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Inducible Nitric Oxide Synthase Immunoreactivity in the Alzheimer Disease Hippocampus: Association with Hirano Bodies, Neurofibrillary Tangles, and Senile Plaques

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Abstract. Inducible nitric oxide synthase (iNOS) is involved in the generation of nitric oxide, a molecule with multiple biological activities. Although iNOS expression may be part of antimicrobial armamentarium, inappropriate expression of iNOS can potentially lead to damage to the host. In this report, we determined the expression of iNOS by immunocytochemistry in the hippocampus of the Alzheimer brains (AD) as well as in young and old normal brains. The results showed localization of iNOS immunoreactivity to Hirano bodies of the AD hippocampus. In addition, small granular iNOS immunoreactive profiles were detected associated with senile plaques and extracellular neurofibrillary tangles. In the hippocampus of control brains, morphologically similar profiles were immunoreactive for iNOS, but in far fewer numbers than in AD hippocampus. The results suggest that iNOS is expressed in a subset of pyramidal neurons in the AD hippocampus, and that iNOS may be involved in the pathogenesis of neuronal degeneration in AD.

Key Words: Alzheimer disease; Hirano body; iNOS; Neurofibrillary tangle; Senile plaque.

INTRODUCTION

Nitric oxide synthase (NOS) is an enzyme involved in the generation of the multifunctional molecule nitric oxide (NO) from L-arginine (for review see 1–5). Among 3 molecular forms of NOS, neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutive and are normally present in the subsets of neurons and endothelial cells in the brain (6, 7). The inducible form of NOS (iNOS) is typically absent in normal tissue, but is induced under pathologic conditions (8–10). Although in animal tissues, iNOS expression occurs primarily in cells of macrophage lineage, in humans, the cell types that express iNOS and the regulation of iNOS expression appear more complex and debatable (1, 11–15).

In many experimental systems, the product of NOS, nitric oxide (NO), has been shown to be involved in numerous biological functions including neurotransmission, learning, and memory (nNOS); vasorelaxation (eNOS); and host defense (macrophage NOS). The role of NO released by activation of iNOS has been more controversial in CNS diseases. In experimental allergic encephalomyelitis (EAE), an animal model for multiple sclerosis, studies have shown that NO can be both detrimental and beneficial depending on the experimental paradigm (see ref 16, 17, for example). In both EAE and multiple sclerosis, astrocytes or microglia/macrophages (or both) have been shown to express iNOS (18–21). Potential for human immunodeficiency virus-1 or its components to trigger iNOS expression in cultured glial cells has been demonstrated (22, 23). Studies of the CNS tissues from patients with HIV-1 encephalitis have shown both presence and absence of iNOS expression (22–24). Report of iNOS expression in Alzheimer disease (AD) is limited to a single study (25). Vodovotz et al reported specific iNOS immunoreactivity in neurofibrillary tangle-bearing neurons in the hippocampus, while other cell types, including glia, lacked iNOS expression. In addition, the presence of nitrotyrosine, a footprint of nitric oxide formation, has been demonstrated in the brains of Alzheimer patients (26, 27).

AD is a chronic neurodegenerative disease of unknown etiology. Neurons in the vulnerable areas of the AD brain undergo synaptic loss and death, and this process is associated with the formation of neurofibrillary tangles (NFTs) and senile plaques (SPs), pathological hallmarks of AD (28–30). The pyramidal neurons in the Sommer's sector of the hippocampus (CA-1) are particularly vulnerable to the neurodegenerative process, and this region is the prime location for NFT and SP formation. In addition to NFT and SP, the hippocampus of the AD patients display 2 additional types of neuronal inclusions, eosinophilic rod-like inclusions (Hirano bodies) and granulovacuolar bodies (31, 32). Hirano bodies are intraneuronal filamentous inclusions primarily composed of actin and actin-associated proteins (33, 34). In addition, epitopes for tau, neurofilament, C-terminal β-amyloid precursor protein (APP), transforming growth factor β3, and recently hippocampal cholinergic neurostimulating peptide (HCNP) have also been localized to Hirano bodies in the hippocampus of the Alzheimer patients (35–37).

