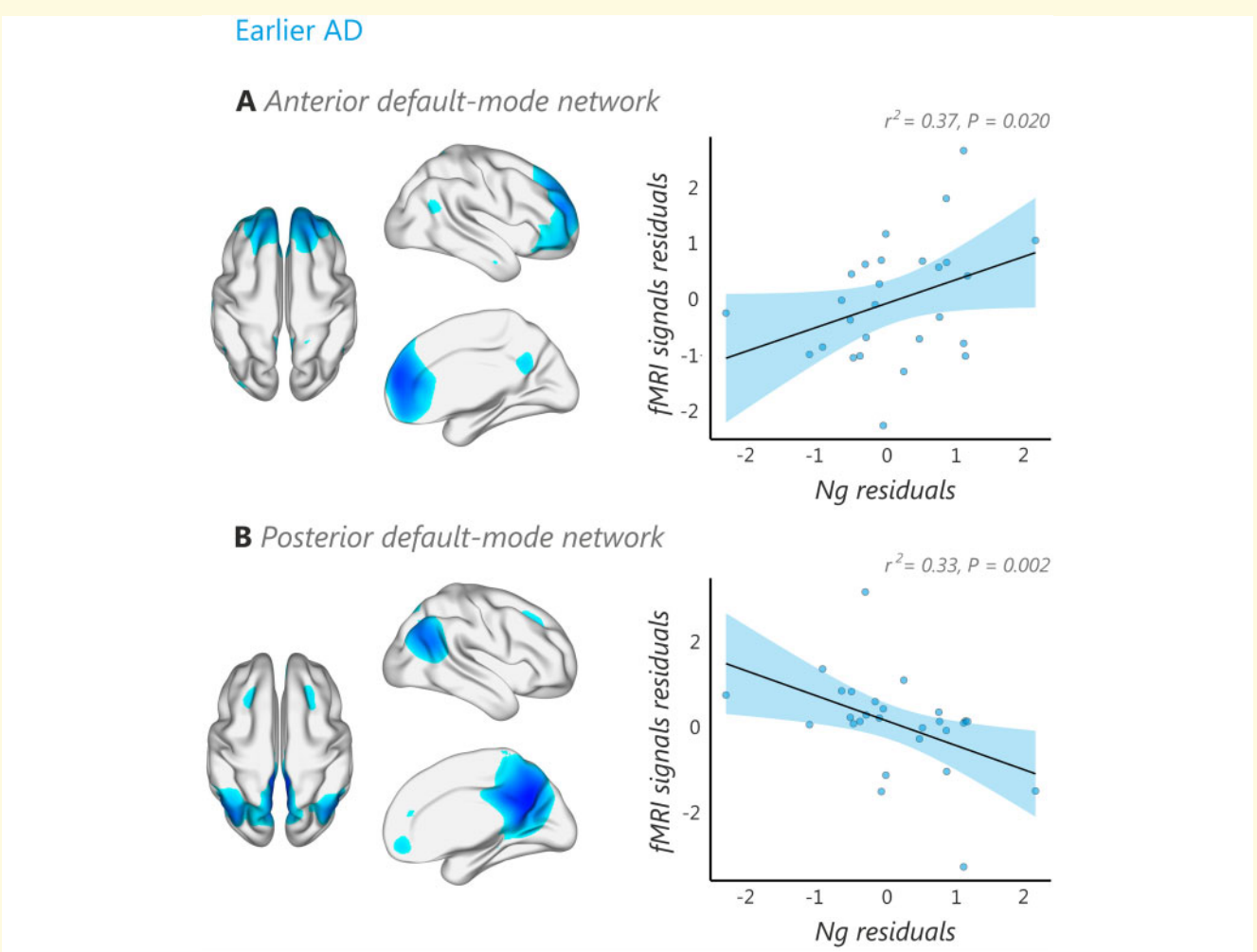

Figure 4 Synaptic loss predicts functional connectivity changes in the default-mode networks. In earlier Alzheimer's disease stages (amyloid-β + Tau– and amyloid-β–Tau–), our linear regression models show that NRGN (Ng) is the marker that correlates best with connectivity increases in the anterior default-mode network (**A**) and connectivity decreases in the posterior default-mode network (**B**). The values presented in the plots are the residuals of functional MRI (fMRI) signals and NRGN after regressing out the effects of age and sex. AD = Alzheimer's disease.

of this analysis showed that NfL was not a mediator of this relationship (0.654, 95% CI: –0.351 to 1.700, $P = 0.190$).

