Inducible Nitric Oxide Synthase in Tangle-bearing Neurons of Patients with Alzheimer's Disease

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Summary

In Alzheimer's disease (AD), affected neurons accumulate β amyloid protein, components of which can induce mouse microglia to express the high-output isoform of nitric oxide synthase (NOS2) in vitro. Products of NOS2 can be neurotoxic. In mice, NOS2 is normally suppressed by transforming growth factor β1 (TGF-β1). Expression of TGF-β1 is decreased in brains from AD patients, a situation that might be permissive for accumulation of NOS2. Accordingly, we investigated the expression of NOS2 in patients with AD, using three monospecific antibodies: a previously described polyclonal and two new monoclonal antibodies. Neurofibrillary tangle–bearing neurons and neuropil threads contained NOS2 in brains from each of 11 AD patients ranging in age from 47 to 81 years. NOS2 was undetectable in brains from 6 control subjects aged 23–72 years, but was expressed in small amounts in 3 control subjects aged 77–87 years. Thus, human neurons can express NOS2 in vivo. The high-output pathway of NO production may contribute to pathogenesis in AD.

Alzheimer's disease (AD)[1] is a chronic form of neurodegeneration characterized by progressive memory loss and other intellectual and emotional dysfunctions leading to severe dementia. The most common form of AD is nondominantly inherited (NDAD), whereas a small percentage of patients suffer from familial AD (FAD). Histopathologically, the most prominent features of AD are neuritic plaques (NP) and neurofibrillary tangles (NFT). Tangle-bearing neurons, which are thought to be degenerating, are found predominantly in the entorhinal cortex, hippocampus, and association cortex. Both NP and NFT can be found in brains of the nondemented elderly, albeit to a lesser extent than in patients with AD (1).

β Amyloid protein (Aβ) accumulates extracellularly, but also accumulates in NFT (1). The appearance of AD-like pathology in mice transgenic for Aβ precursor protein (2) supports the hypothesis that Aβ contributes to the pathogenesis of AD. Aβ-derived peptides triggered nitric oxide (NO) production from nitric oxide synthase (NOS) type 1 (3), a NOS isoform expressed constitutively in cerebellar neurons. In vitro studies have implicated NOS1 in neuronal injury associated with excitotoxicity and ischemia (4, 5). Together with cytokines, Aβ-derived peptides also induced expression of the high-output isoform, NOS2, in mouse microglia (6). NOS inhibitors protect neurons and oligodendrocytes from injury caused by inflammatory cytokines, bacterial products, and/or Aβ in vitro (5–7), suggesting that the injury is dependent on the induction of NOS2.

TGF-β1, a potent suppressor of the expression of NOS2 (8–10), is neuroprotective in various contexts (11–14) and suppresses the NO-dependent toxicity inflicted by activated microglia on oligodendrocytes (7). TGF-β1 is decreased in microglia of rats of increasing age concomitantly with increased NOS activity (15). We and others recently observed that expression of TGF-β1 is deficient in brains of patients with AD (16, 17). Reasoning that this decrease in TGF-β1 might be permissive for an increased expression

[1] *Abbreviations used in this paper:* Aβ, β amyloid; AD, Alzheimer's disease; FAD, familial AD; NDAD, nondominant AD; NFT, neurofibrillary tangles; NO, nitric oxide; NOS, NO synthase; NP, neuritic plaques.

Y. Vodovotz and M.S. Lucia contributed equally to this work.

Table 1. *Characteristics of Patients and Control Subjects*

Donor	Age/sex	Classification	Duration*	Medications	Cause of death or other diagnoses
			yr		
1	23/M	Control	—	None	Anaphylaxis
2	35/M	Control	—	None	Viral pneumonia
3	62/M	Control	—	Unknown	Prostate cancer
4	67/M	Control	—	Unknown	Gastrointestinal hemorrhage
5	68/M	Control	—	Unknown	Cardiac arrest
6	72/M	Control	—	Unknown	Ruptured aneurysm
7	77/M	Control	—	Unknown	Ruptured aneurysm
8	87/F	Control	—	Unknown	Unknown
9	87/M	Control	—	Unknown	Gastrointestinal hemorrhage
10	47/M	FAD	8	Dilantin, Synthroid, antidepressants	Unknown
11	59/M	FAD	8	None	Pneumonia
12	81/F	FAD	23	Synthroid	Pneumonia
13	61/M	NDAD	12	None	Ethanol intoxication, cardiac arrest
14	63/M	NDAD	14	None	Pneumonia
15	65/F	NDAD	5	Dilantin	Unknown
16	65/M	NDAD	Unknown	None	Unknown
17	69/M	NDAD	10	Dilantin	Pneumonia
18	72/M	NDAD	1.5	None	Acute myocardial infarction
19	73/M	NDAD	7	Ativan, Inderal, coumadin	Cardiac arrest
20	79/M	NDAD	6	Unknown	Pneumonia

*With respect to signs or symptoms of AD.

of NOS2 in response to Aβ and/or other stimuli, we examined AD and control brains for NOS2.