In this report, we used a polyclonal antibody specific to human iNOS to determine immunoreactivity for iNOS in the hippocampus of AD. The results demonstrate iNOS immunoreactivity in Hirano bodies and the tau-negative granular neuritic components associated with SP and NFT. The association of iNOS immunoreactivity with abnormal neuronal alterations in AD suggests that iNOS

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TABLE
Summary of Alzheimer Patients (AD) and Normal Control Brains

Case	Age/sex	Diagnosis	[1]iNOS+ "classical" Hirano bodies (HB)	[1]iNOS+ "granular" inclusions
XC-5499	88/F	AD	++++	+++
XC-5500	81/F	AD	+	++
XC-5338	77/F	AD	+	+
XC-5337	74/M	AD	+++	±
XC-5494	62/M	AD	+++	±
A95-82	35/F	Normal	[2]rare	−
A96-16	41/M	Normal	rare	−
A95-100	40/M	Normal	rare	−
A96-40	41/M	Normal	rare	−
A98-306	81/M	Old Normal	rare	rare
A98-236	94/M	Old Normal	rare	−
A98-308	102/F	Old Normal; Focal hemorrhage	−	−
A98-283	83/F	Senile Changes	rare	−

[1] The iNOS-immunoreactive inclusions consisted of classical rod-like Hirano bodies, as well as smaller granular structures that are associated with SP and NFT. These INOS+ profiles in AD hippocampus were quantified in an arbitrary scale ranging from − (absent) to ++++ (maximum).

[2] INOS-immunoreactive profiles in normal control brains were few in number corresponding to rare Hirano bodies in serial sections examined with H&E stain.

may be involved in the pathogenesis of neuronal degeneration in AD.

MATERIALS AND METHODS

Human CNS Tissue

Sections of the hippocampus from the 5 patients with AD and 4 young and 4 old normal controls were obtained from the neuropathology archives of the Albert Einstein College of Medicine, (Bronx, NY), and from Mayo Clinic Jacksonville, (Jacksonville, FL). The age and sex of these patients are list in the Table. The postmortem intervals in all cases were within 24 hours.

Antibodies

Rabbit IgG to human iNOS peptide was purchased from Santa Cruz (Santa Cruz, CA) and is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 1135–1153 mapping at the carboxy terminus of human iNOS. This antibody was extensively characterized in tissue culture and in human brain (7, 11, 38). TG3 was a generous gift of Drs. Inez Vincent and Peter Davies, Albert Einstein College of Medicine (39). TG3 is a mouse IgM that recognizes phosphorylated tau epitope (S231) and is used as a specific marker for NFT and neuritic components of the SP. Rabbit antibody to human brain NOS (bNOS or nNOS) was purchased from Incstar (Stillwater, MN) (7). Astrocytes and microglia were labeled with rabbit anti-GFAP antibody (Bio Genex, San Ramon, CA) and anti-human HLA-DR (LN3: mouse IgG2b from ICN Pharmaceuticals, Irvine, CA). Anti-human interleukin-1α was purchased from Cistron (Pinebrook, NJ) and was used following the protocol of Griffin et al (40) as an additional marker of activated microglial cells in AD brain.

