Inhibitory synapse loss and accumulation of amyloid beta in inhibitory presynaptic terminals in Alzheimer's disease

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Abstract
Background and purpose: Synapse degeneration in Alzheimer’s disease (AD) correlates strongly with cognitive decline. There is well-established excitatory synapse loss in AD with known contributions of pathological amyloid beta (Aβ) to excitatory synapse dysfunction and loss. Despite clear changes in circuit excitability in AD and model systems, relatively little is known about pathology in inhibitory synapses.

Methods: Here human postmortem brain samples (n = 5 control, 10 AD cases) from temporal and occipital cortices were examined to investigate whether inhibitory synapses and neurons are lost in AD and whether Aβ may contribute to inhibitory synapse degeneration. Inhibitory neurons were counted in all six cortical layers using stereology software, and array tomography was used to examine synapse density and the accumulation of Aβ in synaptic terminals.

Results: Differing inhibitory neuron densities were observed in the different cortical layers. The highest inhibitory neuron density was observed in layer 4 in both brain regions and the visual cortex had a higher inhibitory neuron density than the temporal cortex. There was significantly lower inhibitory neuron density in AD than in control cases in all six cortical layers. High-resolution array tomography imaging revealed plaque-associated loss of inhibitory synapses and accumulation of Aβ in a small subset of inhibitory presynaptic terminals with the most accumulation near amyloid plaques.

Conclusions: Inhibitory neuron and synapse loss in AD may contribute to disrupted excitatory/inhibitory balance and cognitive decline. Future work is warranted to determine whether targeting inhibitory synapse loss could be a useful therapeutic strategy.

Keywords
Alzheimer, amyloid, inhibitory, synapse
INTRODUCTION

Alzheimer’s disease (AD) is the most common cause of dementia in the elderly, affecting approximately 50 million people worldwide [1]. Brain changes in AD begin years before symptom onset and include accumulation of amyloid beta (Aβ) peptide in extracellular plaques, aggregation of tau protein in neurofibrillary tangles and neuropil threads, gliosis and extensive loss of neurons and synapses [2]. Of the neuropathological changes that occur in AD, synapse loss correlates most strongly with cognitive decline [3–5].

To date, most of the work examining synapse degeneration in AD has been focused on excitatory synapses, which clearly degenerate extensively in AD, particularly near amyloid plaques [6,7]. In an unbiased proteomic study of synaptic fractions from human AD and control temporal and occipital cortex, proteins involved with glutamatergic signalling were previously observed to be significantly decreased whilst no changes were detected in inhibitory synaptic proteins [8]. Epileptiform activity and seizures are observed in some people with AD and network hyperactivity is observed in animal models of amyloidopathy, leading to testing of anti-epileptic drugs as potential treatments [9]. Whilst it is unlikely that epilepsy is a causative factor in developing AD, there is ample evidence to suggest that pathological changes in AD brain lead to disrupted excitatory/ inhibitory balance. This could involve disruption to inhibitory synapse structure or function, but there is some controversy about the role of inhibitory synapse damage in AD with some studies finding decreases in inhibitory synapse density or function [10–14], some finding increases [15–17], some finding a mix of increases/decreases depending on marker or disease stage [18,19] and some finding no change [20,21] (Table 1).

In model systems including acute rodent brain slices, in vivo injection of Aβ into rodent brain, and transgenic mouse models with plaque pathology, there is abundant evidence showing that oligomeric Aβ causes excitatory synapse dysfunction and loss [6,7,25–27]. Our previous data using high-resolution array tomography imaging suggests that accumulation of oligomeric Aβ within individual pre-synaptic and postsynaptic terminals is associated with excitatory and total (excitatory and inhibitory) synapse loss around plaques in human temporal cortex of AD patient samples [6,28,29]. There are contradictory results examining inhibitory synapse loss or dysfunction downstream of Aβ in model systems with some studies finding increased inhibitory postsynaptic potentials [15] or γ-aminobutyric acid (GABA) conductance [16] whilst others found decreased inhibitory postsynaptic potentials [13] and impaired inhibitory signalling [11]. Some of these discrepancies could be due to differences in model systems or ages, but discrepancies remain even in very similar studies. For example, in transgenic APP/PS1 mice, one group observed an increase in inhibitory synapses in young mice before pathology developed and a later stage loss of inhibitory synapses [19], whilst in a very similar APP/PS1 model another group found no change in inhibitory synapse density at the same late age (12 months) [20]. These two APP/PS1 studies examined different brain regions (hippocampus and somatosensory cortex, respectively) with slightly different techniques. From these data, the role of Aβ in inhibitory synapse changes remains unclear in animal models and has not been explored yet in detail in human brain. In this study, high-resolution array tomography imaging was used to test the hypothesis that inhibitory synapses degenerate in human AD brain and that pathological forms of Aβ may contribute to this process.

