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Modular and laminar pathology of Brodmann's area 37 in Alzheimer's disease

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Abstract

Previous studies suggested a relationship between severity of symptoms and the degree of neurofibrillary tangles (NFTs) clustering in different areas of the cortex in Alzheimer's disease (Lee et al.). The posterior inferior temporal cortex or Brodmann's area (BA37) is involved in object naming and recognition memory. But the cellular architecture and connectivity and the NTF pathology of this cortex in AD received inadequate attention. In this report, we describe the laminar distribution and topography of NFT pathology of BA 37 in brains of AD patients by using thionin staining for Nissl substance, thioflavin-S staining for Neurofibrillary tangles (NFTs), and phosphorylated tau (AT8) immunohistochemistry. NFTs mostly occurred in cortical layers II, III, V and VI in the area 37 of AD brain. Moreover, NFTs appeared like a patch or in cluster pattern along the cortical layers III and V and within the columns of pyramidal cell layers. The abnormal, intensely labeled AT8 immunoreactive cells were clustered mainly in layers III and V. Based on previously published clinical correlations between cognitive abnormalities in AD and the patterns of laminar distributed NFT cluster pathology in other areas of the brain, we conclude that a similar NFT pathology that severely affected BA 37, may indicate disruption of some forms of naming and object recognition related circuits in AD.

Keywords

Neurofibrillary tangles; posterior inferior temporal cortex; Brodmann's area 37; phosphorylated tau; laminar distribution; memory impairment; object recognition

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Neurons in the cerebral cortex are organized horizontally into lamina and vertically into columns and modules. These organized neurons, with similar response properties, form fundamental anatomical and physiological units of the cortex (Fujita et al., 1992, Saleem et al., 1993, Tanigawa et al., 1998).

In Alzheimer's disease, the characteristic neuropathological lesions of neurofibrillary tangles (NFTs) exhibit a specific laminar and regional distribution in different areas of brain. (Hof and Morrison, 1990, Hof et al., 1992, Hof, 1997). The modular/columnar organized NFT pathology of the hippocampus, the entorhinal cortex and surrounding limbic structures in AD has been described in the literature. (Van Hoesen GW and Solodkin A, 1993; Van Hoesen et al., 2000). The NFTs pathology in hippocampus specifically affect the regional cells of origin of afferent and efferent neuronal fibers (Hyman et al., 1984, Van Hoesen et al., 1991). Prominent loss of pyramidal neurons in layers III, V and VI in visual and auditory association cortices also occurs in AD (Hof and Morrison, 1990, Van Hoesen and Solodkin, 1993, Joyce et al., 1998). This multi-layered NTFs pathology may be due to the affected feed forward and feedback corticocortical axons arising from layers II-VI of the cortex (Van Hoesen, 1995).

Previous studies have shown that the AD-related disorders are strongly related to the NFT formation in cortical areas of multimodal association and the NFTs in inferior temporal cortical areas signals more severe memory impairment and impending signs of dementia (Hof et al., 1990; Hof 1997). The positron emission tomography and electrophysiological measurements have designated posterior inferior temporal cortex, mapped as Brodmann's area 37 (BA 37), for object naming and visual object recognition memory (Tanaka, 1997, Harasty et al., 1999; Haxby et al., 2001 and Nakamura et al., 2000). But the NTFs neuropathology and neuronal connectivity studies of BA 37 received inadequate attention in the literature. In the present investigation, we focused on BA 37 considering that progression of NTFs neuropathology in this cortical area may have neurobehavioral consequences in AD patients

EXPERIMENTAL PROCEDURES

Human brain samples

AD brains were obtained from 10 individuals at autopsy (University of Iowa Deeded Body Program) with duration of dementia from 3–15 years (AD cases are summarized in Table 1) and age-matched control brains were obtained at routine autopsy, from patients dying without any history of neurological or psychiatric illness.