Immunocytochemistry

Five-μm-thick serial paraffin sections were first examined with hematoxylin and eosin (H&E) stain, and adjacent paraffin sections were examined with immunocytochemistry. Sections were immunostained following the published protocol (41, 42). Briefly, following deparaffinization, sections were immersed in distilled water and boiled for 7 minutes (min) set at "high" in the microwave oven for antigen retrieval. Following incubation with 3% hydrogen peroxide for 30 min, sections were covered in 10% normal goat serum in phosphate buffered saline for 1 hour (h) to block nonspecific binding. The dilutions of antibodies were as follows: anti-iNOS 1:100–1:250; anti-GFAP at 1:250; anti-LN3 at 1:5; anti-IL-1 at 1:200; anti-bNOS at 1:200; TG3 at 1:5; and MC2 at 1:5. Incubations with primary antibodies were done in phosphate-buffered saline containing 10% normal goat serum for 16 h at 4°C. Secondary antibodies were peroxidase-conjugated or alkaline phosphatase-conjugated, isotype-matched mouse Ig or anti-rabbit IgG at 1:250 to 1:400 dilution (all from Southern Biotechnology, Birmingham, AL), for 2 h at room temperature. Color was developed with diaminobenzidine (DAB) as substrate. Sections were counterstained with Meyer's hematoxylin. As a specificity control for iNOS immunoreaction, sections were incubated with iNOS antibody preabsorbed with specific iNOS peptide provided by the company (Santa Cruz), and the staining was compared with that in parallel sections reacted with antibody absorbed with vehicle only.

Double Immunocytochemistry

For double immunocytochemistry for iNOS/TG3, iNOS/GFAP, and iNOS/LN3, sections were first immunoreacted for iNOS as described above, then processed for the second immunoreaction using either a peroxidase-conjugated secondary

antibody matched with Vector VIP Substrate Kit (purple) or an alkaline phosphatase-conjugated antibody with Vector Fast Red (red) as chromogen. Double immunostained sections were counterstained with fast green in some cases. All photographs were taken using a Leica microscope equipped with Nomarski optics.

RESULTS

iNOS Immunoreactivity is Detected in the Hippocampus of AD Patients

Single immunocytochemistry for iNOS demonstrated immunoreactive inclusions in the hippocampus from all 5 Alzheimer brains. The summary of the clinical profile and the pathology of these patients are shown in the Table. Five cases differed in the number of iNOS reactive inclusions but all cases demonstrated immunoreactivity in predominantly 2 types of structures. One was associated with neuronal soma or neuropil, and oval or rod-shaped, characteristic of Hirano bodies (Fig. 1). As known for the distribution of Hirano bodies in AD, the iNOS-immunoreactive inclusions of Hirano bodies were located primarily within the pyramidal layer of the Sommer's sector (CA-1), and rarely in the stratum lacunosum (32). The second type of iNOS-immunoreactive profiles were groups of small granular dot-like structures that were associated with senile plaques and neurofibrillary tangles, especially so-called "ghost tangles" (Fig. 1). The second type of iNOS-immunoreactive profiles were distributed predominantly within the CA-1 region of the hippocampus, but also within the subiculum, molecular layer of the dentate fascia, and the end-plate region (CA-4). The number of either type of iNOS immunoreactive profiles varied from case to case (Table). Although the second type of inclusions were associated with SP and NFT, double labeling demonstrated that they were not positive for TG3, demonstrating that iNOS$^+$ processes were separate from those that contained TG3 epitopes (Fig. 2). Neurons with granulovacuolar bodies did not stain for iNOS (Fig. 2). Double immunostain for astrocytic protein GFAP and iNOS also revealed that these 2 stains did not overlap, demonstrating that iNOS immunoreactivity was not present in reactive astrocytes (Fig. 2). Similarly, immunostain for reactive microglia with anti-class II MHC or anti-interleukin-1 demonstrated presence of activated microglia in the AD hippocampus, but no iNOS immunoreactivity in these microglial cells (data not shown).

As a specificity control, peptide absorption studies were performed and showed that the iNOS immunoreactivity was completely absorbed by preincubation of the antibody with the control iNOS peptide (Fig. 1). Immunostaining of the adjacent sections for bNOS revealed staining of rare (normal appearing) neurons, consistent with the frequency of NOS neurons detected in paraffin-embedded adult human brain (7). None of the iNOS reactive structures in AD brains showed immunoreactivity for bNOS (data not shown). As additional positive and negative controls, cultures of cytokine-stimulated and unstimulated human fetal astrocytes and microglia (11) were subjected to formalin fixation and paraffin embedding, then immunostained for iNOS, to control for the alteration of iNOS epitope during tissue processing. The results showed that iNOS immunoreactivity in cultured astrocytes was preserved following paraffin embedding (data not shown). Thus, our study demonstrated that the immunoreactivity detected in the AD brains was specific to iNOS.