Materials and Methods

Patients. The patient population was drawn from that used in a previous study (16). NDAD was considered to be present in eight subjects (mean age 69 yr; range 62–79 yr) who met the National Institute of Neurological and Communicative Disorders and Stroke/AD and Related Disorders Association criteria for probable AD (18) without FAD. FAD brains were obtained from three patients (47, 59, and 81 yr old) with early-onset dementia who had a genetic abnormality on chromosome 14q24.3 (16, 17). The patients with FAD were not first- or second-generation relatives, but they were all from the NIH2 or FAD3 families, which have a very distant common relative. All brains from the NDAD and FAD groups met Consortium to Establish a Registry for AD (CERAD) pathological criteria for AD (19). Control brains (n = 9) were obtained from individuals (mean age 64 yr; range 23–87 yr) who exhibited no cognitive or neurological disorders (Table 1). Brains were removed after a postmortem delay of 1.5–36 h and processed as described (16).

Antibodies. NO53, a rabbit antiserum raised against the modified human NOS2-derived peptide C-R-Nle-Orn-SLEMSAL, was described (20). Binding of NO53 can be competitively inhibited in radioimmunoassay, immunocytochemistry, or immunoblot by the cognate peptide YRASLEMSAL, which incorporates SLEMSAL from human NOS2, Tyr for iodination, Arg for solubility, and Ala as a spacer. The control (truncated) peptide YRASLEMSA, lacking the COOH-terminal Leu, is 10,000-fold less effective at binding NO53 by radioimmunoassay and does not block NO53 in immunocytochemical or immunoblot assays (not shown). Thus, YRASLEMSA served as a blocking control. Purified recombinant human NOS2 was injected into mice, hybridomas were produced by standard methods, and their supernatants were screened against purified human NOS2 by ELISA. The resulting mAbs were further screened by immunoblot (see below).

Two were specific for NOS2: 1E8-B8 (IgG1 κ) and 21C10-1D10 (IgG2b κ) (Research & Diagnostic Antibodies, Richmond, CA). These were used either in the form of culture supernate or ascites. Rabbit anti-human NOS1 was raised against the peptide Cys-RLRSESIAFIEESKKDTDEVSFSS, in which Cys modifies the COOH-terminal sequence of huNOS1. Rabbit anti-human NOS3 (C-20, polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA) was raised against amino acids 1183–1202 of huNOS3.

Immunoblot Analysis. The specificity of NO53 (20), 1E8-B8, and 21C10-1D10 for NOS2 was tested by immunoblot analysis of human renal epithelial 293 cells (American Type Culture Collection, Rockville, MD) transiently transfected by the calcium phosphate method with the cDNA of human NOS2 (21) (a gift of Dr. T. Billiar, University of Pittsburgh, Pittsburgh, PA), human NOS1 (22), or human NOS3 (23) (both gifts of P. Marsden, University of Toronto, Toronto, Canada) in the pcDNAI or pcDNAI/Amp vector (Invitrogen, San Diego, CA). Mock transfection served as a control. Lysates were electrophoresed under denaturing, reducing conditions on pre cast 4–20% polyacrylamide gels (Novex, San Diego, CA) in the case of immunoblots with NO53 or on 7.5% polyacrylamide gels in the case of immunoblots with the other antibodies, transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH), and probed with NO53 at a dilution of 1:10,000; with anti-huNOS2 mAbs at 1:400 or 1:500 dilution of ascites; anti-huNOS1 at 1:5,000 dilution; or with anti-huNOS3 at 1:1,000 dilution. Bound rabbit Ig was detected with goat anti-mouse IgG coupled to horseradish peroxidase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) in the case of NO53 or with donkey anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Corp., Arlington Heights, IL) in the other cases, whereas mouse Ig was detected with sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham Corp.). Both types of Ig were revealed by enhanced chemiluminescence, using kits from Amersham Corp. in the case of NO53 or Dupont (Boston, MA) for the rest.

