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Loss of non-phosphorylated neurofilament immunoreactivity in temporal cortical areas in Alzheimer's disease

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Abstract

The distribution of immunoreactive neurons with non-phosphorylated neurofilament protein (SMI32) was studied in temporal cortical areas in normal subjects and in patients with Alzheimer's disease (AD). SMI32 immunopositive neurons were localized mainly in cortical layers II, III, V and VI, and were medium to large sized pyramidal neurons. Patients with AD had prominent degeneration of SMI32 positive neurons in layers III and V of Brodmann areas 38, 36, 35, 20; in layers II and IV of the entorhinal cortex (Brodmann area 28); and hippocampal neurons. Neurofibrillary tangles (NFTs) were stained with thioflavin-S and with an antibody (AT8) against hyperphosphorylated tau. The NFT distribution was compared to that of the neuronal cytoskeletal marker SMI32 in these temporal cortical regions. The results showed that the loss of SMI32 immunoreactivity in temporal cortical regions of AD brain is paralleled by an increase in NFTs and AT8 immunoreactivity in neurons. The SMI32 immunoreactivity was drastically reduced in the cortical layers where tangle-bearing neurons are localized. A strong SMI32 immunoreactivity was observed in numerous neurons containing NFTs by double-immunolabelling with SMI32 and AT8. However, few neurons were labeled by AT8 and SMI32. These results suggest that the development of NFTs in some neurons results from some alteration in SMI32 expression, but does not account for all, particularly, early NFT related changes. Also, there is a clear correlation of NFTs with selective population of pyramidal neurons in the temporal cortical areas and these pyramidal cells are specifically prone to formation of paired helical filaments. Furthermore, these pyramidal neurons might represent a significant portion of the neurons of origin of long corticocortical connection, and consequently contribute to the destruction of memory-related input to the hippocampal formation.

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Keywords

Alzheimer's disease; non-phosphorylated neurofilament protein; neurofibrillary tangles; entorhinal cortex; perirhinal cortex; hyperphosphorylated tau protein

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease affecting the cognitive memory function due to specific neuropathological changes, including the shrinkage and loss of neurons in the cerebral cortex. The two hallmark lesions of this disease, neurofibrillary tangles (NFTs) and senile plaques were described first by Alzheimer in 1907. The lesions are usually found in regions of the hippocampal CA1, entorhinal cortex, perirhinal cortex and other limbic structures. Neurofibrillary tangles contain the abnormally hyperphosphorylated forms of tau protein that invade and destroy cortical neurons in AD. A large number of studies have revealed that NFTs correlated well with the clinical expression of dementia in AD (Arriagada et al., 1992, Bierer et al., 1995, Gomez-Isla et al., 1997, Mitchell et al., 2002, Guillozet et al., 2003). However, NFTs do not affect AD brain uniformly. NFTs occur earlier and with much higher density in the superficial layers of perirhinal and entorhinal cortex than in most areas of the neocortex (Arnold et al., 1991a). Earlier studies also have shown the selective regional variability's of NFT density within the hippocampal CA1, where the NFTs are in a larger number (Bobinski et al., 1997, Fukutani et al., 2000). NFTs target selective populations of neurons, and particularly, specific layers of the cortex. Numerous studies have demonstrated a drastic loss of SMI32 immunoreactive pyramidal cells in the frontal, inferior temporal and visual cortices in AD (Hof et al., 1990, Hof and Morrison, 1990, Bussiere et al., 2003a, Bussiere et al., 2003b, Giannakopoulos et al., 2003, Ayala-Grosso et al., 2006). They also suggest neurofilament protein associated changes in neuronal cytoskeleton lead to NFT pathology in AD (Morrison et al., 1987, Hof et al., 1990). In addition, the loss of pyramidal neurons, containing non-phosphorylated neurofilament protein, is associated with the brain atrophic changes in AD (Hof et al., 1990, Morrison and Hof, 2002) and has been correlated with memory and cognitive impairment in the disease progression.