Finally, the third analysis tested whether the relationship between NfL and global cognition was mediated by tau instead and this proved to be significant (4056.8, 95% CI: 2274.4 to 6099.5, $P < 0.001$).

Altogether, these results suggest that the relationship between amyloid-β and tau is mediated by synaptic changes, in line with our initial hypothesis. In contrast, contrary to what we had predicted, the relationship between NfL and global cognition is mediated by tau pathology.

Discussion

The aggregation of amyloid-β and tau in brain regions serving memory and cognition are thought to be responsible for a sequence of events that lead to clinical Alzheimer's disease (Hardy and Selkoe, 2002). However, a better understanding of the *in vivo* downstream effects of amyloid-β and tau in human subjects is needed. Our findings agree with the hypothesis that amyloid-β and tau are associated with synaptic dysfunction and axonal degeneration, respectively. These changes correlate with memory, global cognition and brain connectivity in a stage-dependent manner, suggesting they may be useful to track disease progression. Although the associations revealed by these data are cross-sectional, the most likely interpretation based on previous animal studies (Zempel *et al.*, 2010, 2013; Rozkalne *et al.*, 2011; Cohen *et al.*, 2013; Spires-Jones and Hyman, 2014; Koffie *et al.*, 2019), is that amyloid-β deposition is followed by synaptic damage, tau aggregation and neurofilament changes (Fig. 6). These findings might open possibilities for new therapeutic targets for Alzheimer's disease based on synaptic and axonal function.