Immunocytochemistry. Immunocytochemical analysis was essentially as described for TGF-β (16). Briefly, tissue sections were deparaffinized at 65°C followed by incubation in xylene and ethanol. After blocking endogenous peroxidase activity in H_2O_2/methanol and nonspecific binding in 5% BSA/1% normal goat serum, sections were incubated overnight at 4°C with NO53 serum at a dilution of 1:10,000, with 1E8-B8 ascites at a dilution of 1:1,000 or culture supernatant at a dilution of 1:50, or with 21C10-1D10 culture supernatant at a dilution of 1:100. Controls for specificity of NO53 were normal rabbit serum; NO53 incubated overnight at 4°C with 15 μg/ml of peptide YRASLEMSAL, or NO53 incubated overnight at 4°C with 15 μg/ml of peptide YRASLEMSA. Bound antibodies were detected with biotinylated goat anti-rabbit or horse anti-mouse secondary antibodies followed by horseradish peroxidase conjugated to streptavidin (Vector Laboratories, Inc., Burlingame, CA). The complexes were visualized using 3,3′-diaminobenzadine (Sigma Chemical Co., St. Louis, MO) as chromagen and $CuSO_4$ as enhancer. Sections were counterstained with Meyer's hematoxylin.

Staining for NFT, NP, and Aβ. To compare NOS2 expression with the presence of NFT and NP, serial 4-μm sections were stained for NOS2 or by Bielchowsky's silver method (24) (American Histolabs, Gaithersburg, MD). Aβ was detected in sections previously stained for NOS2 with NO53 by counterstaining for 4 min with 1.0% aqueous thioflavin S (Polysciences Inc., Warrington, PA). Slides were then rinsed with 80% ethanol followed by water, mounted with Uvak mountant (BioMedical Specialties, Santa Monica, CA), and viewed under fluorescent light. Stained sections were imaged with a video capture board (PixelHr24; Perceptics, Knoxville, TN) connected to an Apple Macintosh Centris 650 computer using Adobe Photoshop 2.5.1.

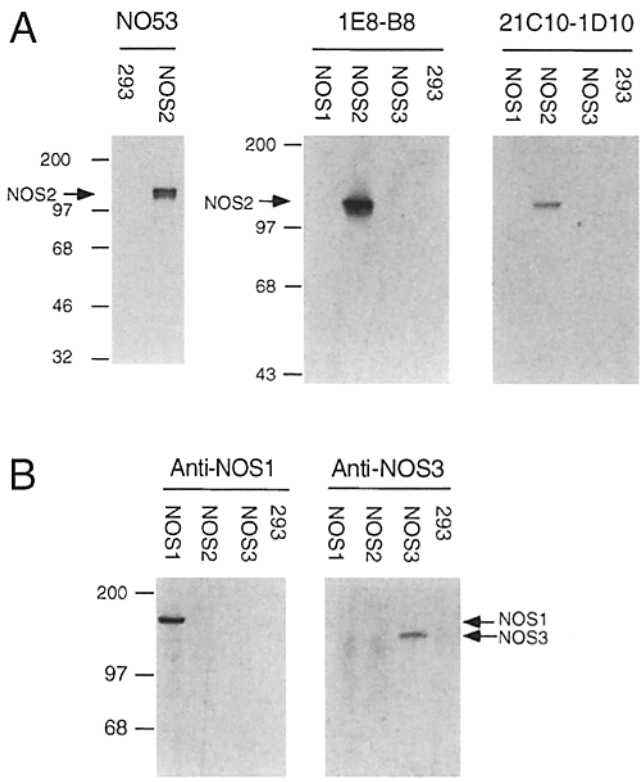
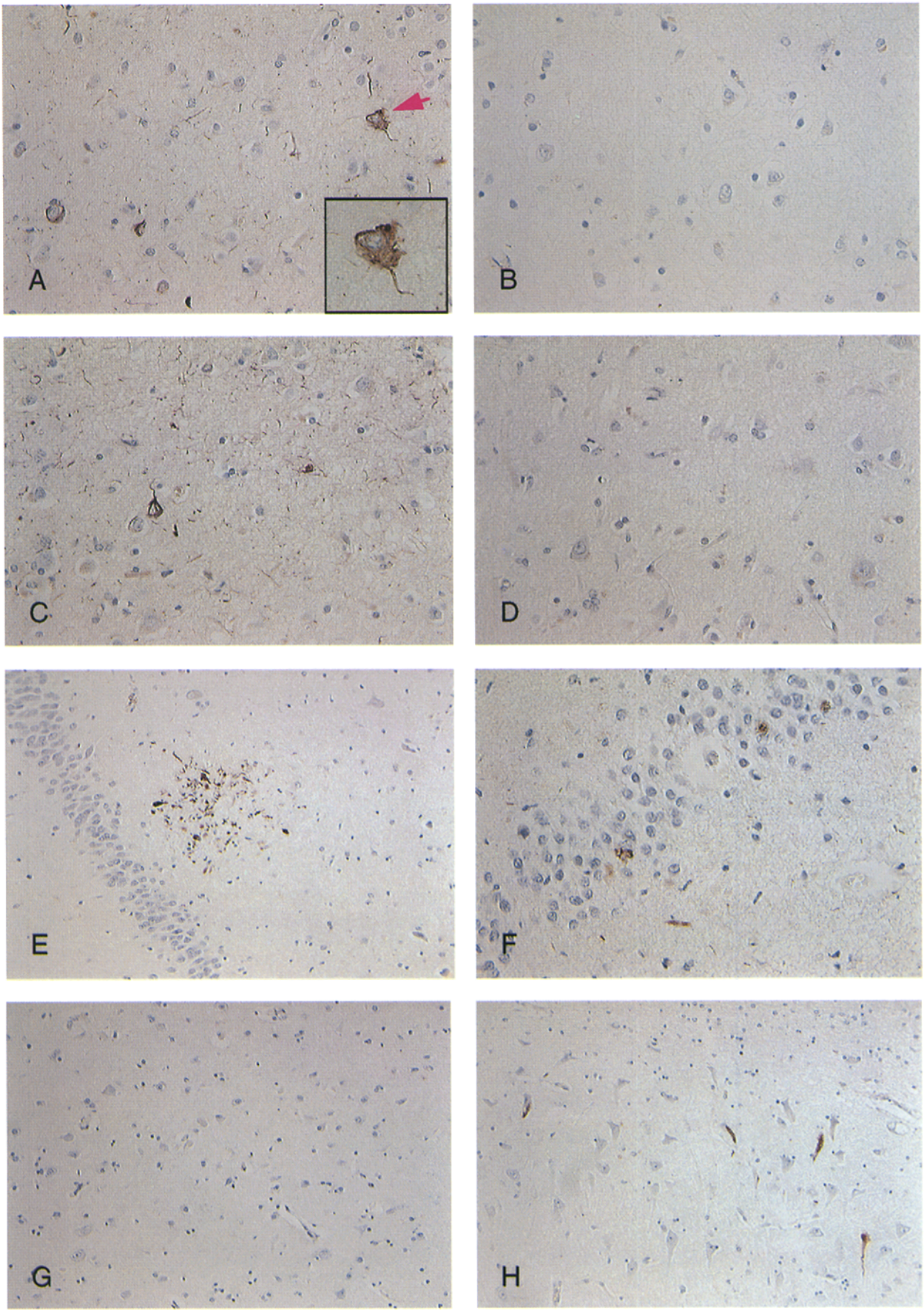


