

nNOS Expression in Reactive Astrocytes Correlates with Increased Cell Death Related DNA Damage in the Hippocampus and Entorhinal Cortex in Alzheimer's Disease

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The immunocytochemical distribution of the neuronal form of nitric oxide synthase (nNOS) was compared with neuropathological changes and with cell death related DNA damage (as revealed by *in situ* end labeling, ISEL) in the hippocampal formation and entorhinal cortex of 12 age-matched control subjects and 12 Alzheimer's disease (AD) patients. Unlike controls, numerous nNOS-positive reactive astrocytes were found in AD patients around β -amyloid plaques in CA1 and subiculum and at the places of clear and overt neuron loss, particularly in the entorhinal cortex layer II and CA4. This is the first evidence of nNOS-like immunoreactivity in reactive astrocytes in AD. In contrast to controls, in all but one AD subject, large numbers of ISEL-positive neuronal nuclei and microglial cells were found in the CA1 and CA4 regions and subiculum. Semiquantitative analysis showed that neuronal DNA fragmentation in AD match with the distribution of nNOS-expressing reactive astroglial cells in CA1 ($r = 0.74$, $P < 0.01$) and CA4 ($r = 0.58$, $P < 0.05$). A portion of the nNOS-positive CA2/CA3 pyramidal neurons was found to be spared even in the most affected hippocampi. A significant inverse correlation between nNOS expression and immunoreactivity to abnormally phosphorylated tau proteins (as revealed by AT8 monoclonal antibody) in perikarya of these CA2/3 neurons ($r = -0.85$, $P < 0.01$) suggests that nNOS expression may provide selective resistance to neuronal degeneration in AD. In conclusion, our results imply that an upregulated production of NO by reactive astrocytes may play a key role in the pathogenesis of AD.

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Key Words: Alzheimer's disease; astrocytes; brain nitric oxide synthase; DNA damage; entorhinal cortex; hippocampus; immunocytochemistry; *in situ* end-labeling.

INTRODUCTION

Nitric oxide (NO) is a diffusible free radical implicated in a wide variety of physiological and pathological processes ranging from vasodilatation and neurotransmission to antimicrobial activity, excitotoxicity, and neurodegeneration (11, 59, 60). NO is synthesized through the conversion of arginine to citrulline by the NO synthases (NOS) (27). In brain, all three isoforms of NOS have been identified and are present in different cell types, and render different functions (44, 61).

The neuronal NOS (nNOS or NOS-I) isoform is generally found to be constitutively expressed in neurons whereas the endothelial NOS (eNOS or NOS-III) is mainly found in endothelial cells. An inducible form of the enzyme (iNOS or NOS-II) is expressed in microglial and astroglial cells (5, 15) as well as in some neuronal cells under the influence of various stimuli (38). Besides being constitutively activated by calmodulin or increases in intracellular calcium levels, nNOS and eNOS are also inducible, but show a longer temporal expression profile than iNOS (20, 34, 40). Activation of nNOS in cultured astroglial cells is furthermore observed after a challenge with calcium ionophores, glutamate, or bradykinin (40). Regulation of expression of the human nNOS gene (NOS1) is complex (69).

A substantial controversy exists considering the presence and role of nNOS in pyramidal neurons of the hippocampus. Some earlier immunocytochemical studies (45, 49, 57), as well as *in situ* hybridization and single-cell expression studies (4, 35), failed to detect nNOS protein or mRNA in hippocampal pyramidal cells. More recent studies, however, reported its presence in rat (8, 16, 64), mouse, and human tissue (13, 14, 56). Several methodological and other reasons may account for these differences (see Discussion).

Although exhibiting protective effects as an antioxidant when properly regulated (67), high concentrations of NO and its intermediates are considered harmful to metabolic processes such as cellular respiration, ion currents, and various enzyme activities (28). They are also known to mediate DNA and transcription damage (28, 30, 48, 53). Since these functions are severely affected in Alzheimer's disease (AD) (68), the role of NO in AD becomes very relevant.

Several lines of evidence suggest an upregulation of NO production in the AD brain as shown by activation of nNOS expression. First, it is postulated that NO mediates NMDA receptor-linked neurotoxicity (10, 24). Second, by forming calcium permeable channels in membranes (63), β -amyloid stimulates the release of NO from a neuronal clone (22). Third, it has been suggested more recently that nNOS expressing neurons of the hippocampus and entorhinal cortex are highly susceptible to degeneration in AD (56).

After our initial observations on nNOS immunoreactivity (55) and DNA fragmentation (33) in AD brains, the aim of our present study was to identify and describe in more detail the relation between the distribution of nNOS and the neuropathological hallmarks, as well as the cell death-associated DNA damage distribution in the hippocampal formation and entorhinal cortex of normal aged subjects and AD patients.

EXPERIMENTAL PROCEDURE

Samples

We selected 12 AD brains (mean age 82 years, range 73–91, SD = 5.9) and 12 normal, elderly human brains (mean age 76 years, range 59–89, SD = 9.5) from the Huddinge Brain Bank (Table 1). For the pathological evaluation, each brain was stained using conventional histological methods (haematoxylin-eosin, cresyl fast violet, luxol fast blue, Congo red, thioflavine-S), silver stainings (modified Bielschowsky, modified Gallyas, methenamine, Campbell-Switzer-Martin), and immunohistochemical detection of phosphorylated tau, β -amyloid, synaptic proteins (Rab-3a, synaptotagmine, synaptophysine), and glial markers (GFAP and ubiquitin). Nineteen brain regions were analyzed: frontal medial gyrus, frontal prepolargyrus, orbitofrontal region, pre/post central region, supramarginal gyrus, medial temporal gyrus, primary/secondary occipital cortex, cingular gyrus, hypothalamus/mammillary bodies, anterior/posterior hippocampus, amygdala, basal ganglia/basal nucleus, thalamus/subthalamus, mesencephalon/nucleus niger, pons/locus coeruleus, medulla oblongata, medulla spinalis/C1&C2, vermis cerebelli, and cortex cerebelli/nucleus dentatus.

The AD group consisted of subjects with a clinically and pathologically confirmed diagnosis of AD, and with no evidence of other major neuropathological alter-

ations, such as vascular dementia or Lewy body disease. The clinical diagnosis of AD was based on combined DSM-IV and NINCDS-ADRDA criteria, while the final neuropathological diagnosis was based on CERAD criteria (39). All subjects in the control group died of nonneurological causes. Medical records were available in all cases and were consistent with an absence of dementia. Thioflavin-S, Congo red, and anti-tau immunocytochemistry showed either no AD lesions or lesions consistent with age-related changes (17).

Methods

Brains were fixed in 4% paraformaldehyde in PBS (pH 7.4) for a period of 2.5 weeks to 15 weeks (see Table 1). The hippocampi and entorhinal cortices were removed and cut in rostrocaudal direction in 3-mm-thick blocks, embedded in paraffin and cut.