METHODS

Human cases

In this study, postmortem human brain tissue from the inferior or middle temporal gyrus (BA20/21) and primary visual cortex (BA17) was examined. Experiments were approved by the Edinburgh Brain Bank ethics committee and the ACCORD medical research ethics committee, AMREC (ACCORD is the Academic and Clinical Central Office for Research and Development, a joint office of the University of Edinburgh and National Health Service Lothian, approval number 15-HV-016). The Edinburgh Brain Bank is a Medical Research Council funded facility with research ethics committee approval (16/ES/0084). Table 2 shows the summary demographics of our cases. Inclusion criteria for AD cases was a clinical diagnosis of AD with neuropathological confirmation and a Braak stage of V or VI. Control cases were selected for age and sex matching with exclusion criteria of no known neurological or psychiatric diseases and Braak stage lower than III. AD and control groups did not differ significantly in age (Wilcoxon test W = 12, p = 0.12) or postmortem interval (Wilcoxon test W = 14, p = 0.20), and both groups contained 40% females. Groups did differ in APOE genotype with no APOE4 carriers in our age-matched control group. For all experiments, the experimenter was blind to case information.

Tissue processing and immunohistochemistry

For determining inhibitory neuron density, tissue blocks from each region of interest were fixed in 10% formalin for at least 24 h and then dehydrated and embedded in paraffin wax. Sections were cut at 4 μm thickness on a Leica microtome and collected on glass slides. Sections were de-waxed and re-hydrated through a series of xylenes and graded ethanol solutions followed by antigen retrieval in 10 mM citrate buffer (pH 6.0) in a pressure cooker for 20 min. Sections were washed in Tris buffered saline (TBS), incubated in 0.3% hydrogen peroxide and 10% methanol in 0.1 M phosphate buffered saline for 10 min, rinsed in phosphate buffered saline, and blocked for 1 h in blocking solution containing 0.2% Tween and 10% normal donkey serum in TBS. Sections were incubated in blocking solution containing glutamic acid decarboxylase (GAD) 65/67 primary antibody at 1:500 dilution overnight at 4°C (Synaptic Systems #198 111). Primary antibody solution was washed off with TBS and secondary antibody solution containing biotinylated anti-rabbit antibody at 1:500 dilution was applied for 1 h (antibody details are in Table 3).
| Subjects                  | Age/disease stage (n) | Brain region                                                                 | Methods                                                                 | Change in inhibitory synapses                                                                 | Ref   |
|--------------------------|-----------------------|------------------------------------------------------------------------------|------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------|
| Human                    | AD Braak III–VI (n = 3) Controls Braak 0–II (n = 7) | Hippocampus CA1 stratum radiatum and pyramidal layer | 3D electron microscopy to count synapses | Loss of overall synapse density No change in proportion of excitatory/inhibitory synapses. Interpreted as loss of both excitatory and inhibitory synapses in AD | [10]  |
| Human                    | Controls (n = 5) | MCI or dementia Braak III–V (n = 5) | Trans-entorhinal cortex layer 2 | 3D electron microscopy to count synapses | Loss of overall synapse density No change in proportion of excitatory/inhibitory synapses. Interpreted as loss of both excitatory and inhibitory synapses in AD | [22]  |
| Human                    | AD Braak IV–VI (n = 6) Controls (n = 6) | Hippocampus, entorhinal cortex (EC), superior temporal gyrus (STG) | Immunofluorescence of GABA transporters BGT-1, GAT-1, GAT-3 | BGT-1 increased in AD in STG, CA2, CA3, DG GAT-1 decreased in AD in EC and STG GAT-3 decreased in CA1, CA3, EC, subiculum | [18]  |
| Human/mouse              | AD Braak I–V (n = 8) Controls (n = 7) APPsw/PS1DE9 amyloidopathy mouse model (n = 6) | Cortex (mix of regions, unclear which were used for inhibitory synapse stain) | Immunofluorescence of plaques, neurons and GAT-1 or VGAT. Confocal imaging. No quantification, only two representative images shown | Loss of inhibitory puncta on neuronal cell bodies only on the side in contact with dense plaques | [23]  |
| Human                    | Braak VI AD (n = 6) Braak III no dementia (n = 6) Controls (n = 5) | Inferior temporal cortex | Immunofluorescence of inhibitory (VGAT) and excitatory (VGLUT) presynaptic terminals | No loss of inhibitory synapses, even near plaques, although excitatory synapses were lost as reported previously | [20]  |
| Human                    | Braak VI AD (n = 5), control (n = 5), Braak VI Down’s syndrome (n = 5) | Parietal cortex | Immunofluorescence of gephrin and PSD95, recordings of microtransplanted synaptic membranes into Xenopus oocytes | No change in layer 1 inhibitory or excitatory synapse density although intensity of staining was lower in AD for these proteins. Excitatory/inhibitory ratio elevated in microtransplanted AD membrane samples | [21]  |
