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## An immunohistochemical study of GABA<sub>A</sub> receptor gamma subunits in Alzheimer's disease hippocampus: relationship to neurofibrillary tangle progression

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### Abstract

Immunohistochemical characterization of the distribution of GABA<sub>A</sub> receptor subunits  $\gamma$  1/3 and 2 in the hippocampus relative to neurofibrillary tangle (NFT) pathology staging was performed in cognitively normal subjects (Braak stage I/II, n=4) and two groups of Alzheimer's disease (AD) patients (Braak stage III/IV, n=4; Braak stage V/VI, n=8). In both Braak groups of AD patients, neuronal  $\gamma$ 1/3 and  $\gamma$ 2 immunoreactivity was preserved in all hippocampal subfields. However, compared to normal controls neuronal  $\gamma$ 1/3 immunoreactivity was more intense in several end-stage AD subjects. Despite increased NFT pathology in the Braak V/VI AD group, GABA<sub>A</sub> $\gamma$  1/3 and  $\gamma$ 2 immunoreactivity did not co-localize with markers of NFT. These results suggest that upregulating or preserving GABA<sub>A</sub> $\gamma$  1/3 and  $\gamma$ 2 receptors may protect neurons against neurofibrillary pathology in AD.

### Keywords

Alzheimer's disease; GABA; excitotoxicity; neurodegeneration; tau

### Introduction

Alzheimer's disease (AD) is characterized pathologically by the presence of neurofibrillary tangles (NFT) and neuritic plaques, and loss of neurons and synapses in selected brain regions, including the hippocampus. Cell loss in AD is in part due to glutamate-mediated

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excitotoxicity<sup>1,2</sup>, which could be countered by compensatory changes in the  $\gamma$ -aminobutyric acid (GABA) inhibitory neurotransmitter system<sup>1</sup>. There are two major classes of GABA receptors, GABA<sub>A</sub> and GABA<sub>B</sub>. GABA<sub>A</sub> receptors are composed of multiple subunits grouped according to sequence similarity into  $\alpha$  ( $\alpha$ 1–6),  $\beta$  ( $\beta$ 1–4),  $\gamma$  ( $\gamma$ 1–4),  $\delta$  ( $\delta$ 1–2),  $\rho$  ( $\rho$ 1–3),  $\epsilon$ ,  $\pi$ , and  $\theta$  subunits<sup>3</sup>. We previously examined alterations in the expression of GABA<sub>A</sub> $\alpha$ 4 and  $\beta$ 5·6 and GABA<sub>B</sub> $\gamma$ 7 receptor subunits in the hippocampus in AD groups with different Braak stages of NFT severity<sup>8</sup>. These studies reported that with increased NFT severity by Braak staging, the  $\beta$ 2/3 subunit protein (but not  $\beta$ 3 mRNA) is stable while  $\alpha$ 1 subunit decreases in the CA1- CA2 hippocampus. These findings indicated that in AD hippocampus, there are complex changes in GABA<sub>A</sub> receptor subunit composition relative to NFT pathology. This warrants continued investigations, as changes in GABA<sub>A</sub> receptors could provide protection against glutamate-induced excitotoxicity, and guide development of therapies targeting specific GABA<sub>A</sub> receptor subunits<sup>1·9</sup>. The present immunohistochemical study tested the hypothesis that GABA<sub>A</sub> receptor  $\gamma$  subunits are altered during the progression of NFT pathology in AD hippocampus.

## Materials and methods

Postmortem hippocampal tissue was obtained from 16 elderly subjects: 12 with a clinical and neuropathological diagnosis of AD (mean age  $\pm$  SD = 77.8  $\pm$  13.9 years) and 4 age-matched controls with no history of dementia (ages 73.3  $\pm$  16.5 years). The mean postmortem delay to tissue collection was not significantly different between control and AD groups (Table 1). All AD subjects were participants in a longitudinal research program maintained by the University of Pittsburgh's Alzheimer's Disease Research Center (ADRC). Clinical diagnosis of AD was based on a standardized ADRC evaluation at a Consensus Conference, utilizing DSM-IV<sup>10</sup> and National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association NINCDS/ADRDA<sup>11</sup> criteria. AD cases number 14 and 15 had old infarcts restricted to cortical and thalamic regions. Controls were cognitively normal subjects received for regular autopsy and showed no evidence of neurological disorders. Neuropathological diagnosis was determined by a certified neuropathologist, and all AD subjects fulfilled CERAD criteria for the diagnosis of "definite" AD<sup>12</sup>. All brains (controls and AD) had NFTs and were assigned Braak scores using neuropathological staging for NFT by Braak and Braak<sup>8</sup>. The four non demented controls were Braak stage I/II with only isolated hippocampal NFT. Four of the 12 AD cases were Braak stage III/IV with moderate hippocampal NFT pathology, and eight AD cases were Braak stage V/VI with severe hippocampal NFT pathology. The three Braak groups are referred to as mildly, moderately or severely affected, referring to the extent of NFT pathology in the hippocampus.