For nNOS immunocytochemistry, one 25- μ m-thick section from each block was deparaffinated and incubated in blocking serum (BS) containing 5% normal goat serum, 1% bovine serum albumin, 0.1% glycine, 0.1% L-lysine, and 0.3% Triton X-100 in PBS (pH = 7.4) for 1 h at room temperature (RT). Sections were then incubated at 4°C for 2–3 days in primary rabbit polyclonal antibody (Auspep Pty., Parkville, Australia—a gift of Dr. Ivica Grkovic) raised against the C-terminal sequence of rat nNOS (amino acids 1409–1429 of primary sequence RSESI AFIEESKK-DADEVFSS) in a dilution of 1:1000 in PBS. This part of the molecule is unique to nNOS. Western blot analysis shows a single band corresponding to nNOS and the antibody shows no cross-reactivity with iNOS or eNOS. After washing in PBS (4 \times 30 min) sections were incubated in biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA), diluted 1:200 in BS for 2 h at RT. Following washing in PBS, sections were incubated in avidin–biotin–peroxidase complex (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA) diluted 1:800 in PBS for 1 h at RT. Finally, peroxidase activity was visualized using the glucose oxidase-nickel method and 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) with 0.02% H₂O₂. For controls, the primary antiserum was omitted. The specificity of the immunostaining was further confirmed with preabsorbed nNOS using the nNOS blocking peptide (1409–1429, Auspep Pty. Ltd., Batch No. K31064, *M*, 2374 as confirmed by mass spectral analysis, purity >80%) in a dilution of 1:500.

In situ end labeling (ISEL) was performed to detect DNA damage, associated with dead or dying cells, according to methodology described earlier (31, 32). Briefly, tissue sections were deparaffinized for 2 \times 15 min in xylene and hydrated to 50% ethanol and distilled water. Subsequently, sections were preincubated with proteinase K (PK) buffer (10 mM Tris/HCl; 2.6 mM CaCl₂; pH 7.0), incubated with 5 μ g/ml PK (Sigma)

TABLE 1
Clinicopathological Data from the AD Patients and Elderly Controls Studied

Case no.	Sex	Age (y)	PMD (h)	Duration of AD (y)	BW (g)	Fix (weeks)	Cause of death	Clinical and pathologic remarks
Alzheimer's disease								
1	F	73	<32	4	1080	11	Bronchopneumonia	
2	M	73	<24	7	950	14	Bronchopneumonia	
3	M	77	<24	3.5	1110	8	Bronchopneumonia	several smaller thalamic infarcts
4	F	80	<32	5	1020	9	Bronchopneumonia	Borrelia infection
5	F	80	<24	6	1200	4.5	Myocardial infarction	
6	M	81	<24	3	1478	13	Bronchopneumonia	lymphoma
7	F	83	<48	5.5	1003	7	Cardiopulmonary insufficiency	
8	F	84	<48	3.5	1384	8	Cardiovascular insufficiency	
9	F	84	<24	3.5	1065	5	Cardiovascular insufficiency	
10	F	88	<24	4.5	1160	7.5	Cancer of the neck	liver metastases
11	F	90	<24	5	850	9.5	Pulmonary embolism	
12	F	91	<24	4	984	10.5	Bronchopneumonia	anomaly of a. cerebri post. sin.
Elderly control subjects								
1	M	59	<24		1360	6	Traffic accident	
2	F	62	n.a.		n.a.	5	Traffic accident	
3	M	68	<24		n.a.	6	Traffic accident	
4	F	71	<24		1210	6	Myocardial infarction	
5	M	75	n.a.		n.a.	5.5	Traffic accident	
6	F	77	<24		1200	12	Myocardial infarction	Braak's argyrophilic grains in entorhinal cortex and hippocampus
7	M	78	<24		1255	8	Cardiovascular insufficiency	
8	F	81	<24		965	9.5	Myocardial infarction	one small, old infarct close to the raphe nuclei in pons
9	F	84	<24		1210	15	Pulmonary embolism	
10	M	84	n.a.		n.a.	2.5	Traffic accident	
11	F	85	n.a.		n.a.	7	Cardiovascular insufficiency	
12	F	89	n.a.		n.a.	8.5	Hyperthermia after urinary infection	diabetes mellitus type II

Note. PMD, postmortem delay; BW, brain weight; Fix, fixation time; n.a., data not available.

in PK buffer for 15 min at RT, washed in distilled water, incubated with terminal transferase (TdT) buffer (0.2 M sodiumcacodylate, 0.025 M Tris/HCl en 0.25 mg/ml Bovine Serum Albumin (BSA); pH 6.6) for 15 min at RT, and incubated for 60 min at 37°C with a reaction mixture that contained: 0.2 μ l TdT (Boehringer-Mannheim)/100 μ l reaction mixture, 1.0 μ l biotin-16-dUTP (Boehringer Mannheim)/100 μ l reaction mixture and cobalt chloride (25 mmol/l; 5% of the final

volume). Incorporation of labeled oligonucleotides was stopped by rinsing the sections in distilled water and PBS (pH 7.4). Endogenous peroxidase activity was blocked with 0.01% H₂O₂ in PBS for 20 min at RT after which sections were washed in PBS and preincubated with PBS/1% BSA for 15 min and incubated with peroxidase-conjugated avidin (ABC-elite kit, Vector Laboratories, Inc.) 1:1000 in PBS/1% BSA overnight at 4°C. Following washing in PBS, labeled DNA was visual-

TABLE 2

An Example of Semiquantitative Assessment of DNA Damage Distribution, nNOS Immunoreactivity of the Pyramidal and Reactive Astroglial Cells, and AT8 Immunoreactivity of the Neurons in the Hippocampus of Typical AD and Control Subjects

		CA1				CA2/3				CA4				GCL				SUB				Cx			
AD subject no. 7																									
block	I	N	n	8	I	N	n	8	I	N	n	8	I	N	n	8	I	N	n	8	I	N	n	8	
1/11	2	0	2	1	—	—	—	—	—	—	—	—	—	—	—	—	0	0	1	1	0	0	3	2	
2/11	3	0	3	1	0	3	0	0	2	0	2	1	—	—	—	—	1	0	2	2	0	0	3	2	
3/11	3	0	3	2	0	2	0	0	3	1	2	1	1	0	0	0	1	0	3	1	0	0	2	1	
4/11	3	0	3	3	0	3	1	1	3	1	2	2	1	0	0	0	1	0	1	2	0	0	4	2	
5/11	3	0	3	2	0	3	0	0	3	1	3	0	1	0	0	0	1	0	1	1	0	0	3	1	
6/11	3	0	3	3	0	3	0	0	3	1	2	0	0	0	0	0	1	0	1	1	0	0	3	2	
7/11	3	0	3	3	0	2	0	1	3	1	3	2	1	0	0	0	1	0	1	2	0	0	2	3	
8/11	3	0	3	2	0	2	1	0	3	1	2	1	1	0	0	1	1	0	2	2	0	0	3	3	
9/11	3	0	3	2	0	2	0	1	2	0	1	0	0	0	0	0	1	0	1	2	0	0	2	2	
10/11	3	0	2	2	0	3	0	0	1	0	1	1	0	0	0	0	1	0	0	2	—	—	—	—	
11/11	3	0	2	2	0	2	0	0	—	—	—	—	—	—	—	—	0	0	1	1	—	—	—	—	
Control subject no. 12																									
block	I	N	n	8	I	N	n	8	I	N	n	8	I	N	n	8	I	N	n	8	I	N	n	8	
1/13	0	0	0	0	—	—	—	0	—	—	—	0	0	0	0	0	0	0	0	0	0	0	0	0	
2/13	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
3/13	0	0	1	0	0	3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	
4/13	0	0	1	0	0	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
5/13	0	0	0	0	0	3	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	
6/13	0	0	0	0	0	3	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	
7/13	0	0	0	0	0	3	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	
8/13	0	0	0	0	0	3	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
9/13	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
10/13	0	0	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
11/13	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
12/13	—	0	0	0	—	—	0	0	—	1	0	0	—	0	0	0	—	0	0	0	0	0	0	0	
13/13	—	0	0	0	—	—	—	0	—	—	0	0	—	—	0	0	—	0	0	0	0	0	0	0	