| Rats treated with oligomeric Aβ | Adult rats 1 week after injection of Aβ or vehicle (n = 4–7 slices/group) | EC injection, DG recording | Whole cell patch clamp in acute slices of DG neurons with performant pathway stimulation | Aβ caused higher inhibitory postsynaptic potentials and lower excitatory postsynaptic potentials in dentate gyrus | [15]  |
| Mice treated with oligomeric Aβ or scrambled Aβ | Adult mice 1 week after Aβ injection (n = 4–5 male mice per group) | Hippocampal injection, recording in CA1 | In vitro whole cell patch clamping | Increased GABAergic conductance mediated by extrasynaptic GABA_A receptors | [16]  |
| Subjects                                      | Age/disease stage (n)                                                                 | Brain region                    | Methods                                                                 | Change in inhibitory synapses                                                                 | Ref  |
|----------------------------------------------|---------------------------------------------------------------------------------------|---------------------------------|-------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|------|
| Mice treated with oligomeric Aβ              | 3–5-month-old mice, n = 14 vehicle treated, n = 7 Aβ treated                          | i.c.v. infusion of Aβ (very high concentration, 0.2 mM) recording in CA1–CA3 | *In vivo* field postsynaptic potentials from the CA3–CA1 synapse in the dorsal hippocampus recorded in freely moving mice | Impairment of inhibitory signalling                                                            | [11] |
| rTg4510 tauopathy mouse model                | 2–6- and 8-month-old rTg4510 and control mice, n = 3–6 per group                     | CA1 hippocampus and cortex for synapse counts, whole brain for PET imaging | Immunofluorescence of inhibitory (VGAT) and excitatory (VGLUT) synapses, PET imaging of inhibitory ([11C]Flumazenil) and excitatory ([11C]ABP688) synapse markers | Tauopathy mice have early inhibitory synapse dysregulation and later excitatory synapse degeneration | [12] |
| J20 amyloidopathy mouse model                | N = 10–14 cells per group (unknown from n mice)                                       | Cortex layer 2–3                 | Intracellular recordings of cortical neurons in acute brain slices       | Reduced amplitude of action potentials of GABAergic cells and reduced spontaneous inhibitory post synaptic potentials in J20 mice | [24] |
| APP<sup>NL-F/NL-F</sup> amyloidopathy mouse model | 1–2-, 4–6- and 10–18-month-old wild-type and APP<sup>NL-F/NL-F</sup> mice, n = 7 per group | CA1, somatosensory cortex layer 2–3 | Intracellular recording in acute slices, immunofluorescence for excitatory (CamKII) and inhibitory (parvalbumin and GAD67) neuron markers | In APP<sup>NL-F/NL-F</sup> mice, inhibitory postsynaptic potentials were decreased and excitatory postsynaptic potentials increased from 1 to 18 months. Excitatory neurons are lost in entorhinal cortex starting at 1–2 months of age, and in CA1 and neocortex at 9–13 months of age. PV positive interneurons were lost at 9–13 months of age in dorsal EC | [13] |
| Osaka mutant APP amyloidopathy mouse model    | 4-, 6-, 8-, 12- and 24-month-old APP, heterozygous APP and wild-type mice (n = 3–4/group) | Hippocampus                      | Western blot for excitatory (VGLUT), inhibitory (VGAT) and total (synaptophysin) synapses, immunostaining of total (NeuN) and inhibitory (parvalbumin) neurons | Osaka mutation carrying APP transgenic mice have loss of GABAergic neurons and synaptic proteins, and aberrant synaptic activity | [14] |
| APP<sup>Swe/PS1</sup>L<sub>166P</sub> amyloidopathy mouse model | 1-, 3-, 8- 12-month-old APP/PS1 and wild-type mice (n = 3–6/group)                   | Hippocampus                      | Western blot and immunofluorescence for inhibitory synaptic protein (gephryn) | In APP/PS1 mice, inhibitory synapses are increased before pathology develops and decreased at 12 months of age when there are robust pathology and cognitive impairments | [19] |
| APP<sup>Swe/PS1</sup>L<sub>166P</sub> amyloidopathy mouse model | 3-month-old male mice (n = 4–6/group)                                                 | Hippocampus                      | Slice electrophysiology (extracellular field potentials), VGAT and gephryn immunofluorescence | In APP/PS1 mice, increased parvalbumin inhibitory synapse density in CA1 and CA3 pyramidal neurons, altered sharp wave–ripple complexes | [17] |
Avidin/biotin amplification was carried out with an ABC Elite kit followed by diamino benzidine peroxidase staining according to the manufacturer’s instructions (Vector Laboratories). Sections were counterstained with haematoxylin for 30 s. Sections were dehydrated through a series of ethanol solutions and xylene and coverslips were mounted with DPX mounting medium.