Brain tissue was processed according to procedures described previously<sup>5·7</sup>. Blocks of tissue containing hippocampus were cut in the coronal plane at the level of the lateral geniculate body and placed in 0.1 mol/L phosphate buffer (PB, pH = 7.4) containing 4 % paraformaldehyde for 48 h at 4 C. Fixed tissue blocks were cryoprotected by immersion into 30% sucrose in PB for several days, and cut at 40  $\mu$ m thickness on a sliding, freezing microtome. For each case, at least one tissue section was stained for Nissl substance to delineate the cytoarchitectural boundaries of the hippocampal fields as defined by Duvernoy<sup>13</sup>.

GABA<sub>A</sub> receptor subunit immunohistochemistry was performed on free-floating tissue sections as described previously<sup>5·7</sup>. At least 3 randomly chosen hippocampal tissue sections from each case were immunohistochemically labeled using polyclonal antisera against the  $\gamma$ 3 subunits (Sc-7371, Santa Cruz, Santa Cruz CA, 1: 25), which recognizes both  $\gamma$ 1 and  $\gamma$ 3 ( $\gamma$ 1/3), and against the  $\gamma$ 2 subunit (345231, Calbiochem, 1: 100) (Fig. 1). As a control for

nonspecific staining, sections were incubated with initial incubation media minus the primary antibody, and otherwise processed as described. No positive staining was detectable in these control sections. Sections from all three groups of Braak staging were processed together. In each case, one immunostained section was photographed and then counterstained with the X-34, a fluorescent amyloid binding dye that is a sensitive marker of amyloid-containing structures in AD brain, including senile plaques, NFT (both intracellular and extracellular “ghost” tangles), dystrophic neurites and neuropil threads, as previously described<sup>14</sup>.

## Results

GABA  $\gamma$ 1/3 immunostaining intensity in Braak stage I/II cases was light to moderate in the cytoplasm and in the neuropil of CA2-4 and DG neurons (Fig. 2A,D). Particularly in CA2-4, cellular  $\gamma$ 1/3 immunostaining was more intense at the junction of the primary dendrite and cell soma (Fig. 2A,D). In the CA1,  $\gamma$ 1/3 immunostaining was relatively weak in pyramidal neurons and smaller multipolar neurons (Fig. 2G), and lightly-immunolabeled dendrites were observed traversing the pyramidal cell layer. In the Braak III/IV cases,  $\gamma$ 1/3 immunoreactivity was indistinguishable from the Braak stage I/II cases (Fig. 2B,E,H). In four of the eight Braak V/VI cases, increased intensity of  $\gamma$ 1/3 immunoreaction in the cell soma was observed in all CA regions (Fig. 2C,F,I).

We next examined the pattern of GABA<sub>A</sub>  $\gamma$ 2 immunoreactivity in the same three Braak staged groups of subjects. In Braak stage I/II (controls) group, moderately intense  $\gamma$ 2 immunoreactivity was detected in neurons and neuropil throughout the hippocampus (Fig. 3A,D,G,J). In all hippocampal fields, neuropil immunostaining was diffuse, while more prominent cellular immunostaining was punctate and filled the entire cell soma, extending into the proximal dendrites. In the dentate gyrus (DG),  $\gamma$ 2 immunoreactivity labeled cell soma and proximal dendrites in the granular cell layer. In the CA fields,  $\gamma$ 2 immunoreactivity was found primarily in pyramidal neurons; a subset of smaller, multipolar neurons was also observed. The  $\gamma$ 2 staining pattern of the Braak III-IV and V-VI severity groups was comparable to that of control Braak I/II cases, except that labeling of dendrites, but not cell bodies, in the CA1 was less prominent in Braak stage V/VI AD cases relative to Braak stage I/II control cases (Fig. 3).