Gross anatomical and neuro pathological examination

The area of posterior inferior temporal cortex is routinely called BA 37. According to Brodmann's areal map of the human cortex, this posterior temporal cortical area is located lateral to the parahippocampal gyrus and the collateral sulcus; it occupies a position immediately adjacent to the posterior parahippocampal gyrus. The borders of this area are not clearly demarcated by functional or anatomical landmarks. The neuro pathological lesions were consistent with AD diagnostic criteria. Nissl-staining was performed to reveal an altered laminar arrangement of neurons and to analyze the neuronal loss and gliosis in BA 37 by visual inspection. Cytoarchitectural comparisons with normal control Nissl-stained series were routinely performed.

Brain tissue preparation

All rapid autopsy brains from AD patients were collected, the temporal lobe was divided in blocks and immersion-fixed in ice-cold 4% paraformaldehyde solution in 0.1 M PBS for 24–48 hr. Fixed tissue blocks containing the hippocampus and adjacent temporal cortices were cut into 50- μ m-thick frozen sections and stored frozen until processed. The thick frozen sections

were further cut into series of thin, 5 μ m, sections on freezing sliding microtome and stored in cryostorage solution until stained with thionin for cytoarchitectonic delimitation.

Thioflavin-S histochemistry

Another parallel series of sections were stained with 1-% thioflavin-S (Sigma, St. Louis, and MO) to survey AD pathology and to identify the laminar localization pattern, density of NFTs, and amyloid plaques in BA 37 of AD. The sections were pretreated in a 1:1 mixture of chloroform (CHCl_3)/absolute ethanol (EtOH) for 10 min and 95% EtOH and 70% EtOH for 10 min then quickly rinsed in water, and incubated in 0.1% Thioflavin-S for 5 min at room temperature in the dark. Finally, the sections were briefly differentiated in 80% EtOH solution and rinsed in water, and mounted with Aquamount. Sections were examined under a Leitz Diaplan fluorescent microscope using a 10x objective. The microscope was equipped with a camera and image analysis software, Neurolucida (MicroBrightField Inc., Colchester, VT), to chart laminar localization and distribution patterns of NFTs in thioflavin-S stained sections.

Immunohistochemistry

A series of free-floating sections from the posterior temporal cortex of each brain was processed for AT8-immunohistochemistry using the avidin-biotin-peroxidase complex (ABC) method as described by Lee et al., 2004. Briefly, the sections were quenched with 0.1% H_2O_2 in 0.1 M PBS containing 0.4% Triton X-100 for 20–30 min. After washing in 0.1 M PBS, the sections were blocked with 5% normal goat serum containing 0.4% Triton X-100 in 0.1 M PBS for 1 hr at room temperature followed by overnight incubation with primary antibody AT8 (1:1000; Innogenetics, Gent, Belgium) at 4°C. After washing, sections were incubated sequentially with biotinylated goat anti-mouse secondary antibody (1:500 dilution; Vector Laboratories, Burlingame, CA), an avidin-biotin peroxidase complex (ABC Elite kit; Vector Laboratories, diluted 1:200 in 0.1 M PBS containing 0.4% Triton X-100), 0.03% 3,3'-diaminobenzidine (DAB) containing 0.25% nickel ammonium sulfate, and 0.01% H_2O_2 in 0.1 M PBS for 5–10 min. The Sections were washed after each incubation steps. To detect any non-specific labeling, the negative control sections were identically incubated without the primary antibody.

Monkey surgery and Fast blue dye injection

Two monkeys were used in this study. All surgical and experimental procedures used were approved by the Institutional Animal Care and Use Committee at the University of Iowa and the surgery and histological procedures performed were as described by Morecraft and Van Hoesen, 1998). Briefly, the retrograde tracer, 3–4% of fast blue dye (4 μ l), was injected into the middle temporal gyrus in brain of monkey anesthetized with Nembutal. Following a 14 days of survival period, monkeys were anesthetized and perfused with 0.9% saline in 0.1 M cacodylate buffer and then a solution of 6% paraformaldehyde in the same buffer. The brains were dissected and post-fixed for 4–6 days at 4 C. Prior to sectioning, the brains were stored overnight in a solution of 30% sucrose in phosphate buffer solution. The 50 μ m thick sections of the brain were collected in series and mounted immediately on glass slides. These series of sections were stained for Nissl substance for cytoarchitectural analysis and for fluorescent data analysis.