Note. Scores of the areas studied represented subjective assessments of staining intensity and numbers of labeled cells, as follows: 0: no labeling observed; 1 (+/-): small number of cells that are only weakly labeled; 2 (+): moderate number of cells that are clearly labeled; 3 (++) : large number of intensely labeled cells present. Abbreviations used: I, ISEL; N, nNOS-ir pyramidal neurons; n, nNOS-ir astroglial cells; 8, AT8-ir neuronal bodies; SUB, subiculum; Cx = temporal cortex; —, not present or determined.

ized by incubation with 0.5 mg/ml DAB (Sigma) in 0.05 M Tris/HCl (pH 7.5) with 0.02% H₂O₂ for exactly 12 min based on earlier pilot experiment (not shown). Color development was stopped by washing in distilled water after which sections were lightly counterstained with methylgreen prior to mounting coverslips. Positive controls were included in every assay consisting of sections from rat prostate, three days after castration (66).

In addition to nNOS and ISEL staining, adjacent sections were stained using AT8 antibody (a monoclonal antibody against the abnormally phosphorylated serine 202 and threonine 205 in human PHF-tau proteins) in dilution 1:1000 (Innogenetics, Zwijndrecht, Belgium, Cat. Code BR-03) (3, 18) and GFAP antibody (to identify astroglial cells, for which GFAP is a specific marker) in dilution 1:100 (Dako, Glostrup, Denmark, cat. code M761). All sections from control and AD subjects were labeled after random selection, under identical experimental conditions and were assessed unaware of the subjects' history or condition.

The numbers of nNOS-, ISEL-, and AT8-positive neurons as well as nNOS-positive and ISEL-positive glial cells were assessed semiquantitatively by two independent observers (Table 2). Obtained scores were compared for each principal field of the hippocampal formation and for the entorhinal cortex by Pearson's product-moment correlation using Statistica Version 5.0 program (Statsoft, Inc.). The value of *P* less than 0.05 was chosen as the criteria for the level of confidence.

RESULTS

In control subjects many pyramidal and non-pyramidal neurons of Ammon's horn were nNOS immunopositive, with CA2 and CA3 regions usually containing a larger number of immunoreactive neurons (Fig. 1a). The staining pattern often terminated abruptly at the border to CA1, without reactivity in CA1 or subicular pyramidal neurons (Figs. 1a and 1b). However, in some control subjects CA1 pyramidal neurons showed weak

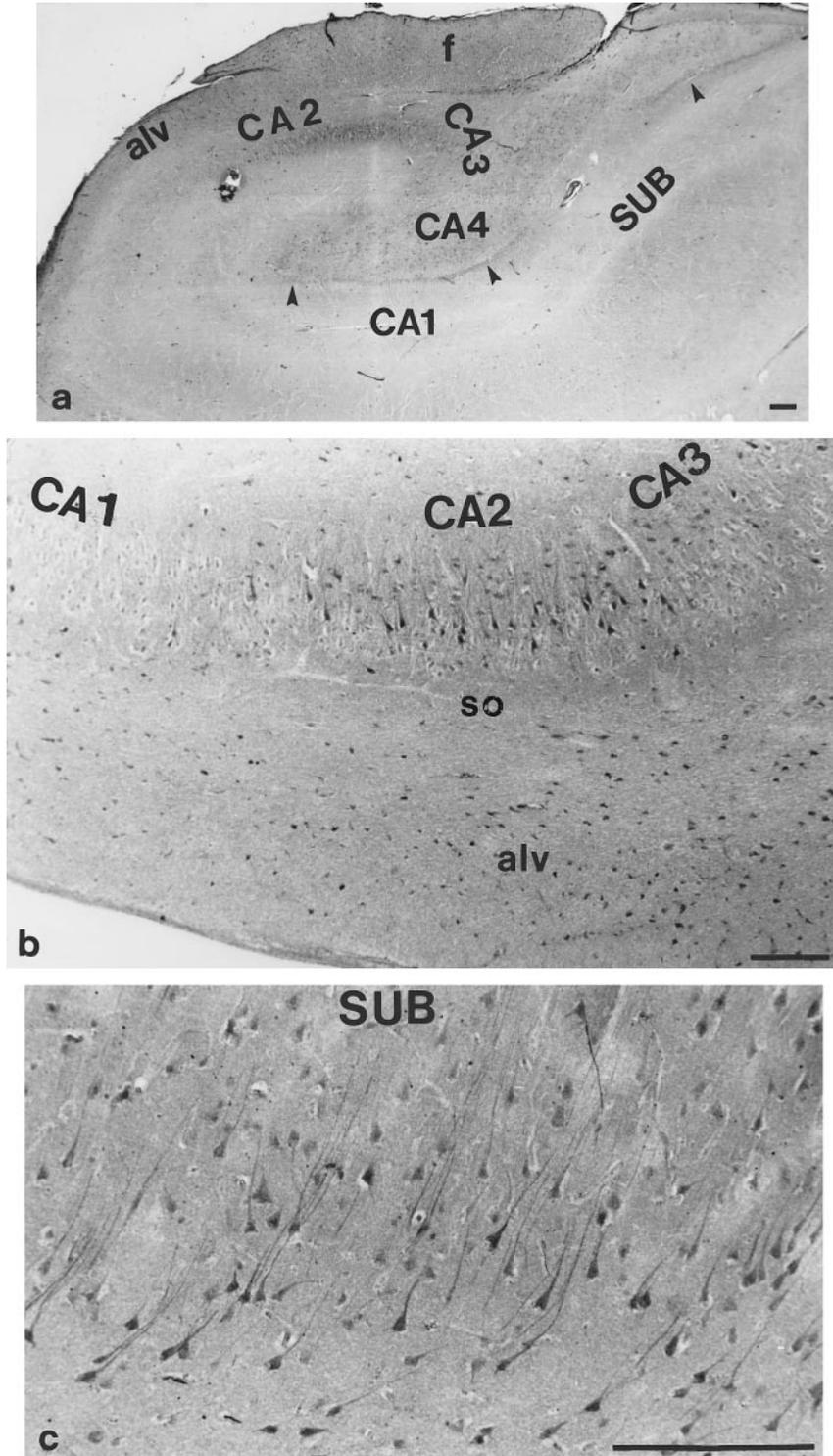


FIG. 1. (a) Distribution of nNOS-immunoreactivity in a control subject. CA2 and CA3 contain a larger number of immunoreactive neurons. (b) Abrupt nNOS staining at the border of CA2 with CA1 in a control subject. (c) Moderate nNOS immunopositivity of the deep subicular pyramidal neurons occasionally found in elderly controls. alv, alveus; f, fimbria; so, stratum oriens; SUB, subiculum. Scale bars, 200 μ m.

to moderate immunopositivity, and a number of deep subicular pyramidal neurons, moderate to strong immunopositivity (Fig. 1c). The CA4 region contained

only a few moderately nNOS positive neurons, while the granule cell layer only occasionally contained faintly labeled cell bodies. Moderate nNOS positivity of