Counterstaining of  $\gamma$ 1/3 and  $\gamma$ 2 immunostained sections with the X-34 (used here to visualize both NFT and amyloid plaques), revealed that neurons immunoreactive to either of the  $\gamma$  subunits did not contain NFT (Fig. 4C-H). This was the case for the majority of the X-34 positive NFT that were of a classic intracellular type, as well as for extracellular “ghost” NFT that were also X-34 positive but with distinct morphological differences (Figure 4G,H, arrow).

Among cases within each Braak (NFT) pathology group, there was substantial variability in the extent and types of hippocampal amyloid plaques, which were frequent in the CA1, CA4, and dentate gyrus, and scarce in the CA2-3; no association of these lesions with either  $\gamma$ 1/3 or  $\gamma$ 2 immunoreactive cells was observed.

## Discussion

This study provides the first immunohistochemical analysis of GABA<sub>A</sub>  $\gamma$  receptor subunits in the human hippocampus staged by the progression of NFT pathology in AD. The overall distribution of GABA<sub>A</sub>  $\gamma$  immunoreactive elements was similar to that from studies of rat hippocampus, where  $\gamma$ 2 immunoreactivity was detected in neuronal soma and principal

dendrites as well as in the surrounding neuropil15, while  $\gamma 1$  and  $\gamma 3$  immunoreactivity was observed in pyramidal cell layers, and only faintly in the neuropil16.

Studies of AD hippocampus using radio-labeled ligand binding to GABA<sub>A</sub> receptors containing  $\gamma$  subunits (especially  $\gamma 2$ ) have produced inconsistent data17–21, and are difficult to compare to the present immunohistochemical study. For example, loss of GABA<sub>A</sub> $\gamma$  protein in AD hippocampus could reflect atrophy related loss of neurons expressing these receptors. However, if remaining cells upregulate  $\gamma$  subunits, as suggested in the present study, the total binding level in the hippocampus would show no change, even in cases affected with more extensive hippocampal cell loss. In our previous studies of hippocampal GABA receptor subunits,  $\alpha 1$  immunoreactivity and  $\beta 3$  subunit mRNA were reduced in the Braak V/VI cases, while  $\beta 2$  subunit mRNA was relatively preserved4·6. Other studies have reported reduction of  $\alpha 5$  protein22 and mRNA23, while no change in  $\beta 1$  protein22. These studies, together with our present observation of  $\gamma$  subunit changes, suggest that each subunit of the GABA<sub>A</sub> receptor undergoes unique responses during the progression of NFT pathology in AD hippocampus. The results of our experiments that employed dual labeling of tissue sections with  $\gamma 1/3$  or  $\gamma 2$  immunohistochemistry and the amyloid binding dye X-34 demonstrated that neurons immunoreactive to  $\gamma$  subunits did not contain NFT. This indicates either a protective role of neuronal GABA<sub>A</sub> $\gamma$  receptor expression, or their loss precedes neurofibrillary degeneration in AD. While we are not aware of a study demonstrating a direct cause-effect link between dysfunction of the hippocampal GABAergic system and NFT formation, it has been hypothesized that the loss of GABAergic inhibitory innervation contributes to excitotoxicity, due to excessive Ca<sup>2+</sup> influx, which can lead to abnormal phosphorylation of tau and its aggregation into NFT 24–26. This warrants future multiple-immunolabeling studies examining the relationship between  $\gamma$  receptor subunit changes and specific markers of different stages in the course of NFT formation (e.g., tau hyperphosphorylation) and neuronal cell death in AD.

Changes in receptor subunit composition during the course of AD could affect regional GABA binding, drug responses and ability to maintain inhibitory tone in the CNS. Currently, the effect of benzodiazepine therapy on psychological and behavioral symptoms in AD patients remains unclear as there have been few controlled clinical trials to date, which have yielded mixed results27. In addition, no study has focused on changes in benzodiazepine sensitivity in AD patients. However, a study by Fastbom et al. (1998) reported a significantly lower incidence of AD in subjects with a history of benzodiazepine use, suggesting a protective role of benzodiazepines in AD, likely through glutamatergic inhibition 28. AD therapies should target the disturbed inhibition-excitation balance, a major driving force for neuronal degenerative changes in AD29.

## Conclusions

The results of the present study suggest that neurons expressing GABA<sub>A</sub> $\gamma$  receptor subunits resist the progression of neurofibrillary degeneration in AD hippocampus. The potential protective effect of these receptors against NFT pathology warrants their further investigation for the benefit of developing novel therapeutic strategies that target the GABAergic system in AD.