Figure 1. Specificity of anti-human NOS2 Abs. Human renal epithelial 293 cells were mock transfected (*293*) or transfected with plasmids containing cDNAs for human NOS1, NOS2, or NOS3. Lysates of the cells containing NOS1 (75 μg), NOS2 (15 μg for NO53 blot; 140 μg for the rest), NOS3 (200 μg) or mock-transfected 293 cells (15 μg for the NO53 blot; 120 μg for the rest) were subjected to SDS-PAGE and immunoblotted with the indicated antibodies. (*A*) Specificity of NO53, 1E8-B8, and 21C10-1D10 for NOS2. (*B*) Recognition of NOS1 and NOS3 by isoform-specific antibodies. Numbers indicate the apparent molecular masses of protein standards in kilodaltons.

Data Analysis. The average number of NOS2-positive neurons per field and average percentage of neurons positive for NOS2 were scored by a pathologist analyzing the data in a blinded fashion in five alternating ×400 fields (i.e., each counted field was followed by an uncounted field) from regions CA1–CA3 of the hippocampus. The same sections were scored for extent of neuropil threads as follows: grade 0 = 0 per ×200 field; grade 1 = 1–5 per ×200 field; grade 2 = 6–20 per ×200 field; grade 3 = >20 per ×200 field. All values are mean ± SEM. Where indicated, data were analyzed for significance by the two-tailed Student's *t* test. The Wilcoxon rank-sum test was used to analyze the statistical significance of differences in the number of NOS2-positive neurons among the groups examined. Neuropil thread staining was analyzed using the Kruskal-Wallis one-way analysis of variance by ranks.