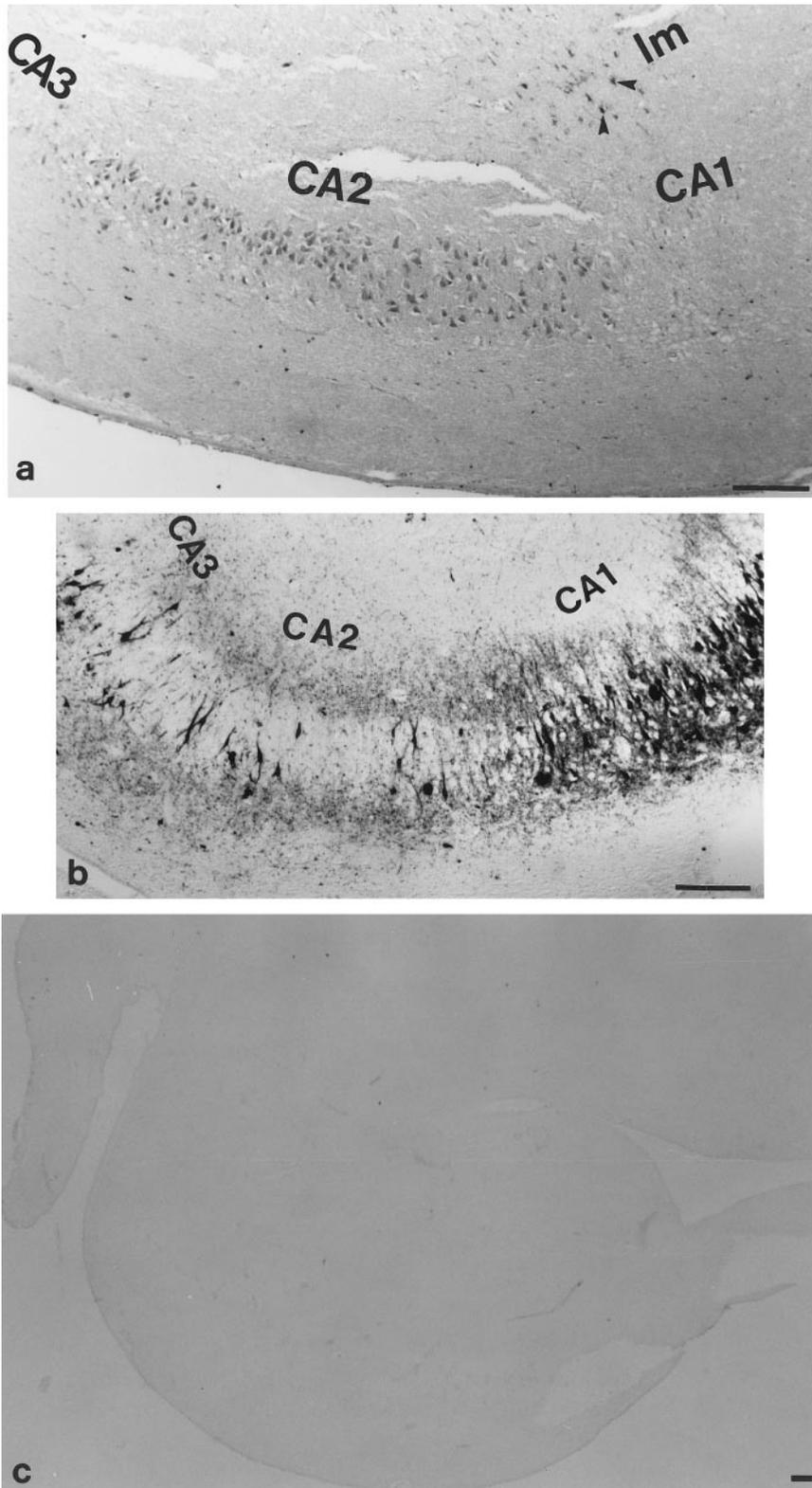


FIG. 2. (a) nNOS immunopositivity of a portion of CA2 pyramidal neurons in an AD patient. Note nNOS positive reactive astrocytes (arrowheads) in the stratum lacunosum-moleculare (Im) in CA1. (b) Adjacent sections showing AT8-negative bodies of spared neurons; moderate to strong AT8 immunoreactivity is seen in their axons, distal dendrites, and surrounding neuropil. (c) Preabsorption control of anti-nNOS showing completely abolished immunostaining of neurons, glial cells, and plaques (black spots are artifacts). Scale bars, 200 μ m.