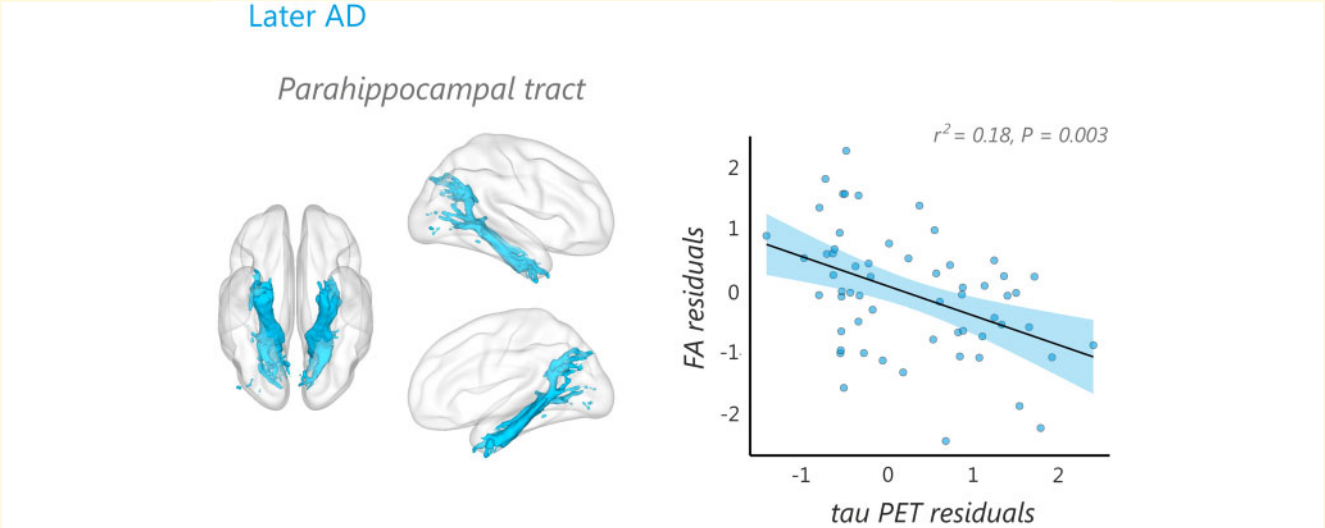

Figure 5 Tau pathology correlates with structural connectivity changes in the parahippocampal tract. In later Alzheimer's disease stages (amyloid-β + Tau + and amyloid-β + Tau–), our linear regression models show that tau PET retention is the measure that correlates best with reduced fractional anisotropy (FA) in the parahippocampal tract (*left*). The values presented in the plots are the residuals of fractional anisotropy values and tau PET levels after regressing out the effects of age and sex.

There is growing evidence that amyloid-β may be part of a mechanism controlling synaptic activity (Fagiani *et al.*, 2019). In particular, it has been shown that amyloid-β production is enhanced by action potential-dependent synaptic activity, leading to increased amyloid-β at synapses and alterations of synaptic excitatory transmission (Palop and Mucke, 2010). These changes can affect both presynaptic and postsynaptic mechanisms of neuronal communication and impair overall brain activity (Gulisano *et al.*, 2019). However, several other studies have also proposed that, compared to amyloid-β, tau might have a more critical role in synaptic dysfunction. This is due to the presence of tau at the synapses in Alzheimer's disease brains (Tai *et al.*, 2012), the fact that tau pathology is more strongly associated with cognitive decline (Nelson *et al.*, 2012) and its ability to spread trans-synaptically between neurons (Pooler *et al.*, 2013).

Thus, motivated by these two different lines of evidence, in this study we sought to untangle the effects of amyloid-β and tau on presynaptic and postsynaptic markers in earlier and later Alzheimer's disease stages, during which amyloid-β and tau could have a differential impact on synaptic loss and axonal integrity. We used SNAP25 and GAP43 as presynaptic markers because of their role in initiating fusion of synaptic vesicles for synaptic communication (Brinkmalm *et al.*, 2014) or synaptic plasticity (Allegra Mascaro et al., 2013), respectively. In addition, we used NRGN as a postsynaptic marker since it is involved in long-term potentiation (Zhong and Gerges, 2010; Zetterberg and Blennow, 2015). We found that increases in SNAP25, GAP43 and NRGN occur already in early Alzheimer's disease stages in amyloid-β + Tau– individuals. The increase in these synaptic proteins was associated with memory impairment and NRGN was specifically associated with functional connectivity changes in the anterior and posterior default-mode networks. It is well known that memory loss is one of the first clinical symptoms of Alzheimer's disease (Carlesimo and Oscar-Berman, 1992). In addition, changes in network hyperactivity and hypoactivity are common in early Alzheimer's disease (Sheline and Raichle, 2013; Huijbers *et al.*, 2015; Jones *et al.*, 2016; Sepulcre *et al.*, 2017), being part of a potentially compensatory mechanism that the brain enables to cope with initial pathology and maintain normal function (Elman *et al.*, 2014). Although reduced connectivity is more expected in the context of a neurodegenerative disorder like Alzheimer's disease, increased connectivity can also occur and may be due to the overactivation of *N*-methyl-D-aspartic acid receptors by glutamate at the synaptic level, which are mediated by amyloid-β (Gouras *et al.*, 1997; Palop and Mucke, 2010). Altogether, these findings would fit a model in which amyloid-β and synaptic loss are the key players in initial memory impairment and early network dysfunction. The assumption that amyloid-β and synaptic damage are linked in Alzheimer's disease is supported by several animal studies showing that neurons lacking the amyloid precursor protein show greater excitatory synaptic transmission (Priller *et al.*, 2006), neuritic outgrowth (Koo *et al.*, 1993) and an increased number of synapses (Steinbach *et al.*, 1998). They also agree with human studies showing that amyloid plaques are associated with both presynaptic and postsynaptic changes in non-demented individuals (Potter *et al.*, 2011), a mechanism that might be potentially regulated by mitochondria (Reddy and Beal, 2008) or cholinergic receptors (Jürgensen and Ferreira, 2010). Thus, our findings confirm previous reports showing the detrimental role of amyloid pathology on synapses. The fact that the