Results

Antibodies. Until recently, a rate-limiting step in evaluation of the possible role of the high-output pathway of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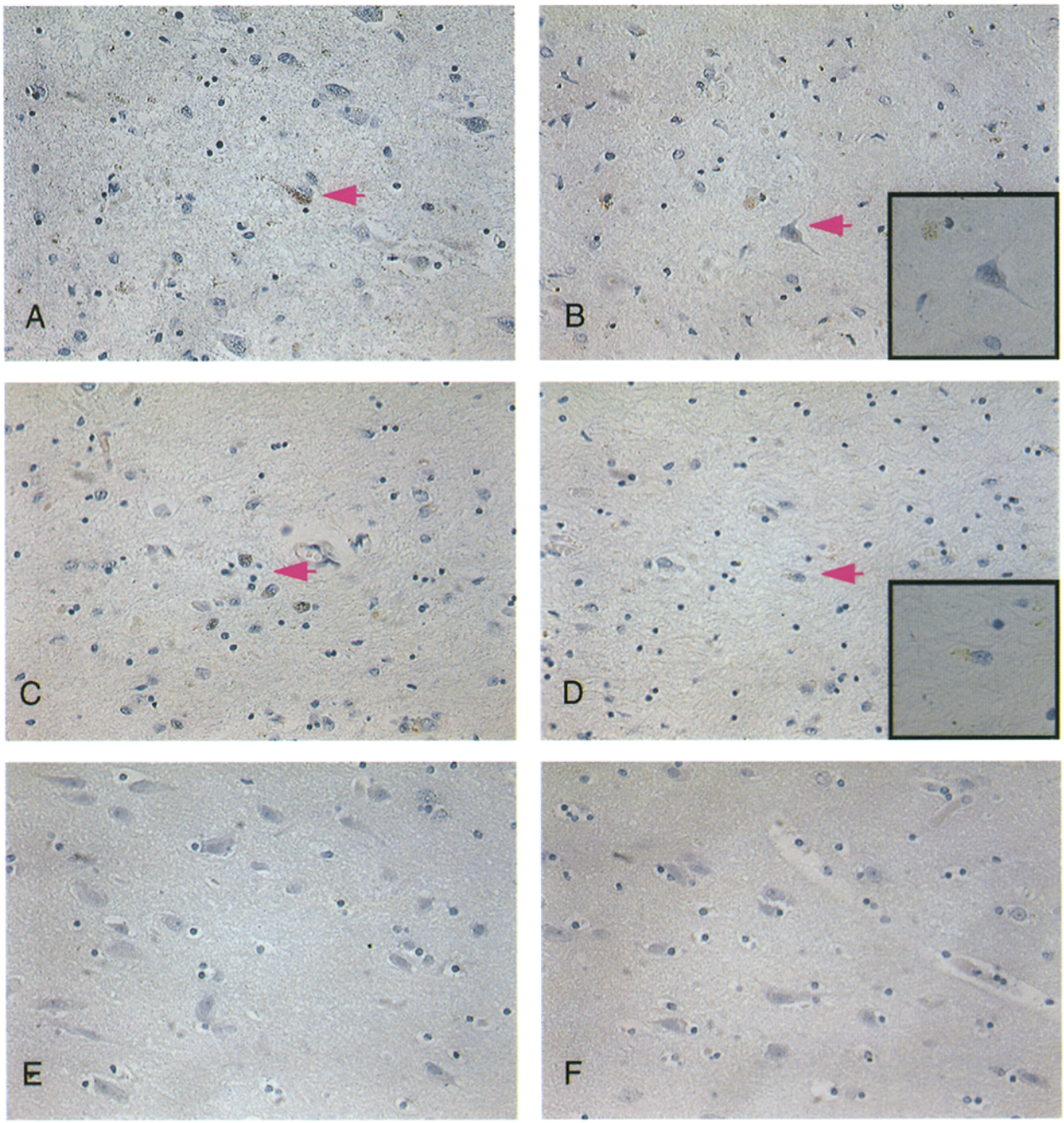

Figure 3. Immunocytochemistry with anti-NOS2 mAbs gives a similar pattern of staining to NO53. (*A*) Hippocampus, NDAD (patient 17), stained with NO53. (*B*) Same as *A* but stained with 1E8B8. (*Inset*) 1E8B8-positive neuron at original magnification of ×1,000. (*C*) Hippocampus, FAD (patient 12), stained with NO53. (*D*) Same as *C* but stained with 21C10-1D10. (*Inset*) 21C10-1D10–positive neuron at original magnification of ×1,000. (*E*) Hippocampus, control (subject 1), stained with 1E8B8. (*F*) Same as *E*, 21C10-1D10. ×400.

NO production in human disease has been the lack of antibodies demonstrably specific for human NOS2. This work made use of NO53, a recently characterized polyclonal antibody specific for a COOH-terminal epitope of human NOS2 (20), and two new mAbs raised against purified recombinant human NOS2. Fig. 1 *A* demonstrates that these reagents immunoblotted no proteins in human epithelial cells that were mock transfected or, in the case of 1E8B8

Figure 2. Immunocytochemistry with NO53. (*A*) Frontal cortex, NDAD (patient 13) stained with NO53. (*Inset*) NO53-positive neuron at original magnification of ×1,000. (*B*) Same as *A* but stained with nonimmune rabbit serum. (*C*) Same region as *A*, but stained with NO53 together with control peptide YRASLEMSA. (*D*) Same region as *A*, but stained with NO53 together with blocking peptide YRASLEMSAL. (*E* and *F*) Senile plaques with dystrophic neurites stained with NO53. (*E*) Hippocampus, NDAD (patient 16). (*F*) Granule cell layer, FAD (patient 12). (*G*) Hippocampus, control (subject 5). (*H*) Hippocampus, elderly control (subject 8). (*A–D, F*) ×400; (*E, G–H*) ×200.