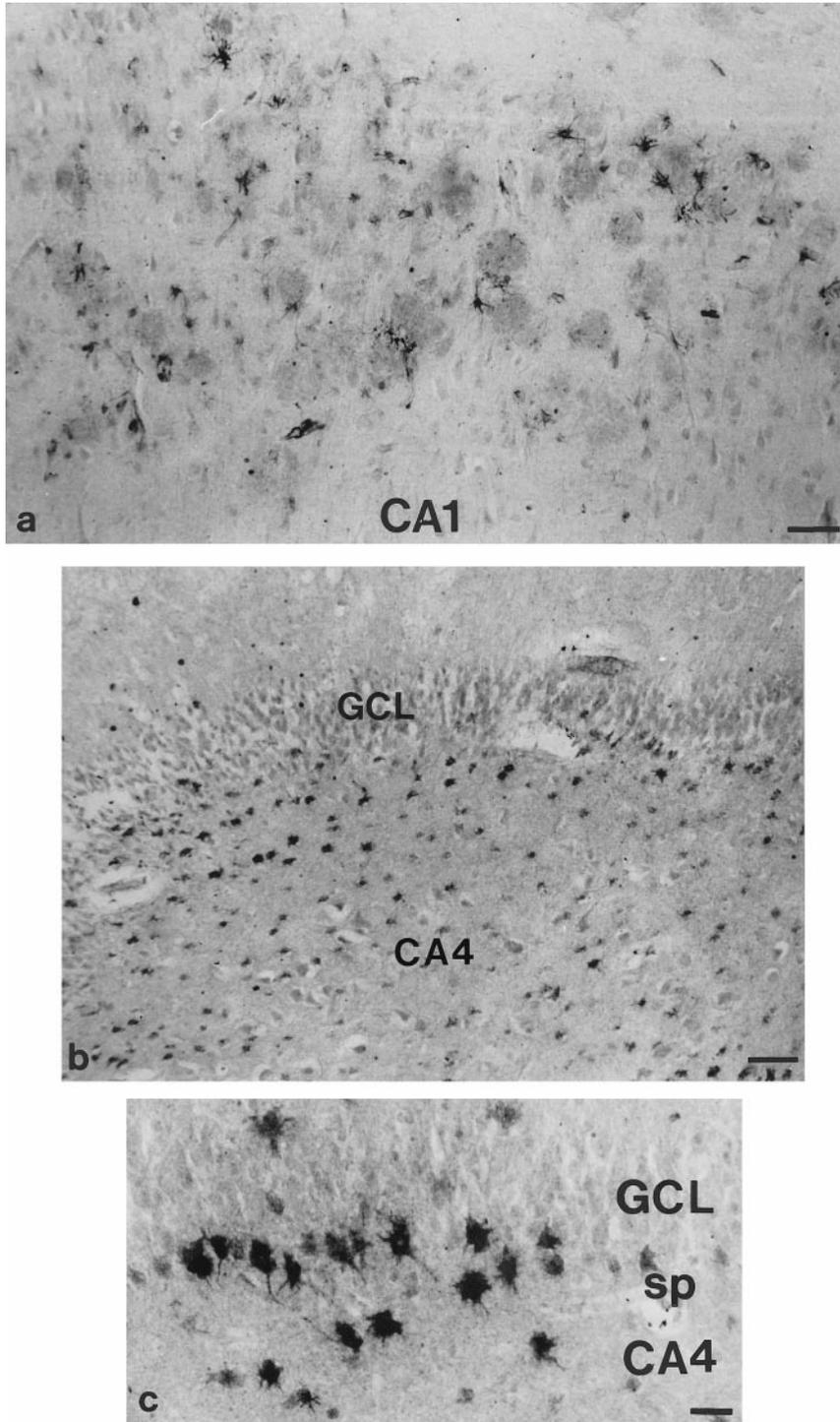


FIG. 3. nNOS-positive reactive astrocytes in AD hippocampus in CA1 (a), CA4 (b), and in the plexiform layer of fascia dentata (c). In CA1 most of the nNOS-positive reactive astrocytes are situated around plaques. Note lightly stained CA1 pyramidal neurons. GCL, granule cell layer; sp, stratum plexiforme. Scale bars, 50 μm in a and b, 10 μm in c.

interneurons was observed in all layers of the hippocampal formation and entorhinal cortex. Throughout all layers, except for layer I, the entorhinal cortex contained occasionally lightly positive nNOS neurons.

In AD subjects, strong nNOS immunoreactivity was again observed in a portion of the CA2/3 neurons (which, based on our subjective observations, was about 40% in most cases) (Fig. 2a), whereas immuno-

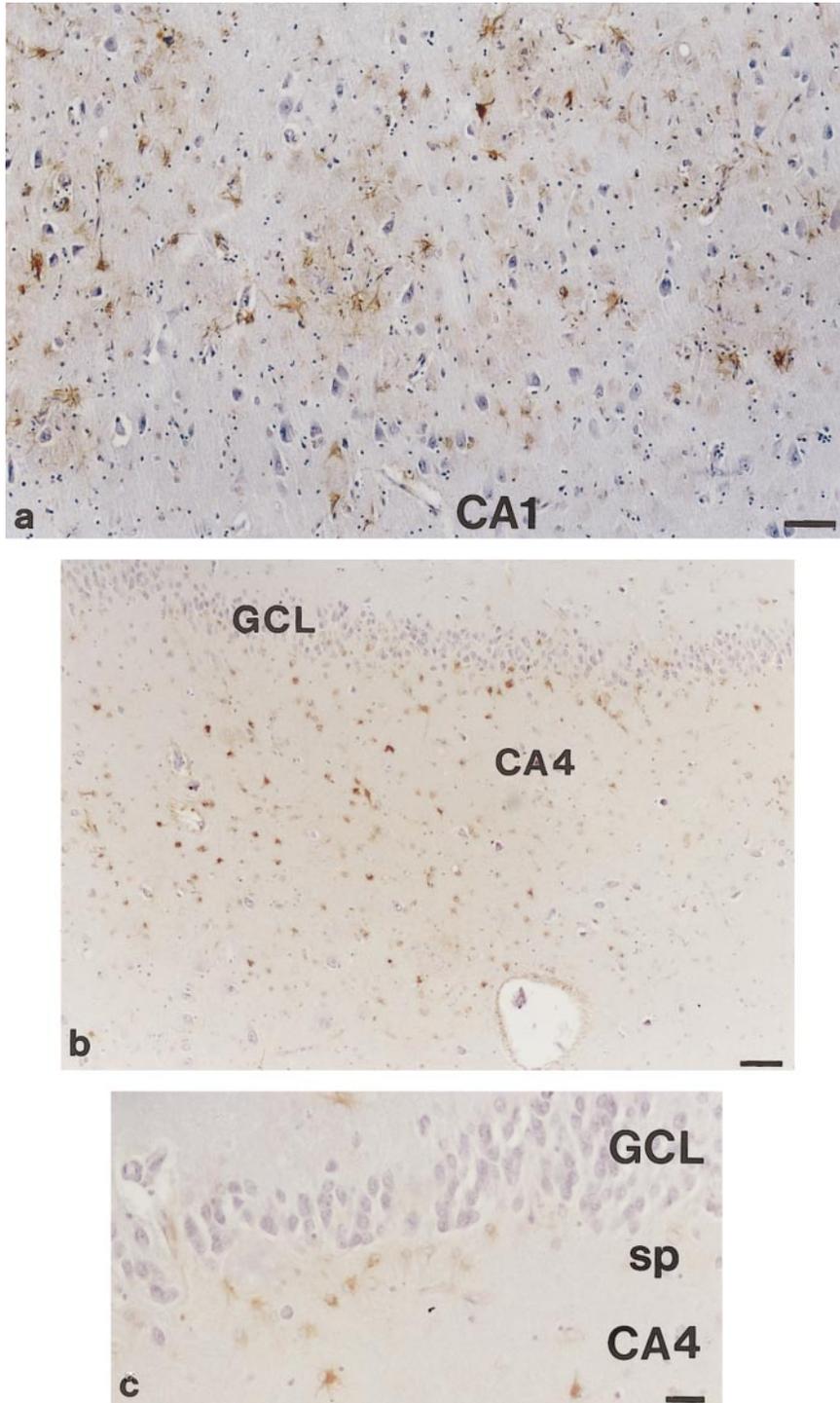


FIG. 4. Corresponding sections to Fig. 3 stained for GFAP. (a) CA1; (b) CA4; (c) plexiform layer of the dentate gyrus. GCL, granule cell layer; sp, stratum plexiforme. Scale bars, 50 μm in a and b, 10 μm in c.

reactivity in CA1 and subicular pyramidal neurons was weak or absent. Even in the most heavily affected hippocampi, these CA2 and neurons of the neighboring part of CA3 were found to be spared and remained nNOS-positive. In adjacent sections, most of the cell

bodies of CA2/3 pyramidal neurons were AT8-negative, while their distal dendrites stained positively (group 2 neurons according to Braak and colleagues (2)) (Fig. 2b). In addition to immunostaining in the neuronal cell body, the nNOS antibody also labeled reactive astro-

glial cells and some plaques, with no evidence of nNOS staining in microglial cells or neurofibrillary tangles. Preabsorption control of anti-nNOS eliminated immunostaining in neurons, glial cells, and plaques (Fig. 2c).

Unlike control cases, in AD brains many β -amyloid plaques were surrounded by numerous nNOS-positive, reactive (protoplasmic) astrocytes, especially in CA1 (Figs. 3a and 4a) and subiculum (Figs. 5a and 5b). Large numbers of GFAP- and nNOS-positive reactive astrocytes were also found in CA4 (Figs. 3b and 4b) and in the plexiform layer of the fascia dentata (Figs. 3c and 4c). Small numbers of only weakly nNOS-positive reactive astrocytes were seen in CA4 of two controls (subjects 4 and 8). In the CA1 sector of three AD and two control hippocampi, close to the CA2 field, reactive astrocytosis consisting of very large, swollen astrocytes, was observed. On adjacent sections these GFAP-positive reactive astrocytes were found to be intensively nNOS positive (Fig. 5c).