INOS Immunoreactivity in Neurologically Normal Control Brains

iNOS immunoreactivity was minimal in 4 young neurologically normal brains and in 4 old normal brains (Table). Similar to the distribution in AD brains, the immunoreactivity was confined to rare Hirano bodies in these individuals, in the stratum pyramidale, or in the stratum lacunosum of the Sommer's sector (Fig. 1). INOS immunoreactive, neurite-like structures were present in 1 out of 4 old normal brains (not shown), but not in young normal brains (Table). The absence or low number of iNOS immunoreactive profiles correlated with the absence or rarity of Hirano bodies in H&E stained adjacent sections.

DISCUSSION

Our study demonstrated that in AD hippocampus, iNOS was localized to a neuronal subset in Hirano bodies and their variants in close association with SP and NFT formation. This was surprising given that neurons are known to express bNOS rather than iNOS. Although the antibodies specific to each isoform of NOS are known to cross react occasionally, we do not believe this was the case in this study. Our previous study of bNOS expression demonstrated that, similar to rodents, a subpopulation of neurons in the cerebral cortex and the hippocampus of *normal* individuals express bNOS/NADPH diaphorase activity (7). Expression of bNOS in AD brains has been examined and shown to be relatively preserved (43, 44). Further support for the specificity of iNOS immunoreaction in our study was provided by the absorption of staining with iNOS peptide and the lack of similar immunoreactivity with bNOS antibody. The search for sequence homology of the iNOS peptide used to generate antibody (C-terminal 19 amino acids: 1135–1153) did not reveal any candidate cross-reactive proteins, and thus the immunoreactivity is probably truly iNOS and not some protein with similar sequences.

It is possible that the granular, dot-like iNOS immunoreactive profiles associated with SP and NFT in our study are variants of Hirano bodies. There is a striking similarity between the iNOS immunoreaction found in