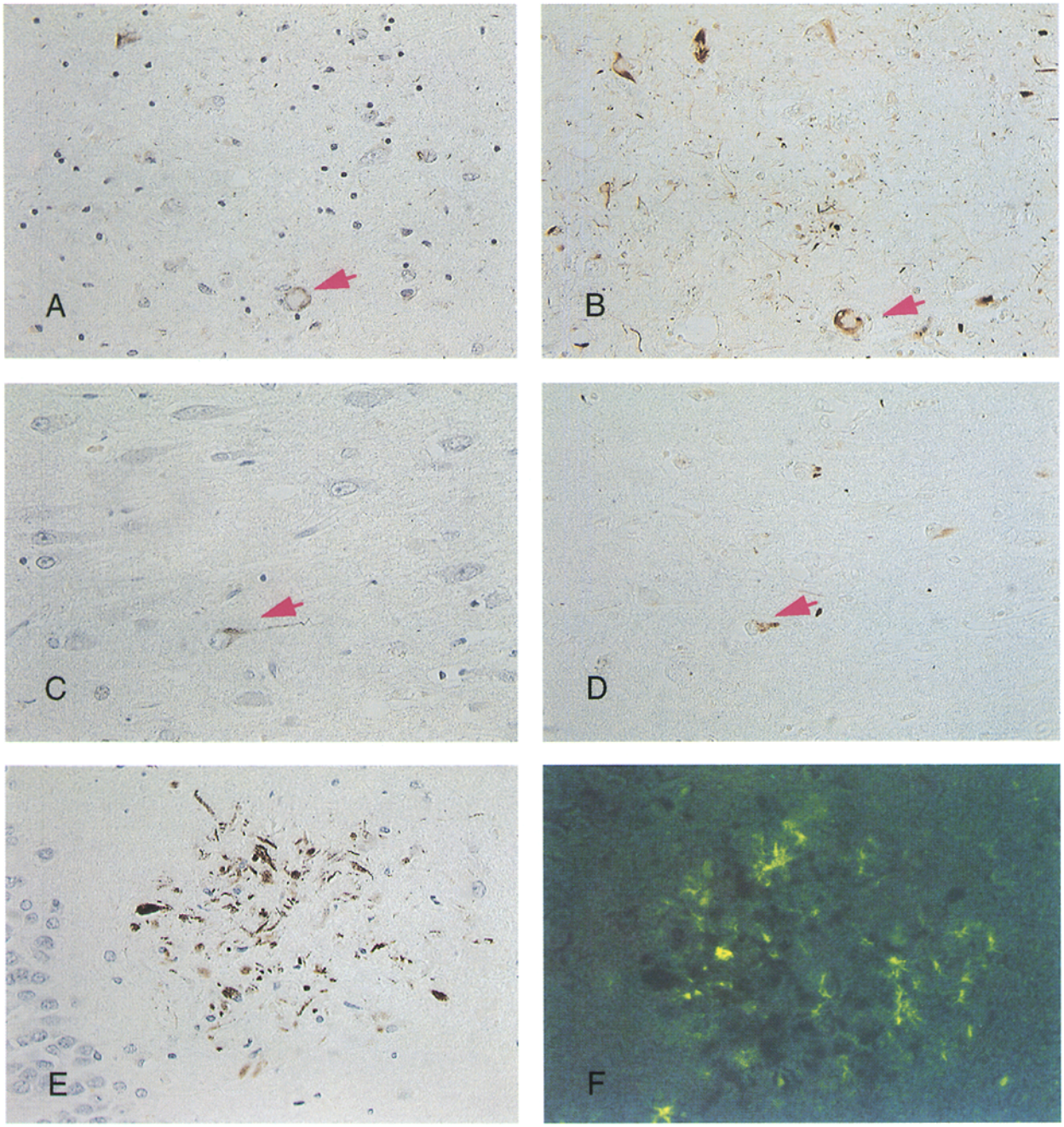

Figure 4. Colocalization of NOS2 with NFT and Aβ. (*A–D*) Serial sections. (*A*) Amygdala, NDAD (patient 16) stained with NO53. (*B*) Same as *A* but with Bielchowsky stain. (*C*) Hippocampus, NDAD (patient 18) stained with NO53. (*D*) Same as *C*, but with Bielchowsky stain. Arrows indicate the same neurons stained by both NO53 and Bielschowsky's reagent. (*E*) Hippocampus (patient 16) stained with NO53. (*F*) Same section as *E*, stained also by thioflavin *S* for the presence of Aβ. ×400.

and 21C10-1D10, transfected with plasmids encoding human NOS1 or NOS3. A previous study has documented that NO53 does not react with NOS1 or NOS3 (20). Expression of the latter proteins was confirmed by reaction with appropriate antibodies as in reference 20 (Fig. 1 *B*). In contrast, in cells transfected with a human NOS2 plasmid, NO53 and both mAbs detected the expected 130-kD band (Fig. 1 *A*).