Similarly to CA1 and subiculum, very weak or absent nNOS immunolabeling in AD was found in neurons of the entorhinal cortex. In five AD cases (i.e., subjects numbers 1, 3, 4, 6, and 7), the entorhinal cortex was almost completely devoid of neurons, and neurofibrillary tangles were fewer in number than usually seen in AD. At these places of overt neuronal loss, particularly in the islands of layer II entorhinal neurons, an intense nNOS immunoreactivity of the hypertrophied astroglial cells was observed in adjacent sections (Figs. 5d and 5e).

The neuronal DNA fragmentation in AD, as shown by ISEL, was prominent mainly in CA1 (Fig. 6a) and CA4 (Fig. 6b), and occasionally also in the subicular areas. In these affected areas, many ISEL-positive glial cells (Fig. 6c) as well as moderate to large numbers of ISEL-positive neurons were frequently found. No clear preference over the rostrocaudal axis was observed. Aside from the CA regions, only occasional labeling was observed in the dentate gyrus and entorhinal cortex, whereas surrounding cortical areas showed no labeling. Only one AD patient lacked ISEL-positive neurons in substantial numbers (subject 9). Exclusively necrotic morphology of ISEL-positive neurons, as opposed to apoptotic morphology, was observed in AD hippocampi, as shown by an absence of nuclear and cytoplasmic condensation, protrusions of the cell membrane, or apoptotic bodies. In controls, ISEL labeling was virtually absent.

Correlational analysis of the semiquantitatively obtained data from adjacent sections revealed that positive neuronal ISEL-labeling in subjects with AD was not anatomically related to the nNOS expression in neurons, but roughly matched nNOS expression in astroglial cells. The correlation was significant in CA1 (Pearson $r = 0.74$, $P < 0.01$) (Fig. 7a) and CA4 ($r = 0.58$, $P < 0.05$) (Fig. 7b). Furthermore, an inverse correlation between neuronal nNOS expression and

AT8 immunoreactivity in CA2/3 neuronal bodies was found ($r = -0.85$, $P < 0.01$) (Fig. 8).

DISCUSSION

Our study provides the first evidence that production of NO in AD is upregulated by nNOS activation in reactive astrocytes. We demonstrate that numerous nNOS-positive reactive astrocytes surround β -amyloid plaques and are present at places of clear and overt neuronal loss (i.e., particularly entorhinal cortex layer II), and in fields with increased levels of DNA fragmentation (CA1 and CA4). We also show that a portion of the intensively nNOS-positive CA2/3 pyramidal neurons are AT8-negative and appear spared in AD.

In response to the stimulation of microglial cytokines IL-1 α and IL-1 β , primary human astrocytes in culture produce substantial amounts of NO, which can result in a marked neuronal loss (7). β -Amyloid has also the strong ability to induce astrocyte activation which, in part, depends on apoE genotype (21, 46). So far, it has been reported that iNOS expression is upregulated in reactive astrocytes adjacent to β -amyloid plaques in AD, suggesting that iNOS may contribute to the pathogenesis of AD (62). Our study revealed that nitric oxide released by reactive astrocytes surrounding β -amyloid plaques is produced by nNOS as well as iNOS. This finding suggests that concurrent activation of iNOS and nNOS in reactive astroglial cells may augment the production of synthesized NO. In support of this view also goes the fact that the amounts of nNOS-positive astroglial cells found in this study (EC > CA1 > CA4 > subiculum) correlate well with the known anatomical distribution patterns of neuronal loss in these regions in AD (19, 54, 65).

Recent findings indicate that in the early stages of AD, reactive astrocytes are present around plaques that do not contain degenerating neurites, and may thus significantly modify the plaque environment (43). This suggests that neurotoxic effect of NO derived from a large number of reactive astrocytes may be an important mediator of the disease progression. Future studies should therefore investigate the possible association between maturation of plaques and nNOS containing reactive astrocytes more closely.

The hippocampal and entorhinal pyramidal neurons that stain weakly with nNOS, or which do not stain at all, are the same cells that show degenerative changes in AD. However, in contrast to the study of Thorns and colleagues (56), we found a strong nNOS staining of the spared CA2 and CA3 neurons. This clear relation between nNOS-positivity and AT8-negativity in the perikarya of these CA2/3 neurons strongly suggests the presence of a selective resistance of this cell population to hyperphosphorylation of tau and degeneration in AD. Therefore, hippocampal pyramidal neurons that express strong nNOS immunoreactivity may be resis-