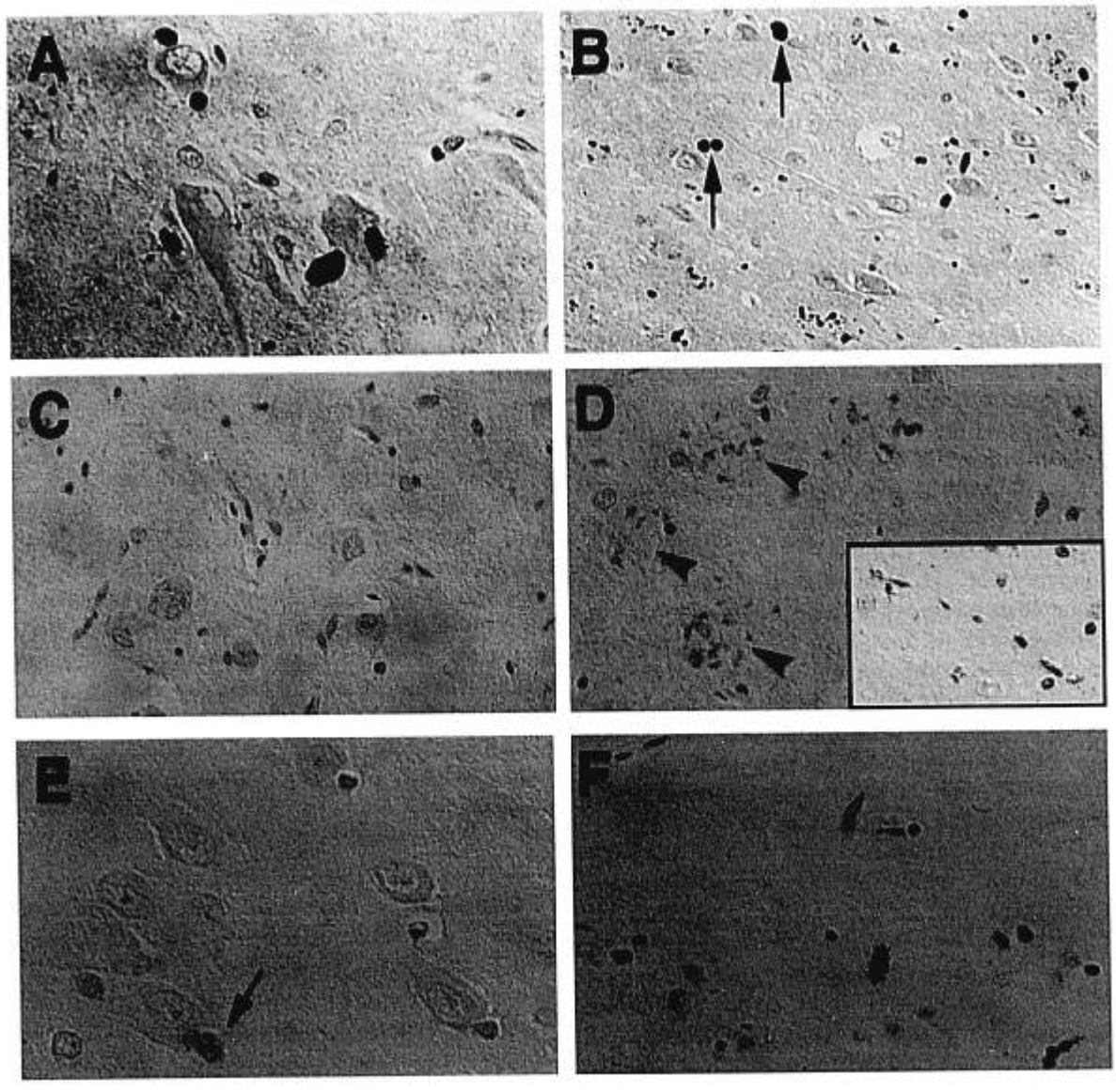

Fig. 1. Expression of iNOS immunoreactivity in the hippocampus of Alzheimer brains and in normal individuals: iNOS immunoreactivity in AD hippocampus is expressed in 2 types of inclusions. (A) and (B) show high and low power figures of single-label iNOS staining demonstrating strong reactivity in Hirano bodies (round and oval structures near neuronal perikarya: arrows), as well as smaller dot-like immunoreactivity in clusters. (C) is a section reacted with an antibody absorbed with iNOS-peptide, demonstrating the lack of staining (specificity control). (D) shows clusters of iNOS-immunoreactive neurites in structures resembling extracellular (ghost) tangles (arrowheads). The inset demonstrates $iNOS^+$ linear profiles probably in a single neuronal process. (E) and (F) show INOS immunoreactivity in the hippocampus of the normal control cases. (E) demonstrates an $iNOS^+$ Hirano body in the stratum pyramidale in an old normal individual. (F) demonstrates a spindle-shaped, $iNOS^+$ structure in the stratum lacunosum of a young individual, consistent with Hirano body. Magnifications: A & C: 600×; B: 300×; D: 800×; E & F: 900×.

this study and the hippocampal cholinergic neurostimulating peptide (HCNP) immunoreactivity in AD hippocampus reported by Mitake et al (37, 45). During an investigation of the expression of HCNP, a peptide growth factor isolated from the rat hippocampus that stimulates cholinergic differentiation of cultured neuronal cells, the authors have found that in human brains, the immunoreactivity for HCNP specifically localizes to the Hirano bodies in the hippocampus of the AD patients and in normal brains. In addition, the authors have found that smaller, granular HCNP-immunoreactive profiles are intermingled with SP and NFT, especially extracellular, "ghost tangles." By immunoelectron microscopy, these small HCNP-immunoreactive structures consisted of paracrystalline filamentous inclusions within neuronal processes (neurites) that are typical of Hirano bodies. Although we did not perform electron microscopy, the light microscopic morphology and the distribution of iNOS-immunoreactive profiles bear similarity to those of HCNP-positive profiles (45). In addition, double labeling