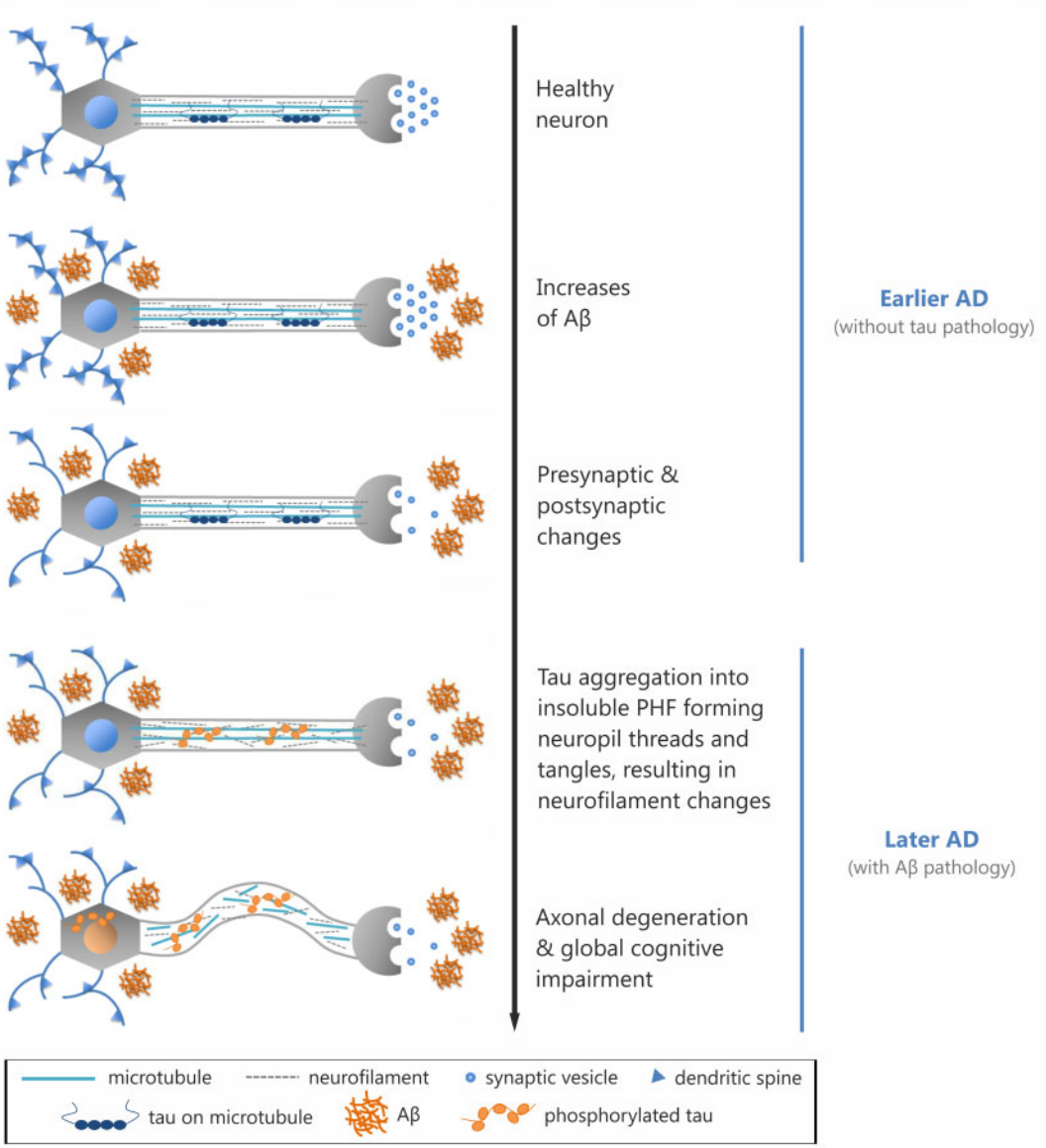

Figure 6 Schematic hypothetical representation of the downstream effects of amyloid-β and tau on synaptic and axonal function in earlier and later Alzheimer's disease stages. In earlier disease stages (amyloid-β + Tau– and amyloid-β–Tau–), the accumulation of amyloid-β into plaques reduces the number of postsynaptic spines and the number of presynaptic vesicles, altering local synaptic communication. In later disease stages (amyloid-β + Tau + and amyloid-β + Tau–), as synaptic changes become more pronounced, tau becomes phosphorylated and aggregates into insoluble paired helical filaments (PHF), which form neuropil threads and neurofibrillary tangles. These changes are followed by the dissociation of neurofilaments from microtubules, axonal degeneration and ultimately global cognitive impairment. Aβ = amyloid-β; AD = Alzheimer's disease.

postsynaptic marker NRGN was the best predictor of amyloid pathology compared to the presynaptic markers SNAP25 and GAP43 could be related to the fact that postsynaptic glutamate receptor trafficking has been shown to be a prime initial target for amyloid-β by several previous studies (Almeida *et al.*, 2005; Roselli *et al.*, 2005; Snyder *et al.*, 2005; Shemer *et al.*, 2006), suggesting that postsynaptic terminals might be more affected by amyloid pathology in early stages of Alzheimer's disease. Thus, altogether, these findings agree with our hypothesis that synaptic markers would be associated with functional connectivity in earlier disease stages and are in line with previous evidence showing that functional connectivity is an early marker that changes in response to amyloid deposition (Sheline and Raichle, 2013; Huijbers *et al.*, 2015; Jones *et al.*, 2016; Sepulcre *et al.*, 2017).