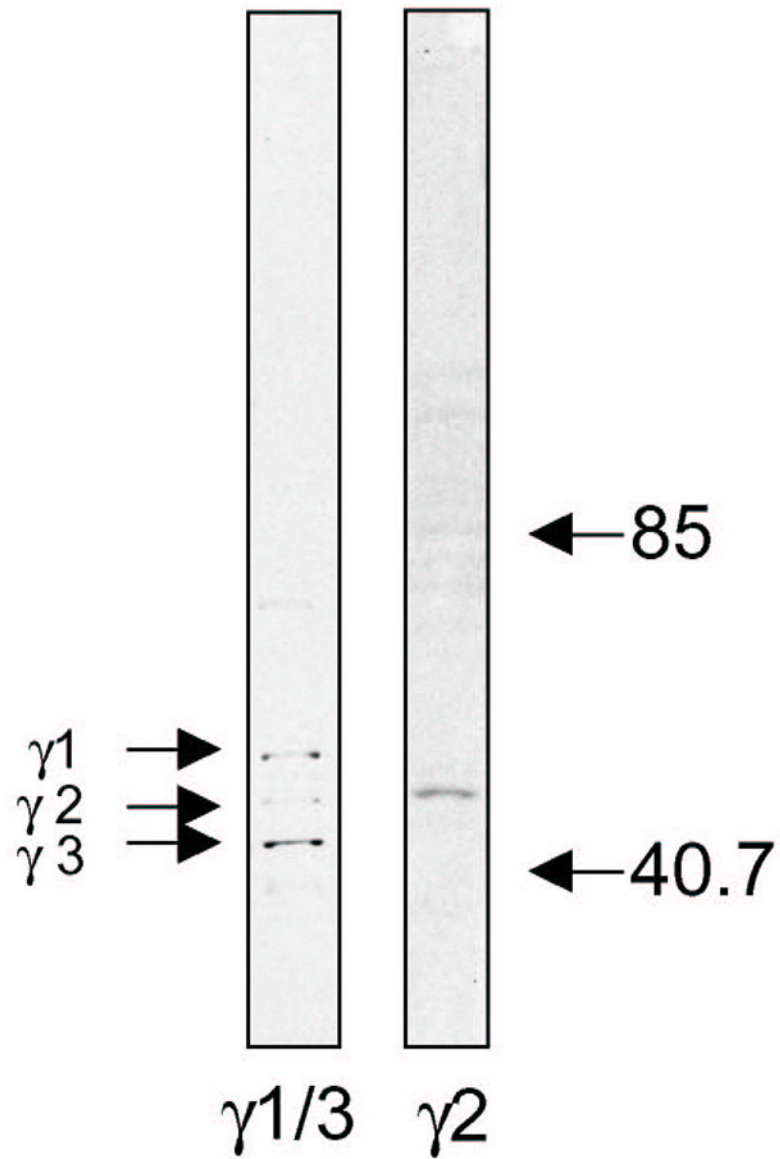
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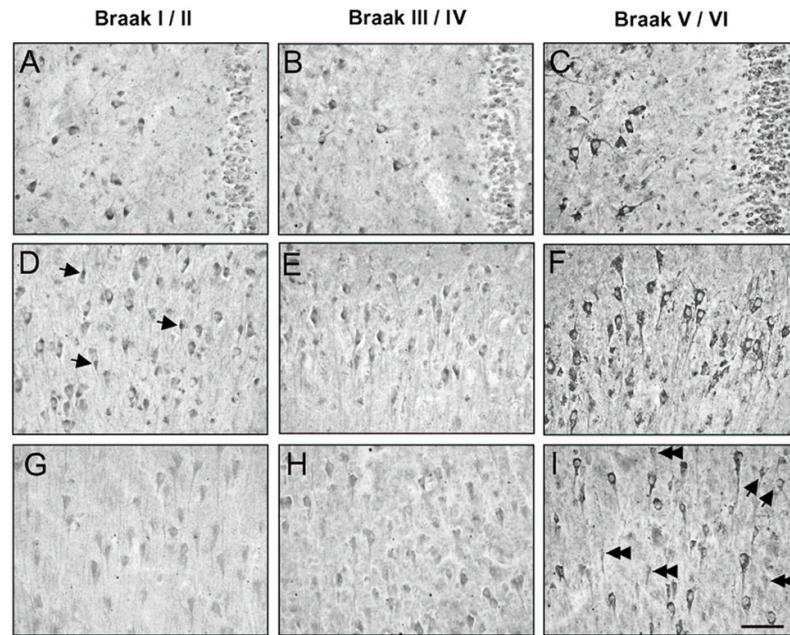