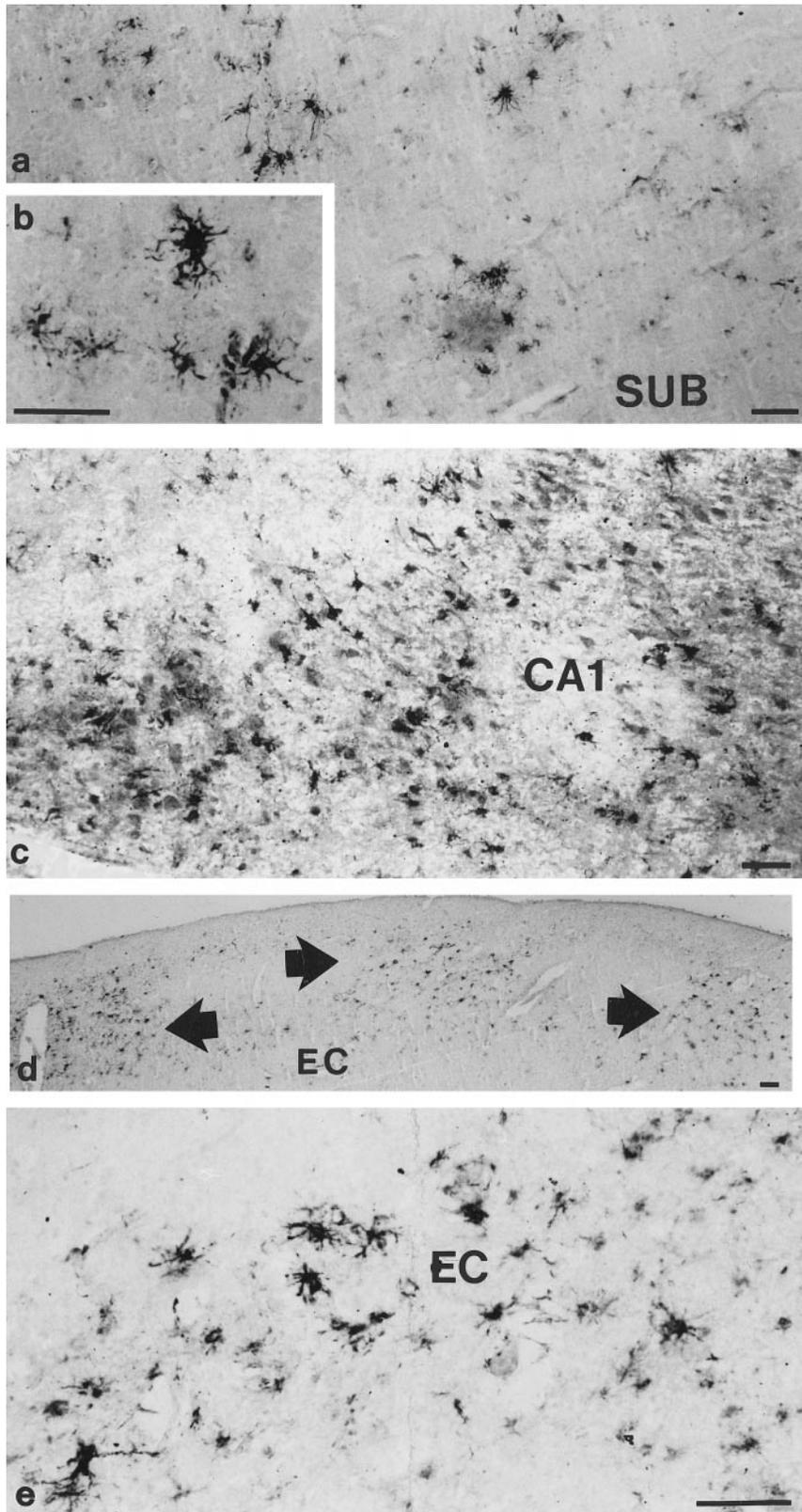


FIG. 5. nNOS-positive reactive astrocytes in AD hippocampus in subiculum at lower (a) and higher (b) magnification. (c) Astroglia of nNOS-positive reactive astrocytes in the CA1 of a control subject. (d and e) Intensive nNOS immunoreactivity of the reactive astroglial cells at the places of overt loss of entorhinal neurons in AD patient (arrows). Scale bars in (a) and (b) 500 μm ; (d) 50 μm . SUB, subiculum; EC, entorhinal cortex. Scale bars, 50 μm .

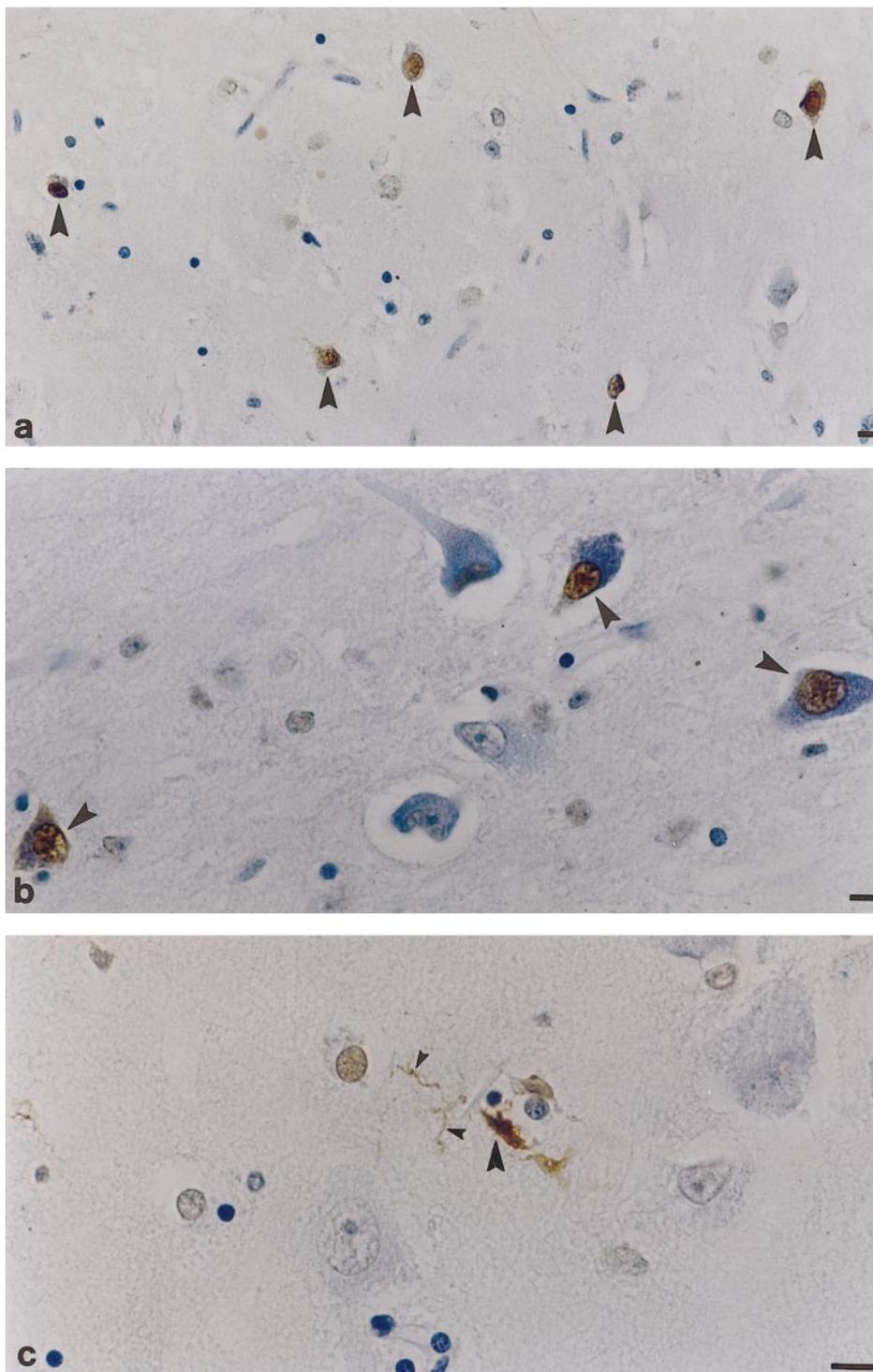


FIG. 6. ISEL-positive neurons (arrowheads) in CA1 (a) and CA4 (b) without signs of membrane blebbing, pronounced nuclear condensation, and apoptotic bodies. (c) The cell body of ISEL-positive microglial cell (large arrowhead). Small arrowheads show ISEL-positive microglial protrusions. Scale bars, 10 μm .

tant to degeneration in AD, whereas those with smaller amounts of this enzyme may not have such a neuroprotective mechanism and would display degenerative changes.

The reactive astrocytosis in the CA1 field of AD subjects 5, 7, and 9 and control subjects 4 and 8, may be related to cardiopulmonary insufficiency or myocardial infarction that was present in these patients. Also, the

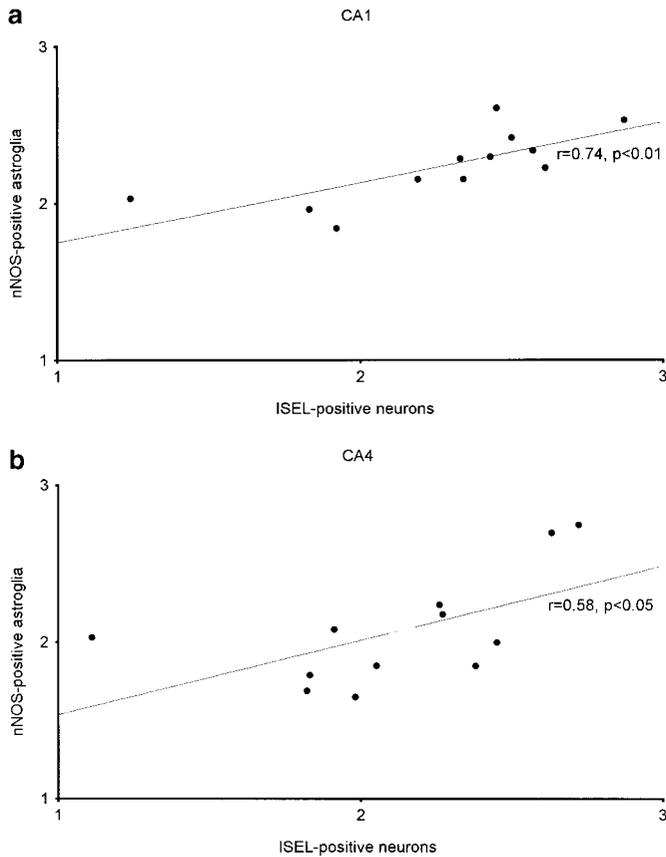


FIG. 7. Correlation between nNOS-ir reactive astroglial cells and ISEL labeling in CA1 (a) and CA4 (b).

small numbers of weakly nNOS-positive reactive astrocytes seen in CA4 of the control subjects 4 and 8 may be related to myocardial infarction. These findings suggest that the increased production of NO that occurs after ischemia (36), may also be, at least in a part, the consequence of nNOS expression induced in astrocytes.