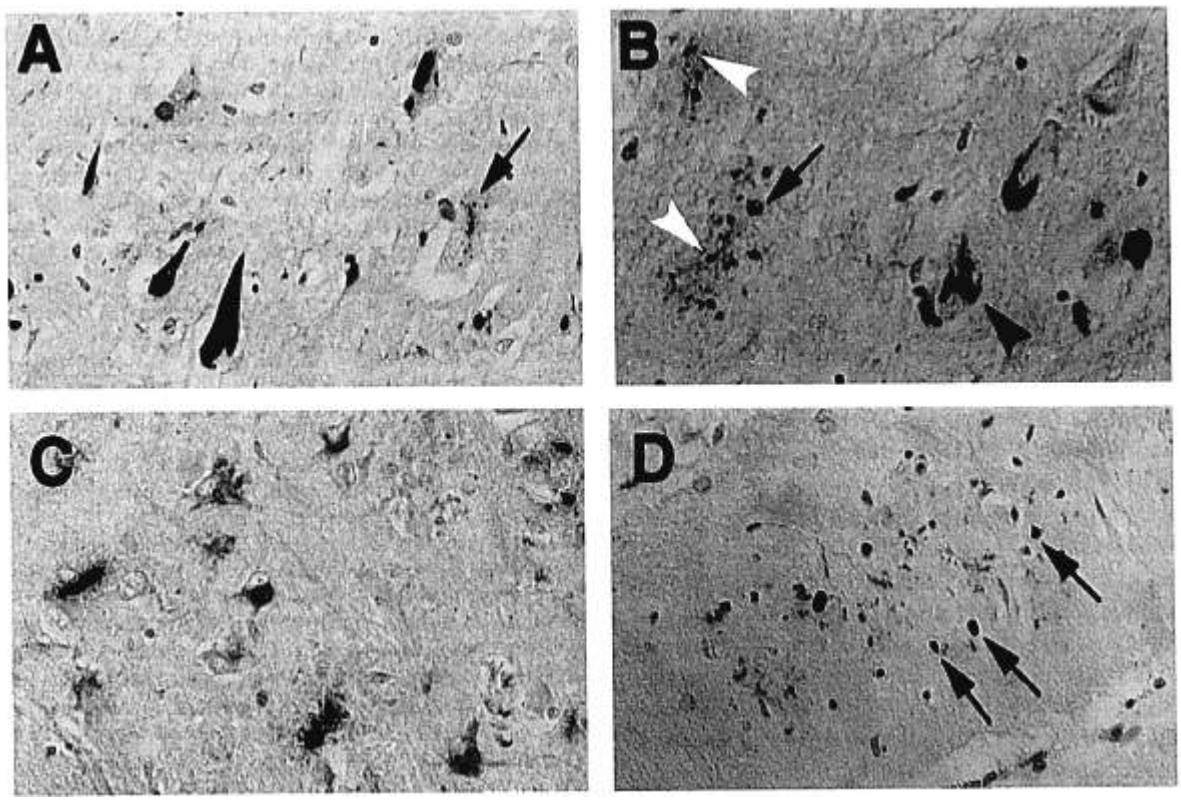

Fig. 2. INOS immunoreactivity in AD hippocampus: double-labeling with tangle and glial markers. (A) is a double-labeling with iNOS (brown) and TG3 (purple) demonstrating TG3 epitope in neurofibrillary tangles and in granulovacuolar bodies (purple dots: arrow), and iNOS epitope in Hirano bodies. The 2 stains essentially do not overlap. (B) is also iNOS/TG3 double-labeling, demonstrating 2 abnormal clusters in the neuropil with both purple dots (TG3 epitope: white arrowheads) and brown dots (iNOS: arrow). Adjacent area has $TG3^+$ tangles and $iNOS^+$ Hirano bodies (black arrowhead). (C) is double labeling for astrocytes (GFAP: brown) and iNOS (purple) demonstrating non-overlapping in staining. (D) is a senile plaque in the molecular layer of the dentate fascia, demonstrating a mixture of $TG3^+$ (purple) and $iNOS^+$ (brown: arrows) neurites. Reactive astrocytes in the AD hippocampus are positively stained for GFAP, while scattered linear Hirano bodies are immunoreactive for iNOS. Magnifications approximately 600× in (A), (C) and (D); 800× in (B).

studies excluded the possibility of colocalization of iNOS with paired helical filament (PHF) tau epitope (TG3) or glial-specific epitopes. These observations support that iNOS expression in Alzheimer hippocampus may be confined to a single type of lesion (Hirano body). Alternatively, the $iNOS^+$ granular profiles could represent another type of lesion described in Alzheimer hippocampus by Munoz and Wang, tangle-associated neuritic clusters (TANCs) (46). TANCs consist of dense aggregates of abnormal neurites each centered by an extracellular (ghost) neurofibrillary tangle rather than an amyloid deposit. Although TANCs are tau-positive (46), and our iNOS-positive granular profiles are largely TG3 (tau)-negative, definite identification of these profiles awaits further investigation.

Our results are similar to the earlier report by Vodovotz et al (25) in that iNOS immunoreactivity was detected in abnormal neuronal inclusions in AD brains, but not in reactive glial cells. Our results are dissimilar, however, in the types of neuronal inclusions in which iNOS immunoreactivity was discovered. Vodovotz et al (25) detected iNOS immunoreactivity in tangle-bearing neurons and in neuropil threads in the AD brains. Although double labeling for PHF tau/iNOS was not performed in that study, given that