For examining inhibitory synapses and synaptic Aβ, samples from each region of interest were fixed and embedded for array tomography as described previously [30,31]. Briefly, small samples of cortex containing all six cortical layers were fixed in 4% paraformaldehyde for 3 h, dehydrated in ethanol and incubated in LR White resin overnight at 4°C. Individual samples were cured in LR White in gelatin capsules overnight at 53°C. Ribbons of ultrathin serial 70 nm sections were cut using a histo Jumbo Diamond knife (Diatome) and an Ultracut (Leica). Ribbons were mounted on glass coverslips, dried on a slide warmer and outlined with hydrophobic pen. Ribbons were rehydrated in 50 mM glycine and antigen retrieval was conducted with 1 mM EDTA, 0.05% Tween 20 solution in a pressure cooker on steam setting for 2 min. Ribbons were rinsed in TBS and blocked in a solution of 0.1% fish skin gelatin and 0.05% Tween in TBS for 30 min at room temperature. Primary antibodies to GAD65/67 (Abcam, ab11070), synaptophysin (R&D Systems, AF5555) and Aβ (6E10 BioLegend SIG-39320) in blocking buffer were applied and incubated overnight at 4°C. Ribbons were washed with TBS and secondary antibodies in blocking solution were applied to label GAD67 with Alexa Fluor 594, synaptophysin with Alexa Fluor 647 and Aβ with Alexa Fluor 488 (antibody details are found in Table 3). 4',6-Diamino-2-phenylindole (DAPI) (1 μg/ml) was used to counterstain nuclei. Ribbons were washed and mounted on glass slides with ImmuMount.

**Stereology estimation of inhibitory neuron density**

Inhibitory neuron density was estimated in GAD65/67 stained paraffin sections using a Zeiss Axiolager Z2 with Stereoinvestigator software (MicroBrightField). Each cortical layer was outlined as a region of interest at 1.5x magnification and tile scans were acquired at 10x magnification for quantification. The area of each layer region of interest was measured, the number of GAD positive neurons in each layer were counted, and the density of GAD positive neurons in each layer for each case was calculated by dividing the number of neurons by the region area.

**Array tomography image acquisition, processing and analysis**

Array tomography ribbons were imaged on an Axiolager Z2 with a 63x1.4 numerical aperture objective and images were acquired in Zen software (Zeiss). A series of images was taken in the same location in the neocortex along ribbons of 9–30 serial sections per ribbon. Images were taken through all cortical layers. Where plaques were
present, an image stack was taken of a plaque and of a plaque-free area in the same cortical layer. Individual images were combined into a stack using Fiji (ImageJ). Using a custom written MATLAB script, images were aligned and segmented, and objects were detected in three dimensions. Alignments were based on rigid and affine registration of the synaptophysin channel which was applied to all other channels from the same stack. Segmentation was done by thresholding image stacks with a semi-automatic local threshold based on mean intensity values in a rolling window. Plaque cores were segmented with a fixed threshold to find the edge of dense plaques for distance from plaque measurements. Objects that were not present in more than one consecutive section were removed to reduce non-specific signal. A neuropil mask was created to exclude blood vessels and cell bodies and the density of objects per cubic millimetre in the neuropil was calculated for each channel. Using a custom Python script, the colocalization between channels was calculated to determine the percentage of synaptophysin stained presynaptic terminals containing GAD65/67, Aβ or both. All custom software can be downloaded from GitHub at https://github.com/ArrayTomographyUsers/Array_tomography_analysis_tool and https://github.com/lewiswilkins/Array-Tomography-Tool.