Our results confirm that increased DNA damage vulnerability is present in the AD hippocampus (29, 31, 50, 51). There was only one AD patient who did not show a substantial number of ISEL-positive neurons, for which no explanation can be given at present. Fixation time or pH could, theoretically, be involved (25, 26, 32). The distribution of DNA fragmentation was particularly prominent in CA1, CA4, and subicular areas, whereas in the entorhinal cortex and dentate gyrus showed only very occasional labeling. Surrounding cortical areas showed no labeling, which suggests a strong anatomical preference of the pathological changes for the hippocampal region. The observed ISEL labeling of microglial cells throughout their cytoplasm and protrusions, often with an intact nucleus, has been observed before (29, 31) and is likely to result from the phagocytosis of fragmented DNA from adjacent dying cells rather than from undergoing apoptosis themselves.

On the basis of ISEL, *in vitro* results, and immunocytochemistry for apoptosis-related proteins (1, 47, 52) several authors have suggested that apoptosis occurs in AD. However, the present study found only necrotic morphology of ISEL-positive neurons in AD, which agrees with the known time kinetics of apoptotic cell death (41, 42, 51). Necrotic cells were in fact defined as ISEL-positive cells that did not display apoptotic morphology. By definition, ISEL-positive apoptotic cells show not only a brown DAB precipitate, but also display clear morphological features, such as an isolated occurrence, strong chromatin condensation, pyknotic nuclei or clear presence of apoptotic bodies (37, 58). Necrotic cells on the other hand fail to show these specific features and rather display a similar size as neighboring neurons, with an evenly stained nucleus and often with clear disruptions of the nuclear membrane visible. In addition, detection of dying cells is supposed to be a rapid, ongoing process and thus represents a turnover state rather than a fixed entity like cell number. Consequently, it reveals considerably smaller numbers of cells per section than are visible with normal staining techniques. The number of dying cells we see is considerably more than in other tissues with only apoptosis present. As it has been calculated that apoptotic morphology is only present for only a few hours in the brain, ranging from 3 to 72 h (9, 23), should very large numbers of cells per thin section of a chronic disease like Alzheimer's be present, this would imply that the entire structure would be fully degenerated in days, which is very unlikely. This also suggests that not apoptosis, but necrosis and increased DNA vulnerability are major phenomena in postmortem AD brain, which is supported by previous studies as well (31, 50).

Whether the enhanced level of DNA breaks found in the AD hippocampus underlies or is the consequence of degenerative phenomena, remains to be established. Apparently, our present findings show that DNA dam-

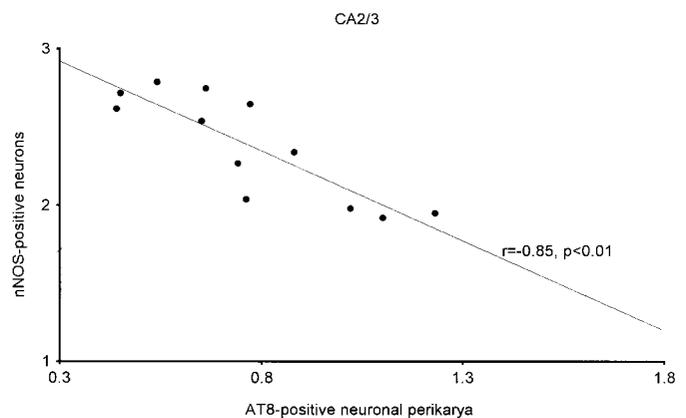


FIG. 8. Correlation between nNOS- and AT8-positivity in CA2/3 pyramidal neurons.

age in the hippocampus of AD brain exhibits a high degree of colocalization with nNOS expression in reactive astrocytes. This finding is in accordance with the colocalization between nitrotyrosine (which is marker for the reaction product of nitric oxide) and neuronal DNA damage (53). It also agrees with the previous study of Lassman and colleagues, who calculated that a major proportion of the ISEL positive cells is astrocyte-related (29). Furthermore, reactive astrocytes that express nNOS are also recently found in the spinal cord of transgenic mice expressing a human Cu/Zn superoxide dismutase mutation (6). This finding indicates that amyotrophic lateral sclerosis (ALS) and AD may share some common pathological mechanisms.

Considering the distribution and proportion of nNOS labeled pyramidal and nonpyramidal cells, our results are in very good concordance with the paper published by Egberongbe and colleagues (14), who used similar experimental conditions (antibody to rat nNOS and 4% paraformaldehyde fixation of the tissue), and (with the exception of CA1 field) the paper of Doyle and Slater (13). In comparison with these and other studies on nNOS expression in normal and AD brain (56), we report here also the occasional nNOS-positivity of the deep subicular pyramidal neurons in normal brains. With respect to differences with the results obtained by the NADPH-diaphorase (NADPH-d), which is widely used as a histochemical marker for NOS (e.g. 49), at this moment the most plausible explanation is that formaldehyde fixation differentially affects NADPH-d staining. Dinerman and colleagues (12) compared the effects on hippocampal NADPH-d staining on rat brains *in situ* with either a 2% glutaraldehyde/0.5% paraformaldehyde mixture or 2% formaldehyde. Alone, the paraformaldehyde produced no NADPH-d staining of pyramidal neurons, whereas glutaraldehyde plus paraformaldehyde fixation resulted in strong NADPH-d staining of pyramidal neurons.

Besides being dependent on type of the fixative used, nNOS immunodetection may also be influenced by ante- and postmortem factors like agonal state and fixation time (13, 14, 45, 56, 64). The reason, e.g., why we failed to always find nNOS positivity in CA1 neurons whereas Doyle and Slater did (13), may lie in the fact that these authors used antibodies raised to protein fragments of human nNOS and short (one week) 2% paraformaldehyde fixation of the tissue. However, since most of these variables are matched for between the groups, they can not explain the present results in subjects with Alzheimer's disease and controls. Another possible explanation may be that nNOS is present, but not active (13), which is not discriminated for by the antibody.

Although the present results in adjacent sections demonstrate convincing evidence, double immunofluorescent-labeling studies could further confirm the nNOS and GFAP colocalization, while future quantita-

tive studies based on unbiased stereological principles may confirm our present semiquantitative observations. In conclusion, the mechanisms of DNA fragmentation by NO-mediated oxidative damage and NMDA-linked neurotoxicity seem to be quite complex. Nevertheless, we think that upregulated production of NO play a causal role in AD pathogenesis, and consequently, nNOS may provide an important therapeutic target. In the future, it would be particularly useful to investigate the role of NO released from astrocytes with respect to the efficacy of antioxidative, neuroprotective and nonsteroidal anti-inflammatory agents in treating AD.

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