In the present study, we performed immunohistochemical methods using antibodies that recognize both non-phosphorylated neurofilaments (SMI32) and abnormally phosphorylated tau protein (AT8) to identify SMI32 containing pyramidal neurons as the vulnerable cell-population in the temporal lobe of AD. In addition, we also determined if AT8 positive NFTs were present in the vulnerable SMI32 containing neurons in AD.

EXPERIMENTAL PROCEDURES

In this study, we examined the temporal cortical areas according to Brodmann's cytoarchitectural nomenclature. In addition, Brodmann's area 36 and 20 were also included. AD brain tissue sections were processed following the procedures described by (Thangavel et al., 2008a). Briefly, free-floating sections of the temporal lobe of AD were stained immunohistochemically using AT8 and SMI32 monoclonal antibodies. No immunostaining was observed in control sections where the AT8 antibody was omitted.

Brain tissue samples

AD brains were obtained from 6 individuals at autopsy (University of Iowa Deeded Body Program, Iowa City, IA, USA) with duration of dementia from 5 to 12 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.

Double Immunohistochemistry

As described previously (Thangavel et al., 2008a), the brain sections were sequentially immunolabeled with the primary antibodies to AT8 and SMI32 then visualized by the avidin-biotin peroxidase complex (ABC) method using chromogens, diaminobenzidine (DAB) and nickel-enhanced diaminobenzidine (DAB/Ni). In brief, the tissue sections were first immunostained with AT8 antibody using the DAB/Ni to develop black-gray color reaction products. In the second cycle, the sections were processed following ABC-method for SMI32 immunoreactivity and the brown color reaction product was developed by DAB without nickel ammonium sulfate.

Thioflavin-S staining

The brain sections were stained with 1-% thioflavin-S (Thangavel et al., 2008a, Thangavel et al., 2008b) to study AD pathology and to identify the laminar distribution pattern, density of NFTs, and APs in the temporal cortical areas of AD. The sections were pretreated in a 1:1 mixture of chloroform (CHCl₃)/absolute ethanol (EtOH) for 10 min, immersed in 95% EtOH and 70% EtOH for 5 min each, rinsed quickly in water, then 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 Aqua mount. Also, some SMI32 immunostained sections were incubated with 1% thioflavin-S for double staining. Both light and fluorescent microscopic observations were performed simultaneously to examine the co-localization of SMI32 immunoreactive neurons and NFTs.

Fluorescence Immunohistochemistry

Sequential double-immunofluorescence labeling was performed essentially as described by (Su et al., 1996). Briefly, the temporal lobe sections were rinsed (3×10 minutes) with phosphate buffer saline (PBS, pH 7.4). To quench endogenous peroxidase activity sections were treated with 0.3% hydrogen peroxide in PBS for 20 minutes. Sections were incubated with 10 % normal goat serum for 1 hour at room temperature to block non-specific staining followed by overnight incubation at 4° C with SMI32 antibody (1:5000 dilutions). Sections were rinsed again with PBS-Triton-X 100 and incubated with FITC-conjugated IgG for 1 hour at room temperature. Sections were rinsed with PBS (3× 10 minutes) and the second blocking step at room temperature in the dark was carried out. After this step, sections were again incubated overnight with AT8 antibody (1:1000 dilutions) at 4° C. Finally the sections were washed with PBS and incubated with CY3 labeled IgG (red fluorescence, Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature in the dark.

Results

SMI32 immunoreactive pyramidal neurons are comprised of long corticocortical projections whose functional impairment is thought to contribute to dementia in AD. Our results show a significant reduction of SMI32 immunoreactive neurons in AD brains. SMI32 immunoreactivity showed in the somata and dendrites of pyramidal neurons and SMI32 neurons were severely degenerated in entorhinal layer II islands and perirhinal column of the AD group in comparison to the normal cases (Fig. 1). Loss of SMI32 immunoreactivity was observed in the AD brain, mainly where AT8 positive NFTs were distributed. NFTs were not uniformly distributed in AD. In early AD cases, some of the SMI32 immunoreactive neurons were not completely altered in entorhinal layer II islands and perirhinal cortex (Fig. 2).