Statistics

Age at death and postmortem interval differences between groups were analysed with Wilcoxon tests after determining that the distributions were not normal with Shapiro–Wilk normality tests. The comparison between groups in all the other studied variables was analysed using linear mixed models including case as a random effect to account for multiple measures per case. Residual plots of the linear mixed effects models were examined and, where these did not meet assumptions of the model, data were transformed with Tukey transformations to meet assumptions. ANOVAs were run on linear mixed effects models with Satterthwaite's method of estimation of degrees of freedom. Post hoc comparisons were performed using Tukey corrected estimated marginal means. All the analyses were performed with R Studio [32] and the scripts and full statistical results can be found in supplementary data with associated spreadsheets available on the Edinburgh DataShare repository https://doi.org/10.7488/ds/3098.

RESULTS

Inhibitory neuron loss in Alzheimer’s disease

To determine whether inhibitory neurons are lost in our AD cases, two brain regions were examined: the inferior/middle temporal gyrus (BA20/21) which is heavily affected by pathology at the end stage of disease and the primary visual cortex (BA17) which accumulates tau pathology only at very late stages of disease and has less pathology than the temporal cortex [2]. Sections from these regions were immunostained with antibodies recognizing GAD 65 and 67. GAD is an enzyme required for the synthesis of GABA and is present in GABAergic inhibitory neurons and synaptic terminals. GAD staining

| ID (BBN) | Diagnosis (clinical) | APOE genotype | Age at death (y) | Sex | PMI (h) | Braak NFT stage |
|----------|----------------------|---------------|------------------|-----|---------|-----------------|
| 001.28406 | Control | 3/3 | 79 | M | 72 | II |
| 001.32577 | Control | 3/3 | 81 | M | 74 | II |
| 001.28793 | Control | 3/3 | 79 | F | 72 | II |
| 001.26495 | Control | 3/3 | 78 | M | 39 | I |
| BBN_19686 | Control | 3/3 | 77 | F | 75 | I |
| Control summary | | 100% APOE3 | 79 [77, 81] | 40% F | 72 [39, 75] | Range I–II |
| 001.28410 | AD | 3/3 | 62 | F | 109 | VI |
| 001.28771 | AD | 3/3 | 85 | M | 91 | VI |
| 001.32929 | AD | 3/3 | 85 | F | 80 | VI |
| BBN_24527 | AD | 3/3 | 81 | M | 74 | V |
| 001.29695 | AD | 3/4 | 86 | M | 72 | VI |
| BBN_25739 | AD | 3/4 | 85 | F | 45 | VI |
| 001.30142 | AD | 3/4 | 88 | F | 112 | VI |
| 001.26718 | AD | 3/4 | 78 | M | 74 | VI |
| BBN_24322 | AD | 3/4 | 80 | M | 101 | VI |
| BBN_24526 | AD | 3/4 | 79 | M | 65 | VI |
| AD summary | | 40% APOE3 | 83 [62, 80] | 40% F | 77 [45, 112] | Range V–VI |

Note: In summary rows, percentages, range or medians [min, max] shown as noted.

Abbreviations: AD, Alzheimer’s disease; BBN, Medical Research Council Brain Bank number; NFT, neurofibrillary tangle; PMI, postmortem interval.
in neuronal cell bodies and in punctate immunoreactivity in the neuropil was observed (Figure 1). Quantification of cell bodies stained with GAD65/67 (Figure 2) revealed significant decreases in inhibitory neuron density in AD (ANOVA of linear mixed effects model $F = 11.73, p = 0.008$), lower densities in temporal cortex compared to visual cortex ($F = 34.96, p = 2.73e-08$) and different densities depending on the cortical layer with the highest densities of inhibitory neurons found in layer 4 ($F = 74.90, p < 2.2e-16$).