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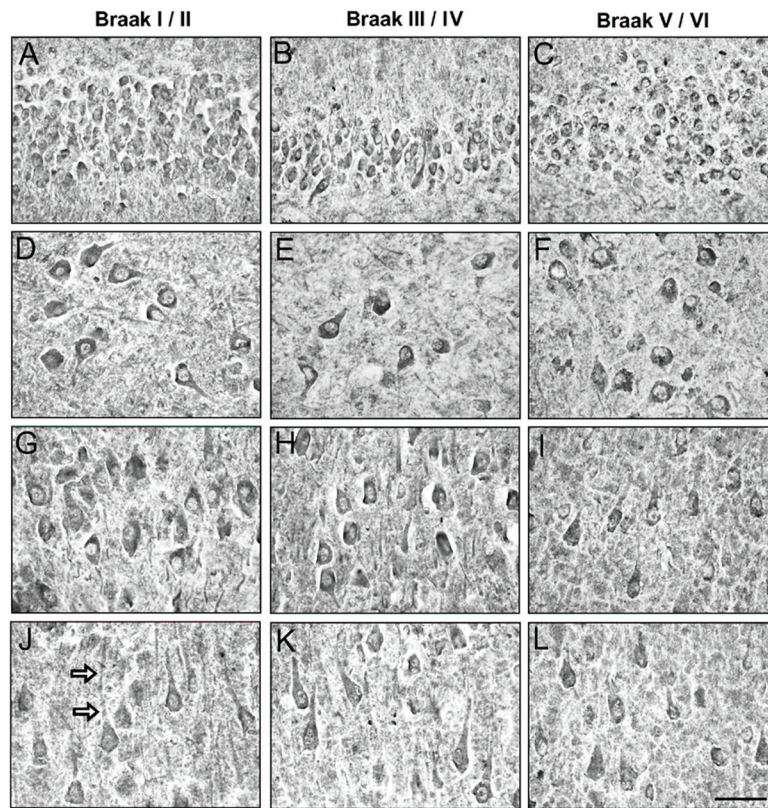