As previously proposed by animal models (Howlett *et al.*, 2008; Hong *et al.*, 2016; Edwards, 2019), in later

Table 1 Cohort characteristics

	Amyloid-β–Tau– (*n* = 37)	Amyloid-β + Tau–(*n* = 35)	Amyloid-β + Tau + (*n* = 53)	Amyloid-β– Tau– versus amyloid-β + Tau–(*P*-value)	Amyloid-β + Tau– versus amyloid-β + Tau + (*P*-value)
Age	74 (52-87)	77 (56-88)	71 (40-88)	0.124	0.003
Sex, male/female	21/16	19/16	28/25	0.833	0.893
Education	12 (7–18)	10 (6–23)	12 (5–20)	0.235	0.264
MMSE	29.0 (27–30)	29.0 (21–30)	23.5 (7–29)	0.070	<0.001
Delayed memory	1.0 (0–5)	3.0 (0–9)	8.0 (0–10)	0.005	<0.001
Cognitively impaired, %	2.7	37.1	92.5	<0.001	<0.001
Global amyloid-β SUVR	0.65 (0.56–0.74)	0.95 (0.76–1.18)	1.02 (0.81–1.31)	<0.001	<0.001
Temporal meta-ROI tau SUVR	1.15 (1.02–1.23)	1.21 (1.05–1.30)	1.80 (1.34–2.84)	0.002	<0.001
SNAP25, pmol/l	16.83 (6.99–25.51)	21.92 (7.27–36.17)	24.82 (5.82–54.66)	0.004	0.180
GAP43, pg/ml	3576.16 (1788–6475)	4272.97 (2009–7926)	5003.62 (1193–9086)	0.016	0.162
NGRN, pg/ml	222.92 (90.98–527.49)	263.45 (90.04–494.03)	300.36 (112.34–547.84)	0.007	0.421
NfL, pg/ml	792.51 (388.44–11 632.0)	1065.10 (470.95–11 759.0)	1429.60 (517.78–13 322.0)	0.395	<0.001
Subsample with rs-fMRI (%)	70.3	82.9	83.0	–	–
Subsample with DTI (%)	56.8	62.9	79.2	–	–

Data are presented as median (range). *P*-values were derived from ANOVA for continuous normally distributed measures, Mann-Whitney tests for continuous non-normally distributed measures and chi-squared tests for categorical measures. DTI = diffusion tensor imaging; MMSE = Mini-Mental State Examination; ROI = region of interest; SUVR = standard uptake value ratio; rs-fMRI = resting-state functional MRI.