Expression of AT8 and SMI32

In tissue sections double-labeled with AT8 and SMI32 antibodies, AT8-immunopositive neurons, dystrophic neurites and plaques were found in the hippocampus, areas 28, 38, 35, 36

and 20. We found numerous AT8-immunoreactive NFTs and high intensity AT8 staining was associated with cytoskeletal changes.

Immunoreactivity for SMI32 was observed in pyramidal neurons of hippocampus and other temporal cortical regions. A higher number of AT8/SMI32 double-labeled neurons were seen in layers III and V of temporal cortex. We found a significant inverse association between AT8 and SMI32 staining in most of the AD cases since, there were high populations of AT8-stained cell bodies and neuropil threads in the presence of low numbers of SMI32 positive neurons. By contrast, AT8 immunoreactivity was normal in SMI32 labeling regions not affected by the neuropathological process of AD.

NFT formation and SMI32 expression were most intense in selective layers and specific areas in the temporal lobe of AD brain. The AT8 and SMI32 immunostaining overlaps were in the entorhinal, perirhinal cortical areas (Fig. 3) and in the hippocampus (Fig. 4). SMI32 aberrantly immunostained the pyramidal cells in the hippocampus. Some pyramidal neurons appeared relatively normal in morphology. Even within the affected areas, there were clear differences between adjacent sub-populations of neurons. AT8 immunoreactivity was seen in association with SMI32 positive cells. SMI32 is distributed in many NFTs. However, SMI32 immunoreactivity was not seen in all NFT bearing neurons. SMI32 expression levels are altered in AD.

Thioflavin-S counterstaining

We investigated the relevance of NFT distribution to the SMI32 immunoreactive neurons in the temporal cortical regions by using the brain sections double stained with SMI32 antibody and thioflavin-S histochemistry. Some SMI32 stained pyramidal neurons contained thioflavin-S stained NFTs (Fig. 5). The entorhinal, perirhinal regions and hippocampus showed more dense distribution of NFTs and the loss of SMI32 immunoreactive pyramidal neurons. In our severe AD case, the SMI32 containing pyramidal neurons were dramatically reduced in the entorhinal and perirhinal regions.

Double immunofluorescence staining

The antibodies SMI32 and AT8 were used to visualize the co-localization of the non-phosphorylated neurofilament protein and tau in the temporal cortex of AD using double immunofluorescence technique. Results show that the SMI32 staining was stronger in pyramidal neurons (Fig. 6). NFTs were mainly occurred in the SMI32 positive pyramidal neurons. In addition, the high expression of SMI32 neurons was more vulnerable in AD. NFTs labeled with AT8 antibody were present in the cell body of SMI32 labeled pyramidal neurons and neuritic processes. Some neurons with AT8 positive staining also showed weak staining with SMI32.

Discussion

We compared early and advanced cases of AD brains with the age matched non-AD control brains. Our results clearly showed a loss of large pyramidal neurons in AD brains. We noted that SMI32 immunoreactive neurons decreased moderately in some areas of the hippocampus and severely in the entorhinal/perirhinal region of the AD group. These results are consistent with the reported loss of SMI32 immunoreactive neurons in the temporal neocortex of AD brain (Hof et al., 1990, Hof and Morrison, 1995, Ayala-Grosso et al., 2006), extensive loss of neurons in the layer II of entorhinal cortex in early and severe AD (Gomez-Isla et al., 1996), and the formation of clustering pattern of NFTs in SMI32 immunoreactive pyramidal neurons that comprise the corticocortical connections in the neocortex (Radenahmad et al., 2003).

Several investigators have described the hierarchical pattern of NFT pathology in some brain areas and different cortical layers exhibiting pathological changes prior to others in AD (Arnold et al., 1991b, Bouras et al., 1993, Thangavel et al., 2008b). In early AD, NFTs are found first in layer II of the entorhinal cortex, perirhinal cortex, the CA1 area of the hippocampus, and the inferior temporal cortex.