RESULTS

Thioflavin-S staining and Cluster analysis

Under microscope, the NFTs were intensively labeled with thioflavin-S containing histochemical stain. In AD brains, substantial numbers of NFTs accumulated throughout the posterior temporal lobe, but a high density of NFTs was registered in the entorhinal and perirhinal cortices of the parahippocampal gyrus (Fig 1C and D) and both, intracellular and

end-stage NFTs or extracellular tangles were also observed in this area. In area 37 of AD brain, NFTs formation was prominently distributed in infra-cortical layers II and III and in supra-cortical layers V and VI. Moreover, the NFTs appeared aggregated and were distributed along the cortical layers III, V and VI arranged in clusters or in patch-like patterns. The same cortical layers were also sparsely populated with cells. The modular and cluster pattern of NTFs prominently seen in layers V and VI of area 37 in an AD brain is illustrated in Figs. 1A and 1B. The NFT cluster size was also measured (Table 2).

Neurolucida Charting

The Neurolucida charting of a cross-section through the inferior temporal cortex in an AD case depicting the topography and laminar distribution of NFTs is presented in Fig 2. The figure shows a moderate NFT distribution in BA 37 and 20, sandwiched between intensely involved medial temporal areas 35, 36 and the lesser involved lateral temporal area 21. In section from BA 37, we observed NFT as clusters or patches appearing in infra-granular layer III and supra-granular layers V and VI. In some cases, the patchy appearances of NFTs were found within the columns of pyramidal cell in layers V and VI of BA 37. The inferior temporal cortex sections from age matched non-AD control subjects were devoid of NFT (Fig 4C).

AT8 Immunohistochemistry

The monoclonal antibody AT8, which recognizes a phosphorylation-dependent tau epitope, gave a strong staining to degenerating clusters of NFTs, neuritis and neuritic plaques. These lesions are also stained intensely by thioflavin S. Numerous NFTs were aggregated in and around the hippocampus (entorhinal cortex, BA 35 and 36, and hippocampal field CA1). However, this accumulation was largely confined to the layers III and V and amyloid plaques were seen in layers I-V. The AT8 immunoreactive lesions in area 37 of AD were aggregated in a cluster and were numerous mainly in layers III, V and VI of this area. The immunoreactivity was less in cortical layer VI of BA 37. In addition, AT8 immunoreactive positive neurons were visible in association with the laminar pattern of NFTs stained with thioflavin-S based histochemistry (Fig. 3). Neurons with neurofibrillary processes and degeneration were detected in layers II-VI of BA 37. Fig. 4(A and B) show the laminar distribution of the NFT clusters in BA37. Neurons with NFTs were predominantly expressed in the two-tiered (bilaminar) pattern that appeared as AT8 immunoreactivity in layers II, III, V, and VI in entire regions of BA 37.

We observed a considerable difference in the laminar distributed pattern of phosphorylated tau protein in different cortical areas of the posterior temporal lobe in AD. The areas in and around the hippocampal formation and parahippocampal gyrus (BA 35 and 36) showed the strongest immunoreactivity to AT 8. The BA 37 was also labeled severely. The distribution of intense AT8 staining transitioned gradually from BA 37 to the weakly stained BA 17 and 18 and between adjoining cytoarchitectonically distinct cortical regions, as is shown in Fig. 3. The staining intensity diminished in BA 21, 22, 41 and 42. Fig. 4 (A and B) reveals the laminar location of the NFT clusters in BA37 of an AD brain.