Alzheimer's disease stages the spread of amyloid-β-induced synaptic damage along the length of the axon, results in phosphorylation, subsequent dissociation of tau from microtubules and the formation of tangles. Since the microtubules are the structures that support the axon, the dissociation of tau from these structures gives place to axonal degeneration (Spires-Jones *et al.*, 2009). Axonal degeneration is thus an important aspect of Alzheimer's disease that can be measured indirectly using NfL, a scaffolding protein involved in the growth of axons (Gaetani *et al.*, 2019), or with fractional anisotropy on diffusion tensor imaging. In this study, we found that NfL was significantly increased in later Alzheimer's disease stages, i.e. in tau-positive individuals with amyloid-β pathology. These increases were associated with global cognition, which normally becomes impaired in more advanced Alzheimer's disease stages. However, contrary to what we expected, tau pathology was the best predictor of reduced parahippocampal white matter integrity, instead of NfL. This finding may be due to the size of the damaged microstructures associated with tau pathology or high NfL levels. Tau proteins can be found in microtubules, which have a diameter of ∼25 nm (Ledbetter and Porter, 1963), whereas neurofilaments are much smaller, being ∼1.42 nm (Gaetani *et al.*, 2019). Although the contribution of cellular components to diffusion anisotropy is not completely understood (DaSilva *et al.*, 2003), there is evidence supporting that certain axonal structures such as neurofilaments and microtubules might play an important role in this process (Beaulieu and Allen, 1994). In addition, other metabolic mechanisms such as axonal transport, which is primarily carried out through the microtubules, has also been suggested to be associated with anisotropy (Beaulieu, 2002). Thus, it is plausible that, compared to neurofilament injury, damage to the microtubules, which are larger in size, would have a greater impact on the integrity of white matter fibres by increasing the number of larger obstacles to directional water diffusion, which is captured by fractional anisotropy on diffusion brain images. Altogether, these findings suggest that, in the presence of amyloid-β pathology, tau may be the main contributor to both increasing NfL levels and structural connectivity loss. In addition, we also found that tau mediates the effects of NfL on global cognition, suggesting that the effects of NfL on cognitive function might not be independent of tau. We note that, in the current study, we did not hypothesize that functional connectivity would be associated with NfL or axonal damage because functional connectivity often occurs between brain areas that are not anatomically connected or linked by axons, as reported in previous studies (Honey *et al.*, 2009; Hermundstad *et al.*, 2013; Park and Friston, 2013; Palmqvist *et al.*, 2017).

Regarding previous literature, our results are in line with a study showing early increases in SNAP25 in presymptomatic stages of familial Alzheimer's disease (Schindler *et al.*, 2019). They also agree with findings in sporadic Alzheimer's disease showing elevated NRGN in amyloid-β + non-demented individuals compared to amyloid-β– subjects (Portelius *et al.*, 2015; Mattsson *et al.*, 2016), an association between GAP43 and amyloid pathology measured using CSF amyloid-$\beta_{1\text{-}42}$ (Sandelius *et al.*, 2019), and finally an association between NRGN with memory (Casaletto *et al.*, 2017) or between NfL and global cognitive decline (Zetterberg *et al.*, 2016; Mattsson *et al.*, 2019). Regarding NfL, although familial Alzheimer's studies suggest that it is an early disease marker (Preische *et al.*, 2019), they have not compared it to synaptic markers within the same sample to establish which changes occur earlier or later over the course of the disease.