Abnormally phosphorylated tau (AT8-immunoreactive), one of the major components of the NFTs in AD, recognizes the earliest stage of hyperphosphorylation in NFTs. Earlier studies have shown AT8 immunostained NFT formation in the entorhinal layers II, III, IV, perirhinal layers II, III, V, hippocampal CA1, and temporal neocortical layers III and V in AD (Augustinack et al., 2002). We also observed the preferential AT8 immunostained NFTs in specific layers and areas of the AD brain. In the early AD group, we observed AT8 immunoreactive NFT formations in layer III and severe alterations of SMI32 immunoreactivity in the perirhinal cortex and the inferior temporal cortex. (Bouras et al., 1993), made a similar observation. Such AT8 immunoreactive NFTs formation in selected layers and loss of SMI32 immunoreactive neurons in these temporal cortical regions were reported to reflect the major disruption of hippocampal circuits and cortico-cortical connections in AD (Bussiere et al., 2003a). The loss of large pyramidal SMI32 immunoreactive neurons is common to AD and other neurological and neurodegenerative disorders. Supporting this notion, a number of studies have shown the selective loss of large pyramidal neurons in deep cortical layers III, V and VI in Huntington and in psychiatric diseases (Sieradzan and Mann, 2001, Macdonald and Halliday, 2002, Law and Harrison, 2003). Similarly, loss of SMI32 positive large pyramidal neurons in the cerebral neocortex and cortico-cortical projection system in AD (Lewis et al., 1987, Hof et al., 1990); a marked reduction in SMI32-containing pyramidal neurons in the temporal neocortex and cortico-cortical projections during the progression of Lowy Body Variant disease (Wakabayashi et al., 1995). Additionally, pyramidal neurons enriched in SMI32 immunopositive neurofilaments connecting the superior temporal and prefrontal cortices of the association neocortex are selectively and severely affected in dementia (Hof et al., 1990, Duong and Gallagher, 1994, Bussiere et al., 2003a, Bussiere et al., 2003b). Therefore, the susceptibility of SMI32 positive pyramidal neurons in AD is an expected reality (Hof et al., 1990, Duong and Gallagher, 1994, Vickers et al., 1994, Morrison and Hof, 2002). In contrast, some of the studies have shown that SMI32-immunoreactive pyramidal neurons were not completely degenerated and a number of neurons were not altered in AD (Shepherd et al., 2001, Radenahmad et al., 2003). Such non-homogenous results are very likely since, SMI32 immunoreactivity is expressed in subpopulations of pyramidal neurons in non-human primates and human brain (Campbell and Morrison, 1989, Hof and Morrison, 1995, Hof et al., 1996a, Hof et al., 1996b, Elston and Rosa, 1997). In this study, we found that degenerating neurons were immunoreactive for AT8 and SMI32 while some neurons were positive for SMI32 only and others stained only for AT8.

In brief, our results show that non-phosphorylated (SMI32 positive) neurofilaments in temporal cortical areas were susceptible to early degeneration in AD and the vulnerability of these SMI32 positive subpopulations of pyramidal cells in AD was associated with co-expression of abnormally phosphorylated (AT8 positive) tau protein.

Conclusion

The regional and laminar NFT distribution in the cortex of AD reveals that NFTs target neurons that give rise to corticocortical association axons and pathology is coupled closely with dementia. Our results showed a loss of long projecting SMI32 immunopositive pyramidal neurons in the temporal cortical regions with associated morphological changes. The temporal cortical neuronal loss suggests early involvement of corticocortical association pathways in AD.