**Fig. 1.** Western blotting shows that Sc-7371 (Santa Cruz) recognizes both  $\gamma 1$  and  $\gamma 3$ , and 345231 (Calbiochem) recognizes  $\gamma 2$  subunit.

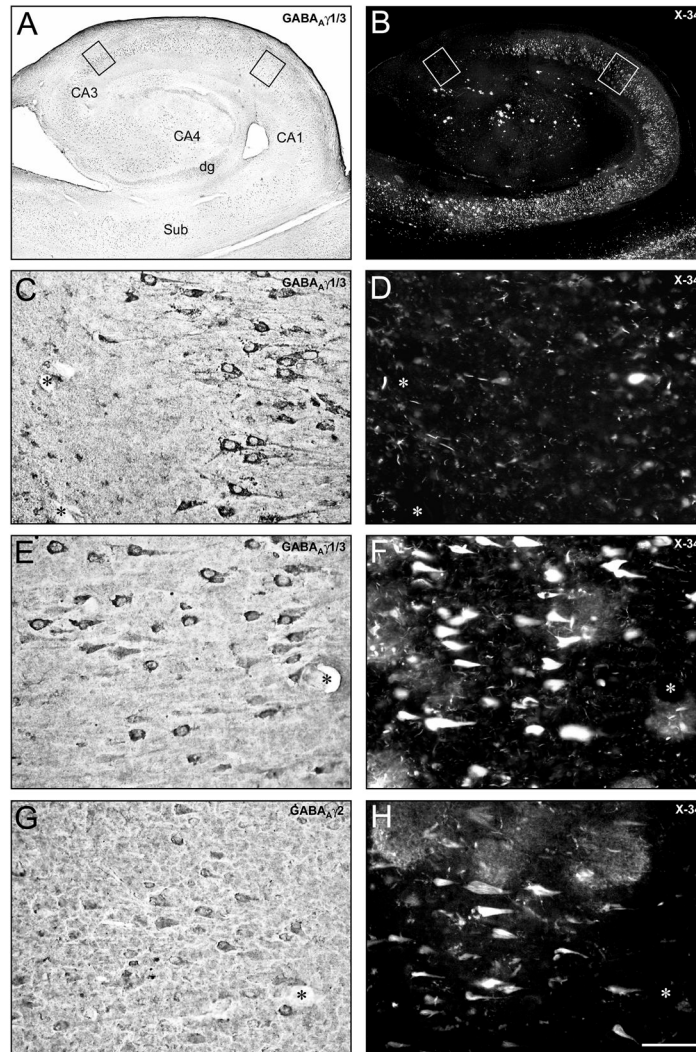


**Fig. 2.** GABA<sub>A</sub>γ1/3 immunoreactivity in CA4/DG (A-C), CA2/3 (D-F) and CA1 (G-I) hippocampus from a representative Braak stage I/II control case (A,D,G), and AD cases in Braak stages III/IV (B,E,H) and V/VI (C,F,I). Arrows in (D) point to concentrations of γ1/3 immunoreactivity in CA2/3 neurons. In the Braak V/VI AD case, increased immunostaining of neuronal cells is evident in all hippocampal regions, filling the entire cytoplasm of large pyramidal cells and smaller multipolar neurons (arrows in I). A subset of CA1 cells (double arrowheads in I) shows γ1/3 immunoreactivity that is comparable to controls. Scale bar = 100 μm.





**Fig. 3.** GABA<sub>A</sub>2 immunoreactivity in DG (A-C), CA4 (D-F), CA2/3 (G-I) and CA1 (J-L) hippocampus from a representative Braak stage I/II control case (A,D,G,J), and AD cases in Braak stages III/IV (B,E,H,K) and V/VI (C,F,I,L). Neuronal and neuropil immunostaining is preserved across severity groups in AD. Arrows in (J) point to the labeled primary dendrite of a large CA1 pyramidal neuron. Such dendritic labeling is less prominent in the Braak stage V/VI case, although the immunostaining intensity of cell bodies is comparable to that seen in the other Braak staged groups across all hippocampal regions. Scale bar = 50  $\mu$ m.



**Fig. 4.** Paired low-power composites (A-B) and high-power (C-D boxed area in CA3, and E-H boxed areas CA1) images of successive GABA<sub>A</sub>γ1/3 (A-F) and GABA<sub>A</sub>γ2 (G-H) immunoreactivity and X-34 histofluorescent amyloid staining in a single hippocampal tissue section from a Braak stage V/VI AD case. GABA<sub>A</sub>γ1/3 immunoreactive cells are present in all hippocampal regions, regardless of the degree of X-34 labeled NFT and plaque pathology, which is particularly abundant throughout the subiculum/CA1 (A-B). In the CA3 region (C-D), most neurons show prominent γ1/3 immunoreactivity, while X-34 labeling is limited to a single NFT (arrow in D) and scarce neuropil threads. In CA1 (E-F), GABA<sub>A</sub>γ1/3 immunostained neurons do not contain NFT, and are present throughout the field despite numerous plaques (arrows in F). Likewise, GABA<sub>A</sub>γ2 (G-H) immunostained neurons do not contain NFT (H). Asterisk matches blood vessels in each high-power field. Scale bar = 7.5 mm (A,B); 75 μm (C-H). CA1-3 = Cornu Ammonis fields; dg = dentate gyrus; Sub = subiculum.

Table 1

Case demographics

	NFT severity (Braak stage)			Total (N=16)	Kruskal Wallis test
	Braak I/II (N=4)	Braak III/IV (N=4)	Braak V/VI (N=8)		
Age	Mean $\pm$ SD (Range) 73.3 $\pm$ 16.5 (57–88)	79.8 $\pm$ 8.4 (72–91)	76.9 $\pm$ 16.5 (48–100)	76.7 $\pm$ 14.2 (48–100)	NS
PMI	Mean $\pm$ SD (Range) 7.38 $\pm$ 1.3 (5.5–8)	4.5 $\pm$ 1.7 (3–7)	4.6 $\pm$ 1.7 (2–8)	5.3 $\pm$ 2.0 (2–8)	NS
BW	Mean $\pm$ SD (Range) 1217 $\pm$ 196 (990–1400)	1217 $\pm$ 74 (1150–1300)	1091 $\pm$ 116 (960–1330)	1154 $\pm$ 139 (960–1330)	NS
Gender	M/F 0/4	3/1	5/3	8/8	

NFT, neurofibrillary tangle; PMI, postmortem interval, BW, brain weight; NS, not significant; P-value cutoff was 0.01