Retrograde tracer fast blue injection into the inferior temporal cortex of monkey

The fast blue (FB) dye injected close to the middle temporal gyrus revealed many columnar clusters of cells that were distributed within the area as well as local projection neurons of the inferior temporal cortex of the monkey. The diffusely distributed FB stained cells were also noted in some areas. Fig. 5 panel B illustrates the fast blue stained pattern of local or within the area projection of neurons to show a laminar distribution and connectivity in this area. The cluster appearance of FB labeled cell was observed mostly in layers III, V and VI; only a few cells were labeled in layer II in the region close to the injection area. The mean diameter of the FB labeled cell clusters in layer III were 160.54 μm and 143.82 μm in layers V and V (Table 3).

Discussion

The columnar or clustered organization of neurons is known to be widespread, and may be universal in primary sensory areas. In AD patients, the greatest NFT inclusions occur in the temporal lobe (Price et al., 1991). These pathological observations resonate well with the memory-related cognitive changes in AD (Hyman et al., 1984, Braak and Braak, 1985, Arnold et al., 1991, Braak and Braak, 1991). Others have shown that the NFTs density in the inferior temporal cortex (Brodmann area 20) relates to the onset of AD and the development of dementia (Hof et al., 1992, Bouras et al., 1994). A modular pattern of NFT pathology was also described in the temporal cortex of AD by Van Hoesen and Solodkin, 1993; Solodkin and Van Hoesen, 1996; Delacourte et al., 1999. Specifically, NFTs were observed as clusters or patches in layers III and V in the temporal lobe areas of brain. Our results also show the modular and laminar distribution pattern of NFTs in BA 37 in AD. These changes were prominent in layers III, V and VI of the BA 37 in AD. We observed a moderate NFT distribution in BA 37 and 20, sandwiched between intensely involved medial temporal areas 35, 36 and the lesser involved lateral temporal area 21.

In normal aging, NFT changes have been described in specific areas of the limbic system and neocortex that undergo selective neuronal degeneration. Also, the NFTs in AD are densely distributed in areas surrounding the entorhinal and hippocampal cortical neurons, whereas other neocortical areas are consistently less affected in normal aging (Price et al., 1991, Price and Morris, 1999). Similarly, the NFTs are not distributed homogeneously in all areas of AD brains. In this study we used the samples only from individuals suffering from severe AD with many years of dementia. While it is not certain that NFTs avoided BA 37 in normally aged brains, our results show a considerable involvement of the NFT pathology in BA 37 of patients with severe AD since, the age matched normal control showed no such abnormality (Fig 4C).

The clinical and experimental studies in humans and non-human primates have recognized the BA 37 as a vital structure for object recognition memory and a form of naming function. In the present study, we have described the cluster or patchy appearance of NFTs and AT8 positive neurons in BA 37 of AD. Also, we compared the localization of corticocortically projecting cells as demonstrated in our retrograde transport studies in monkey cortex and their laminar distribution pattern in human cortex are very similar to that of NFT distribution in BA 37 of AD patients. In addition, our findings suggest that cluster or patchy appearance of NFTs in infra-granular layers and supra-granular layers of BA 37 may possibly cause disruption in the feed forward and feedback and/or efferent cortical pathways resulting in disruption of language and memory impairments related to some forms of naming and object recognition in AD.

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Abbreviations

AD	Alzheimer's disease
NFT	neurofibrillary tangles
BA 37	Brodmann's area 37

AT8	abnormally phosphorylated tau
PET	positron emission tomography
FB	fast blue
ABC	avidin biotin-peroxidase complex
PBS	phosphate buffer saline
DAB	diaminobenzidine