NOS2 Immunoreactivity in AD Brains. In brains of all eight patients with NDAD, NO53 stained clusters of neurons as well as plaquelike areas of neuropil in a focal pattern. Expression of NOS2 was most pronounced in tangle-bearing neurons and neuropil threads within the hippocampus, parahippocampal gyrus, amygdala, and frontal cortex (Figs. 2 and 3). All three patients with FAD expressed NOS2 in neuronal cell bodies, albeit fewer than in patients with

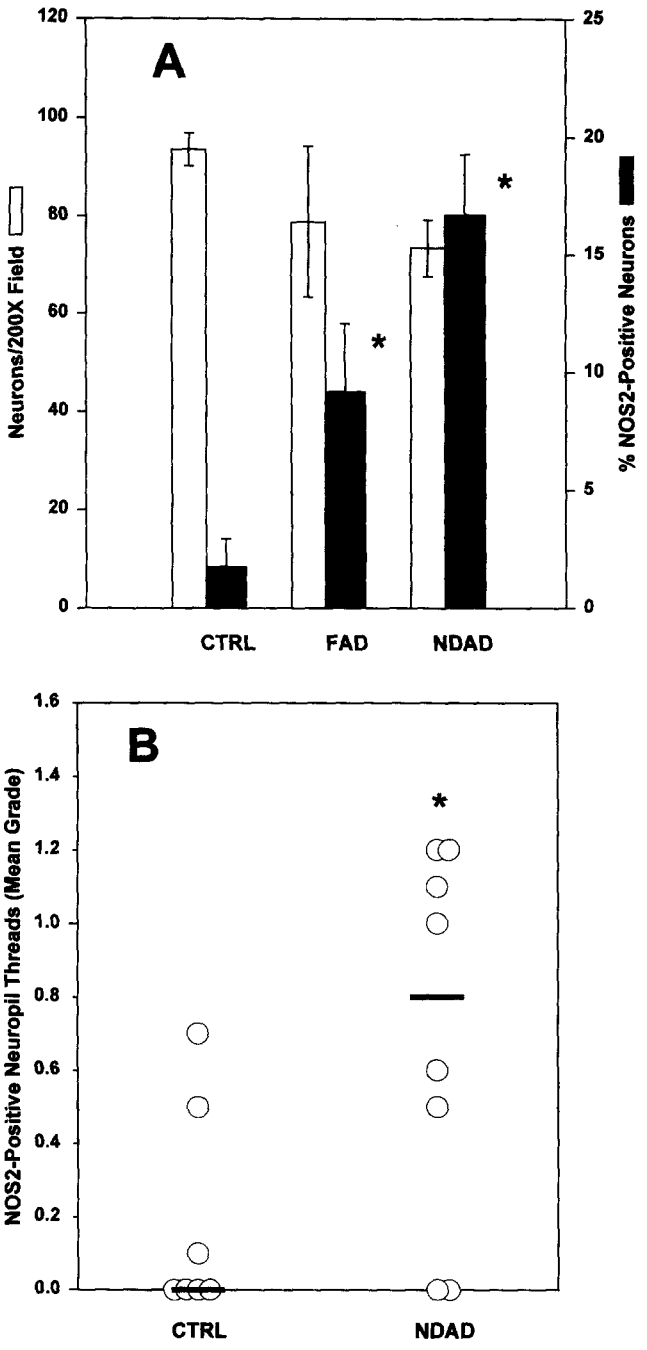

Figure 5. Quantitation of expression of NOS2. Sections from control subjects (*CTRL*; $n = 8$) and patients with FAD ($n = 3$) or NDAD ($n = 8$) were stained with NO53. (*A*) Average number of neurons in five alternating ×400 fields in regions CA1–CA3 of the hippocampus (*open bars*) and average percentage of NOS2-positive neurons in the same fields (*solid bars*) (means ± SEM). Asterisk denotes statistically significant difference from control subjects ($P < 0.05$) by the Wilcoxon rank–sum test. (*B*) Same sections as in *A* were analyzed for extent of NOS2-positive neuropil threads and scored as described in Materials and Methods. Open symbols represent means of 10 contiguous fields in the hippocampus. Horizontal bars represent median values. Asterisk denotes significant difference from control subjects ($P < 0.05$).