**Inhibitory synapses loss and accumulation of synaptic Aβ in AD**

High-resolution array tomography imaging was used to examine inhibitory synapse density and the accumulation of oligomeric Aβ within presynaptic terminals. Ribbons of 70 nm serial sections were stained with antibodies to Aβ (6E10), GAD65/67 and presynaptic vesicle protein synaptophysin (Figure 3). Puncta containing both synaptophysin and GAD staining were counted as inhibitory synapses. As reported previously [6,29], loss of total synapses (synaptophysin puncta) in the vicinity of plaques in AD cortex was observed (Figure 4a,b; ANOVA on linear mixed effects model effect of plaque distance $F = 5.772, p = 1.05e-6$). Inhibitory synapse density is decreased by 21% in AD cases compared to controls (Figure 4c; ANOVA on linear mixed effects model $F = 7.419, p = 0.021$). There is a significant effect of brain region with overall lower inhibitory synapse density in temporal cortex ($F = 10.798, p = 0.001$) and an interaction between disease and brain region with more loss in AD cases in visual cortex than in temporal cortex ($F = 7.202, p = 0.007$). Inhibitory synapse loss is most pronounced near plaques (Figure 4d, significant effect of plaque distance $F = 2.90, p = 0.006$).

To determine whether oligomeric Aβ within inhibitory presynaptic terminals may be contributing to synapse loss, colocalization of synaptophysin, GAD and 6E10 staining was examined (Figure 5). As previously observed, there is accumulation of Aβ in a subset of synapses in AD (1.2% of synapses contain Aβ) which is highest near plaques with 13.5% of synapses within 5 μm of a plaque containing Aβ (Figure 6a, significant effect of disease $F = 16.43, p = 0.001$; Figure 6b, significant effect of plaque distance $F = 59.12, p < 2.2e-16$). A small subset of inhibitory synapses also contain Aβ in our cohort, which is also highest near plaques with 0.7% of inhibitory synapse within 5 μm of a plaque containing Aβ (Figure 6c,d, significant effect of plaque distance, Tukey transformed data, $F = 2.90, p = 0.006$).

**DISCUSSION**

Synaptic function is the key for healthy cognition, and loss and dysfunction of synapses are associated with cognitive decline in AD [7,33]. Previous data strongly implicate excitatory neurons and synapses as vulnerable to degeneration downstream of AD pathology [2,6,8,34], but less is known specifically about the degeneration of
The occurrence of seizures in people with AD and the animal model data indicating altered excitatory/inhibitory balance highlight the need to understand pathological changes in both excitatory and inhibitory synapses in disease [9,35–37]. Here inhibitory neuron and synapse densities were examined in postmortem brain samples from people with AD and control subjects in two brain regions: inferior/middle temporal gyrus which is affected early in the disease process and primary visual cortex which is affected much later in disease. Both inhibitory neuron loss and inhibitory synapse loss were observed in AD.

Inhibitory neuron loss has been reported in both AD and mouse models of amyloidopathy (e.g., [38]). Previous studies examining inhibitory synapses in human AD brain report conflicting results with brain region and even sub-region differences (Table 1). The only
known study examining a similar brain region to the two looked at here found no loss of inhibitory synapses even near plaques in AD in the inferior temporal cortex [20]. Both our study and the work by Mitew et al. [20] examined inhibitory presynaptic terminals, theirs stained with vesicular GABA transporter, GAD65 or GAD67 separately and ours co-stained with presynaptic marker synaptophysin and inhibitory maker GAD. Mitew et al. did observe loss of inhibitory synapses within fibrillar plaque cores, but not in the periphery as measured here. One potential reason for the discrepancy between these studies is the more refined method used here with higher axial resolution and the ability to count inhibitory synapses only when they contain both a presynaptic marker and a marker of inhibitory neurons. Our total synapse density in control subject temporal cortex ($1.04 \times 10^9$ synapses/mm$^3$) is comparable to previous data using electron microscopy to calculate synapse density in the same brain region, which found approximately $1 \times 10^9$ synapses/ mm$^3$ with notably higher densities in male than female subjects [39]. These data lend credence to the array tomography technique to examine synapse density and point to the importance of including sex in our statistical models despite gender matching our cases and controls. Contrary to these previous findings, sex effects on synapse density were not observed in our study.