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Abbreviations

AD	Alzheimer's disease
SMI32	non-phosphorylated neurofilament protein
AT8	abnormally hyperphosphorylated tau protein
NFTs	neurofibrillary tangles
ABC	avidin-biotin peroxidase complex
DAB/Ni	nickel enhanced diaminobenzidine
EtOH	ethanol
PBS	phosphate buffer saline
IgG	immunoglobulin

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Fig. 1. Images showing SMI32 immunoreactivity in the entorhinal cortex of age-matched control (A), AD (B) brain, and (C) higher magnification area from (B). Note, the darkly stained layer II island cells, somata and dendrites of pyramidal neurons in layers III, V and VI of the entorhinal cortex (A). Also, note the loss of SMI32 immunoreactive pyramidal neurons in entorhinal cortex of AD brain (B, C). Abbreviation: EC, entorhinal cortex. Cortical layers are indicated by Roman numerals.

Fig. 2.

Digital images showing SMI32 immunoreactivity in the entorhinal and perirhinal cortical regions of an early AD brain (A) and severe AD case (B). Note the bi-laminar pattern of SMI-32 immunoreactivity in the early AD case. Also, loss of SMI32-immunoreactive neurons in the entorhinal islands of this severe AD case (B). In A, note the patchy appearance of SMI32 immunostaining in the layer II of entorhinal cortex and columnar pattern of SMI32 pyramidal neurons in layer III of perirhinal cortex. The border between the entorhinal cortex and the perirhinal cortex is marked by a solid line in A. In B, arrows marked both banks of the collateral sulcus to show the perirhinal cortex. The obvious loss of SMI32 immunoreactive pyramidal neurons in these areas is shown in this image. Abbreviations: PRC, perirhinal cortex; HC, hippocampus; EC, entorhinal cortex; ITC, inferior temporal cortex. Roman numerals identify the different cortical layers.

Fig. 3.

Image showing double immunostaining of AT8 (black) and SMI32 (brown) immunoreactive cells in the perirhinal cortex of an early AD brain (A) and higher magnification area (B). SMI32 immunoreactive neurons (brown) and AT8 immunoreactive NFTs (black) are distributed like a columnar pattern in the perirhinal cortex from an AD case. SMI32 immunoreactive pyramidal neurons are co-localized with AT8 antibody indicated by arrowhead. Some neurons stained only with AT8 antibody highlighted by arrowhead. Somata of the pyramidal neurons and the dendritic processes are stained darkly with SMI32 antibody (brown) indicated by arrow. Abbreviation: PRC, perirhinal cortex. Cortical layers are highlighted by Roman numerals.

Fig. 4. Double immunostaining of SMI32 immunoreactivity (grey/black; small arrows) and AT8 stained NFTs (brown; large arrows) in the hippocampus of a severe AD case. Low (A) and high (B) magnification images. Note, weak or less numbers of SMI32 positive cells are located in region showing more NFTs and many SMI32 containing immunoreactive neurons are in the area showing a low density of NFTs. Arrowhead shows the presence of NFTs in some of the SMI32 immunoreactive neurons.

Fig. 5. Double labeling for thioflavin-S and SMI32 immunostaining in the entorhinal cortex of AD brain. Note the presence of thioflavin-S stained NFTs in the layer II island of the entorhinal cortex (small arrow). Some of the NFTs are labeled with SMI32 antibody. Arrowheads highlight thioflavin-S stained NFT contained SMI32 positive processes. Abbreviation: EC, entorhinal cortex.

Fig. 6.

Double labeled fluorescence images of the temporal cortex pyramidal neurons in an AD case are shown in A, SMI32/FITC green; B, AT8/CY3 red; and C (merged). Note that some of the SMI32 labeled cell bodies are immunoreactive to AT8 positive neurons, also labeled with SMI32 (thin arrow), are yellow in merged image (C), while some of the neuronal processes lacked AT8 immunoreactivity. Some neurites, positive only for AT8, are pointed by arrowhead (C). Asterisk shows only for SMI32.

Table 1

Summary of cases

Case	Age (Yrs)	Sex	Duration of illness (Yrs)
1	85	F	5
2	73	F	10+
3	87	F	10
4	83	M	12+
5	87	M	10+
6	84	M	8
7	92	F	Non-AD
8	87	F	Non-AD
9	89	M	Non-AD
10	85	M	Non-AD