NDAD (Figs. 2 *F* and 3, *C* and *D*). Staining was most extensive in the oldest FAD patient examined (patient 12; see Table 1). This patient also exhibited staining in scattered neuropil threads throughout the hippocampus. No staining was obtained in any subject with normal rabbit serum, as illustrated in Fig. 2 *B*, nor with NO53 incubated with an excess of the cognate peptide, as illustrated in Fig. 2 *D*. NO53 staining was unaffected by the control (truncated) blocking peptide (Fig. 2 *C*). mAbs 1E8-B8 and 21C10-1D10 gave essentially the same staining pattern as NO53 in serial sections, though staining of neuropil threads was less pronounced with the mAbs than with NO53 (Fig. 3, *A–D*).

NOS2 Immunoreactivity in Brains from Neurologically Normal Subjects. Younger (23–72-yr-old) control subjects expressed no NOS2 protein in the cerebellum, frontal cortex, hippocampus, amygdala, or entorhinal cortex (Figs. 2 *G* and 3, *E* and *F*). Older (77–87-yr-old) control subjects expressed NOS2 in rare neurons in the frontal cortex, hippocampus, amygdala, or entorhinal cortex. Subject 8, an 87-yr-old woman who did not fit the CERAD criteria for AD (Table 1), expressed NOS2 in neurons, NFT, and NP in the medial temporal lobe (Fig. 2 *H*). An 87-yr-old man (subject 9) expressed NOS2 in scattered neurons and neuropil threads in the amygdala (not shown). No hippocampal sections were available for staining in this case. The mAbs 1E8-B8 and 21C10-1D10 likewise stained no cells in control brains from subjects <77 yr of age (Fig. 3, *E* and *F*).

Presence of NOS2 in Neurons Bearing Neurofibrillary Tangles in Areas Expressing Aβ. Neuronal staining for NOS2 in AD cases appeared to involve neurons containing NFT (Figs. 2 *A* and 3, *A–D*). To test this impression, adjacent 4-μm sections of hippocampus, amygdala, and frontal cortex were stained for NOS2 or with Bielchowsky's silver-based method for NP and NFT. Considering the average diameter of pyramidal neuronal cell bodies in these regions (~20 μm), it was anticipated that the same neuron could be represented in both sections. As shown in Fig. 4, *A–D*, NOS2 appeared to be expressed predominantly in neurons containing NFT. However, not all NP and NFT stained for NOS2. NOS2 was expressed in focal areas in which Aβ was also expressed (Fig. 4, *E* and *F*).

Enumeration of NOS2-positive Neurons. We next quantitated the proportion of total neurons that were NOS2 positive within hippocampal regions CA1–CA3 in cases where these regions were well represented. Although the total number of neurons in these regions was slightly lower in patients with NDAD and FAD than in controls, 1.7 ± 1.2% of neurons ($n = 8$) stained positive in controls, 9.2 ± 2.9% ($n = 3$) in FAD, and 16.5 ± 2.3% ($n = 8$) in NDAD (Fig. 5 *A*). The density of neuropil threads within the hippocampus that stained positive for NOS2 was significantly increased in patients with NDAD (median grade = 0 for controls and 0.8 for NDAD, $P < 0.05$; Fig. 5 *B*).

Discussion

In the human central nervous system, NOS2 has been detected in glial cells during cytomegalovirus infection

(25), in extracted brain tissue from a patient with AIDS-associated cerebritis (26), in reactive astrocytes (27) and extracted brain tissue (28) from patients with multiple sclerosis, and in brain microvessels from patients with AD (29). To our knowledge, the evidence herein is the first to localize NOS2 to neurons in human brain. NOS2-positive neurons were detected in 100% of the AD brains studied. NOS2 was undetectable in brains from neurologically normal subjects <77 yr old, but was expressed in rare neurons in those of more advanced age. In contrast to NOS2, NOS1 was reported to be expressed normally in brains of patients with AD (30, 31). The cerebellum, site of greatest expression of NOS1 (4, 5), did not stain with the antibodies used in this study, further evidence of their specificity.

In vitro, cytokines, microbial products, and Aβ peptides, usually in combination, have induced NOS2 in rodent and human microglia and astrocytes (5–7). However, in brains of patients with AD, we did not observe NOS2 in any cells or structures other than neurons and neuropil, although the antibodies used are capable of detecting NOS2 in nonneuronal cells, such as macrophages (20). Astrocytes and microglia were not immunoreactive, and we did not confirm the reported microvascular localization (29). Tangle-bearing neurons stained most intensely. The restricted localization suggests that at least one factor essential for expression of NOS2 was probably present only within neurons or only active upon neurons; especially those that contained, or came to contain, aggregates of Aβ. Because of the expression of NOS2 and Aβ in the same foci (Fig. 4, *E* and *F*), we hypothesize that Aβ in conjunction with other inflammatory stimuli may have induced an enzymatically active NOS2. We further suggest that the deficiency of TGF-β1 (16, 17) may have been permissive for sustained expression of NOS2, and that the possible overproduction of NO in AD neurons may have been detrimental (5) rather than protective (32). It may be feasible to test some of these ideas in animal models.

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