In fact, previous studies by our group comparing different CSF and plasma markers across the same subjects suggest that the changes in NfL in early disease stages are rather subtle, arguing against NfL being a reliable early marker of Alzheimer's disease (Palmqvist *et al.*, 2019, 2020). To our knowledge, there are no previous studies assessing the relationship between changes in synaptic and axonal CSF markers with measures of tau aggregation into insoluble paired helical filaments captured by tau PET. The studies published so far have mainly used CSF measures of tau (Mattsson *et al.*, 2016; Sutphen *et al.*, 2018; Sandelius *et al.*, 2019), which mostly reflect soluble tau forms that change in earlier stages of Alzheimer's disease at least partly in response to amyloid pathology (Sato *et al.*, 2018; Mattsson-Carlgren *et al.* 2020). Thus, our study has the advantage of measuring a constellation of synaptic and axonal markers in individuals who also underwent amyloid PET and tau PET, which are thought to reflect the formation of neuritic plaques (Ikonomovic *et al.*, 2008) and tangles (Sato *et al.*, 2018; Smith *et al.*, 2019) that characterize earlier and later stages of Alzheimer's disease (Braak and Braak, 1991).

It should be noted that our cross-sectional design relies on the assumption that amyloid-β deposition is followed by tau pathology, which is in line with current models of Alzheimer's disease (Jack *et al.* 2010, 2013*a*, 2014, 2018). Future studies assessing longitudinal changes in SNAP25, GAP43, NRGN, NfL in relation to longitudinal amyloid-β PET and tau-PET imaging would be needed to understand their dynamic trajectories over the course of Alzheimer's disease, as described in the previous models (Jack *et al.*, 2010, 2013*a*, 2014, 2018). In addition, studies assessing the relationship between cognitive resilience and synaptic integrity (Arnold *et al.*, 2013; Boros *et al.*, 2017; Latimer *et al.*, 2019) or synaptic changes in primary age-related tauopathy (PART) (Crary *et al.*, 2014) would also be useful to further understand the implications of synaptic and axonal loss. Finally, note also that we used tau PET imaging instead of CSF tau to assess later disease stages in the current study. This choice was driven by recent evidence showing that CSF tau reflects mainly soluble tau forms, which change in earlier stages of Alzheimer's disease at least partly in response to amyloid pathology, in contrast to tau PET, which most likely reflects the formation of tau into paired helical filaments (Sato *et al.*, 2018; Mattsson-Carlgren *et al.*, 2020), correlating strongly with neurofibrillary tangles (Smith *et al.*, 2019) and being therefore a better late disease marker. To ensure that our results were not driven by a specific threshold to define tau-positivity, we replicated all of our findings using a less stringent cut-off of 1.22, which can be found in Supplementary material.

In summary, the results of our *in vivo* study agree with the hypothesis that amyloid-β and tau have toxic effects on synaptic function and axonal integrity, respectively, linking these events over the course of Alzheimer's disease. By combining several established and innovative methods, we show that early disease stages are characterized by amyloid-induced synaptic damage, memory impairment and functional connectivity changes, whereas later disease stages are characterized by tau-associated axonal damage, global cognitive decline and reduced anatomical connectivity. While we recognize that the current techniques do not have the resolution to assess the molecular mechanisms of amyloid-β and tau at the single neuron level, these results are consistent with several animal studies and current disease models that place synaptic dysfunction and axonal degeneration as central events in the pathogenesis of Alzheimer's disease.

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Competing interests

K.B. has served as a consultant or at advisory boards for Abcam, Axon, Biogen, Lilly, MagQu, Novartis and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform

company at the University of Gothenburg. H.Z. has served at scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed and CogRx, has given lectures in symposia sponsored by Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. O.H. has acquired research support (for the institution) from Roche, Pfizer, GE Healthcare, Biogen, AVID Radiopharmaceuticals and Euroimmun. In the past 2 years, he has received consultancy/speaker fees (paid to the institution) from Biogen and Roche. All other authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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