References

- Arnold SE, Hyman BT, Flory J, Damasio AR, Van Hoesen GW. The topographical and neuroanatomical distribution of neurofibrillary tangles and neuritic plaques in the cerebral cortex of patients with Alzheimer's disease. *Cereb Cortex* 1991;1, 103–116. [PubMed: 1822724]
- Bouras C, Hof PR, Giannakopoulos P, Michel JP, Morrison JH. Regional distribution of neurofibrillary tangles and senile plaques in the cerebral cortex of elderly patients: a quantitative evaluation of a one-year autopsy population from a geriatric hospital. *Cereb Cortex* 1994;4, 138–150.
- Braak H, Braak E. On areas of transition between entorhinal allocortex and temporal isocortex in the human brain. Normal morphology and lamina-specific pathology in Alzheimer's disease. *Acta neuropathologica* 1985;68, 325–332. [PubMed: 2986405]
- Braak H, Braak E. Neuropathological staging of Alzheimer-related changes. *Acta neuropathologica* 1991;82, 239–259.
- Fujita I, Tanaka K, Ito M, Cheng K. Columns for visual features of objects in monkey inferotemporal cortex. *Nature* 1992;360, 343–346. [PubMed: 1594039]
- Hof PR. Morphology and neurochemical characteristics of the vulnerable neurons in brain aging and Alzheimer's disease. *Eur Neurol* 1997;37, 71–81. [PubMed: 9387802]
- Hof PR, Bierer LM, Perl DP, Delacourte A, Buee L, Bouras C, Morrison JH. Evidence for early vulnerability of the medial and inferior aspects of the temporal lobe in an 82-year-old patient with preclinical signs of dementia. Regional and laminar distribution of neurofibrillary tangles and senile plaques. *Arch Neurol* 1992;49, 946–953.
- Hof PR, Morrison JH. Quantitative analysis of a vulnerable subset of pyramidal neurons in Alzheimer's disease: II. Primary and secondary visual cortex. *The Journal of comparative neurology* 1990;301, 55–64. [PubMed: 1702452]
- Hyman BT, Van Hoesen GW, Damasio AR, Barnes CL. Alzheimer's disease: cell-specific pathology isolates the hippocampal formation. *Science* 1984;225, 1168–1170.
- Joyce JN, Myers AJ, Gurevich E. Dopamine D2 receptor bands in normal human temporal cortex are absent in Alzheimer's disease. *Brain Res* 1998;784, 7–17.
- Lee G, Thangavel R, Sharma VM, Litersky JM, Bhaskar K, Fang SM, Do LH, Andreadis A, Van Hoesen G, Ksiezak-Reding H. Phosphorylation of tau by fyn: implications for Alzheimer's disease. *J Neurosci* 2004;24, 2304–2312. [PubMed: 14715934]
- Price JL, Davis PB, Morris JC, White DL. The distribution of tangles, plaques and related immunohistochemical markers in healthy aging and Alzheimer's disease. *Neurobiology of aging* 1991;12, 295–312.
- Price JL, Morris JC. Tangles and plaques in nondemented aging and “preclinical” Alzheimer's disease. *Annals of neurology* 1999;45, 358–368. [PubMed: 10401779]

- Saleem KS, Tanaka K, Rockland KS. Specific and columnar projection from area TEO to TE in the macaque inferotemporal cortex. *Cereb Cortex* 1993;3, 454–464.
- Tanigawa H, Fujita I, Kato M, Ojima H. Distribution, morphology, and gamma-aminobutyric acid immunoreactivity of horizontally projecting neurons in the macaque inferior temporal cortex. *The Journal of comparative neurology* 1998;401, 129–143.
- Van Hoesen GW. Anatomy of the medial temporal lobe. *Magn Reson Imaging* 1995;13, 1047–1055.
- Van Hoesen GW, Hyman BT, Damasio AR. Entorhinal cortex pathology in Alzheimer's disease. *Hippocampus* 1991;1, 1–8. [PubMed: 1669339]
- Van Hoesen GW, Solodkin A. Some modular features of temporal cortex in humans as revealed by pathological changes in Alzheimer's disease. *Cereb Cortex* 1993;3, 465–475.

Fig. 1.