NFTs and neuropil threads are composed of PHF tau (28, 39), it would have shown overlapping distribution of iNOS and PHF tau. In contrast, our study demonstrated iNOS in Hirano bodies and tau-negative granular profiles associated with NFT and SP. Since we used a commercial rabbit antibody to the C-terminal sequence (C19) of human iNOS protein, whereas the former study used different antibodies (NO53 and 2 other monoclonals against human iNOS), the differences may have resulted from the differences in the fine specificity of antibodies used in these studies. Aberrant formation of nitric oxide in AD brains has been further supported by the study of Smith et al, which detected increased immunoreactivity for nitrotyrosine in AD brains suggesting that widespread peroxynitrite-induced damage was present in AD brains (26).

In various rodent tissue culture paradigms, it has been shown that the component of senile plaque, β-amyloid,

can enhance iNOS expression in microglia and that microglial iNOS can contribute to neurotoxicity (47). In addition, we have found that in cultured *human* glial cells, specific cytokine stimulation leads to induction of iNOS in these cells (11, 48). In contrast to rodent microglia, human microglia lacked the ability to express iNOS, and for iNOS induction in human astrocytes interleukin-1 was an essential stimulant, a conclusion supported by studies of iNOS regulation in other human cell types (49, 50). Thus, it is of particular interest that neither the Vodovotz study nor our study immunolocalized iNOS to microglia or astrocytes in AD brains, while glial cell activation was clearly evident by the expression of class II MHC, interleukin-1 and increased GFAP in these brains. The mechanisms of iNOS activation in neuronal cells of AD brains is unknown, but these findings suggest that the regulation of iNOS in vivo is subject to additional cell-cell and cell-matrix interactions, in addition to species- and cell type-specific regulations observed in vitro. Finally, the close proximity between β-amyloid, interleukin-1$^+$ microglia, PHF-tau (TG3 epitope), and iNOS found in this and other study supports that iNOS may be involved in the cascades of events that lead to glial and neuronal activation in AD. Further studies are needed to clarify that role of iNOS in the pathogenesis and potential therapies of AD.

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