Inhibitory synapse loss was also observed in AD brain in the visual cortex, which is more pronounced than the loss in the temporal cortex. This is somewhat surprising as temporal cortex contains more pathology than visual cortex even at the end stages of disease [8]. A 30% higher inhibitory synapse density was observed in visual cortex compared to temporal cortex in control subjects, similar to our previous case study on a control subject from the Lothian Birth Cohort 1936 [40]. In monkey primary visual cortex, there is age-related loss of both excitatory and inhibitory synapses in layer 3 [41].

![Representative images of array tomography staining. Amyloid beta (6E10, cyan), GAD65/67 (magenta) and presynaptic terminals (synaptophysin, yellow) were stained on array tomography ribbons of ultrathin serial sections. The images shown are maximum intensity projections of five consecutive 70 nm sections from aligned, segmented stacks from AD and control cases in both visual cortex and temporal cortex. Scale bar 100 μm](Colour figure can be viewed at wileyonlinelibrary.com)
**FIGURE 4** Plaque-associated total and inhibitory synapse loss in AD. Quantification of array tomography images of synaptophysin puncta (a), (b) reveals plaque-associated synapse loss (both excitatory and inhibitory) in AD. Inhibitory synapses labelled with both GAD65/67 and synaptophysin (c), (d) are also lost in AD with a significant effect of distance from plaque. Dotted lines in (b) and (d) show the mean of control synapse density. (c) Asterisk shows post hoc Tukey test \( p = 0.002 \). (b), (d) Asterisks indicate values significantly different from 20–40 \( \mu \)m in post hoc Tukey tests [Colour figure can be viewed at wileyonlinelibrary.com]

**FIGURE 5** A\( \beta \) is found within presynaptic terminals. Three-dimensional reconstructions of five serial sections of array tomography ribbons stained with synaptophysin (yellow), 6E10 (cyan) and GAD65/67 (magenta) reveal a subset of inhibitory synapses (GAD positive synaptophysin puncta, arrowheads) which contain A\( \beta \) in AD cases (6E10 and GAD positive synaptophysin puncta, arrows). Channels shown in each reconstruction are labelled on the top row. Scale 500 nm [Colour figure can be viewed at wileyonlinelibrary.com]
INHIBITORY SYNAPSE LOSS IN ALZHEIMER’S

Here a small subset of inhibitory synapses containing Aβ in human visual and temporal cortices was observed with the most accumulation near amyloid plaques. Whilst excitatory synapses were not directly measured in this study, it can be inferred that the synaptophysin stained synapses that do not colocalize with GAD65/67 are excitatory. Under that assumption, an overall 5% loss of excitatory synapses and 20% loss of inhibitory synapses were observed in AD in this study (with more loss near plaques), indicating that inhibitory synapses may be particularly vulnerable to degeneration. This would be expected to alter the excitatory/inhibitory balance in these brain regions. On the other hand, 0.85% of excitatory synapses and 0.07% inhibitory synapses in our study were positive for Aβ. This is proportionally over 10-fold more excitatory than inhibitory synapses positive for Aβ, leading us to speculate that, whilst it is possible that Aβ damages inhibitory synapses, this may not be as important as the damage caused to excitatory synapses. Use of postmortem tissue allows only a snapshot of disease and Aβ could play a role in inhibitory synapse loss at earlier disease stages than were examined here. Both microglia and astrocytes have been implicated in refinement of synaptic circuits and in excitatory synapse degeneration in AD [42]. There are very few data regarding a potential role of glia in inhibitory synapse loss, which will be an important area of future research.

In conclusion, our current data show that inhibitory synapses degenerate in two brain regions in AD and point to the need for future work to understand the mechanisms of inhibitory synapse loss. The loss of inhibitory synapses may contribute to cognitive decline and could be beneficial to target with therapeutics.

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CONFLICT OF INTEREST

TS-J receives funding from two industry collaborators and is on the Scientific Advisory board of Cognition Therapeutics, Alzheimer’s...
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**AUTHOR CONTRIBUTIONS**

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Tara Leigh Spires-Jones: Conceptualization (equal); Formal analysis (lead); Methodology (lead); Resources (lead); Supervision (lead); Writing-original draft (lead).

**DATA AVAILABILITY STATEMENT**

R scripts for statistics, data spreadsheets, and raw images can be downloaded from the Edinburgh DataShare repository (https://doi.org/10.7488/ds/309).

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