Panel A. The low magnification photomicrograph of thioflavin-S-stained section of the inferior temporal cortex of an AD case showing the clusters of NFTs in layers III, V and VI. Note, the dense location of pathology in layers III, V and VI and the lesser involvement of layers II and IV. Also, note the modular and cluster patterns in layer V and VI. **Panel B.** Showing the high magnification of NFT clusters in layer V and VI of the inferior temporal cortex stained with thioflavin S. The arrow heads indicate the NFT clusters in both panel A and B. The AD case exhibits high NFT densities in layers V – VI of area 37. **Panel C.** Showing Thioflavin S histochemical staining of the entorhinal cortex of an AD brain. **Panel D** is higher magnification image from **C**. Neurofibrillary tangles are present in a laminar distribution in the entorhinal cortex involving the characteristic clusters or patches of neurons in layer II and the pyramidal neurons of layer IV in severe AD brain. Layers are indicated by Roman numerals.

Fig. 2.

Neurolucida map of coronal section through the posterior temporal lobe in an AD case depicting the topography and laminar distribution of NFTs. Note, that NFT distribution in area 37 and its neighbor, area 20 is moderate, sandwiched between the intense involvement of medial temporal areas (35 and 36) and lesser involvement of more lateral temporal cortex (area 21). The bilaminar pattern in layers III and V-VI and the intense quantities in layer V. Abbreviations: LV=lateral ventricle, CA1, CA3= Hippocampal subfields, S= Subiculum, PrS= Presubiculum, PaS= Parasubiculum, CS= Collateral Sulcus, LOS= Lateral occipitotemporal sulcus, ITS= Inferior temporal sulcus, STS= Superior temporal sulcus, 20, 21, 35, 36, 41, 42= Brodmann's areas.

Fig. 3.

Coronal section at the level of later geniculate nucleus from AD case showing anterior area 37. AT8 immunostaining showing the laminar distribution. Note the bilaminar patterns in layers III and V–VI of area 37 and intense distribution of AT8 positive staining in layer V. The neural and neuropil immunoreactivity of layers III, V and VI in the temporal visual cortices and its absence in layer IV. Abbreviations: LV=lateral ventricle, CA1, CA3= Hippocampal subfields, S= Subiculum, PrS= Presubiculum, PaS= Parasubiculum, CS= Collateral Sulcus, LOS= Lateral occipitotemporal sulcus, ITS= Inferior temporal sulcus, STS= Superior temporal sulcus, 20, 21, 35, 36, 41, 42= Brodmann's areas. Cortical layers are showed by Roman numerals.

Fig. 4.

Immunostaining for hyperphosphorylated tau (AT8) in area 37 of AD brain (A and B). Low (A) and (B) magnification views of AT8 immunostaining demonstrate tau cluster pathology in layers III, V and VI (arrows) of the area 37 of an AD case. B is higher magnification of A. Note the absence of tau pathology in the inferior temporal cortex of control brain (C). Layers are showed by Roman numerals. Scale bars, 250 μ m (A and C) and 100 μ m (B)

Fig. 5.

Panel A. Photograph showing a Fast Blue injection site. **Panel B.** showing the retrograde labeled neurons with Fast Blue in the inferior temporal cortex in monkey. The distribution of retrograde labeled neurons appeared as discontinuous clusters located in layers III, V and VI in the region adjacent to the injection site. Note the dense distribution of fast blue labeling neurons in layers V and VI of inferior temporal cortex. Layers are showed by Roman numerals.

Table 1

AD Case Demographics

Case	Age (years)	Sex	Duration of illness (years)	Brain Weight (gm)
A559	79	F	11	956
A569	89	M	10+	1630
A584	79	F	11	860
A596	82	M	12	1180
A599	85	M	5	1050
A630	83	M	5	986
A631	61	M	6+	1265
A660	82	M	4	1224
A666	78	M	12	1060
A669	88	F	3	950

Table 2
NFT cluster size in cortical layers III, V and VI of AD case

Case	Layers	Mean diameter (μm)
A561	III	99.0906
	V & VI	133.3148
A599	III	195.5402
	V & VI	183.044

Table 3

Fast blue labeled clusters or patches in cortical layers in the inferior temporal cortex of monkey

Layers	Mean diameter (μm)
III	160.5442
V & VI	143.8153