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## In Situ Labeling of Dying Cortical Neurons in Normal Aging and in Alzheimer's Disease: Correlations with Senile Plaques and Disease Progression

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**Abstract.** We examined the degeneration of neocortical neurons in normal aging and Alzheimer's disease (AD) using terminal transferase (TdT)-mediated deoxyuridine triphosphate (d-UTP)-biotin nick-end labeling (TUNEL), a method that identifies DNA strand breaks and constitutes a positive marker for dying neurons. TUNEL was positive in neurons, glia, and microglial cells in AD but not in younger or age-matched cognitively characterized controls. Neuronal labeling in AD was most conspicuous in cortical layer III in the early stages of the disease and became more widespread as the disease progressed. In addition, we observed TUNEL of lamina III neurons in a subset of older subjects who had normal cognition but abundant neocortical senile plaques. In concert, the availability of a direct marker of dying neurons allows for specific correlations of cell death with other neuropathological markers as well as clinical variables. Observations from the present study suggest that the death of cortical neurons precedes the symptomatic stage of AD and evolves in parallel with the clinical progression of the disease and that there appears to be an association between the degree of cell death and the severity of senile plaques.

**Key Words:** Amyloid; Cell death; Neocortex; NFT; Senile plaques; TUNEL.

### INTRODUCTION

Alzheimer's disease (AD), the most common dementia of late life (1), is characterized clinically by cognitive and behavioral abnormalities (2). Neuropathological features of AD include cerebral atrophy,  $\beta$ -amyloid protein (A $\beta$ ) deposits/senile plaques (SP), neurofibrillary tangles (NFT), and loss of synapses (3-6). Until recently, the assessment of neuronal degeneration in AD has been indirect and based on various morphometric methods to estimate the number of remaining neurons in affected regions of brain. Using these approaches, investigators have demonstrated loss of nerve cells in individuals with AD in the basal forebrain (7), locus coeruleus (8, 9), hippocampus (10-12), entorhinal cortex (ERC) (13), and neocortex (14-16). Morphometric studies of the cerebral cortex have suggested that pyramidal neurons of layers III and V are at risk in AD (16), a notion supported by the prominence of NFT in neurons of those layers (17).

In the present investigation, we use terminal transferase (TdT)-mediated deoxyuridine triphosphate (d-UTP)-biotin nick end labeling (TUNEL), a method for the detection of the 3' hydroxyl groups of fragmented DNA in degenerating cells and neurons (18-21). TUNEL allows the direct identification of dying cells and has been proposed as a specific method for in situ labeling of cells undergoing programmed cell death (PCD) or apoptosis. Beyond its still debated role as a specific marker for apoptotic neurons, TUNEL has proved to be an extremely sensitive method to evaluate the anatomical distribution and temporal course of neuronal death in animal models (22-27) and in human infectious (28) and neurodegenerative disorders (i.e. Huntington's disease (29) and AD (30-33)). Previous studies using TUNEL or in situ end labeling in AD have demonstrated degeneration of cortical neurons, astrocytes, oligodendrocytes, and microglial cells. In the present study, TUNEL was used to identify patterns of neuronal degeneration in the cerebral cortex of individuals with AD at various stages of the evolution of the disease and in a group of cognitively characterized older controls from the Baltimore Longitudinal Study of Aging (BLSA), some of whom, despite normal cognitive evaluation, had the neuropathological features of AD.

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### MATERIAL AND METHODS

#### Subjects

We examined autopsy brain tissues from twelve control subjects and 9 AD patients (Table). Clinical diagnoses of AD followed NINCDS-ADRDA criteria (2). Ten control subjects, >65 years of age, were participants in the BLSA and had thorough cognitive and neurological evaluations, the majority within one year prior to death. The two young controls (7 months and 21 years of age) did not have cognitive evaluations, but had no history of neurological disorder or dementia. We also studied

TABLE 1  
Tunel of Neocortex and ERC in Control and AD Cases

Group	Case #	Age (yrs) sex	MMSE- BIMC scores	Post- mortem delay (hrs)	Brain weight (grams)	Neo- cortical SP/ mm <sup>2</sup>	Tunel**		Cause of death
							Neo- cortex	ERC	
Young controls	5780	21 M	n/a	24	1,290	0	-	-	trauma
	5827	7/12 M	n/a	18	650	0	-	-	trauma
Older controls without significant neuropath- ology	818	93 M	28-2	19	1,320	0	-	-	pneumonia, sepsis
	1146	90 M	30-0	24	1,516	4	-	-	pneumonia
	1033	74 M	29-2	35	1,550	5	-	-	aortic dissection
	1092	81 F	27-2	8	1,092	0	-	-	pulmonary embolus, cerebral infarct
	1061	77 F	30-5	48	1,210	3	-	-	complications of diabetes mellitus
Older controls with pa- thology of AD	1103	69 M	29-0	23	1,110	7	-	-	lung carcinoma
	862	94 F	25-2	13	1,270	0	+	++	pneumonia
	1141	91 M	30-0	16	1,410	>25	+	+	prostate carcinoma
Early AD	1139	87 M	28-3	11	1,230	>25	++	+++	pancreatic cancer
	767	81 M	30-1	72	1,160	>25	+	-	cardiac arrest
	890	97 M	24-8	11	1,200	24	+	+++	pneumonia
	976	90 M	n/a-10	8	1,380	>25	++	+	n/a
Advanced AD	1101	80 F	26	11	1,410	>25	n/a	+	renal infarct, peritonitis
	916	86 F	24-7	4	1,040	11	-	+	pneumonia, sepsis
	1044	80 F	1-n/a	32	980	>50	++	+++	consistent with pneumonia
	1049	89 F	0-n/a	23	1,070	>50	++	++	consistent with pneumonia
	1024	70 M	3-n/a	9	1,120	>50	-	-	cardiac arrest
1081	59 M	<10-n/a	7	1,100	>50	+	++	cardiac arrest	
1135	59 M	4-n/a	19	1,130	>30	++	+++	consistent with pneumonia	

\* Neocortical SP/mm<sup>2</sup> represents the highest density of diffuse and neuritic SP throughout the neocortex.

\*\* TUNEL: -, absent; +, sparse (<5 neurons/mm<sup>2</sup>); ++, moderate (6-20 neurons/mm<sup>2</sup>); +++, frequent (>20 neurons/mm<sup>2</sup>). Abbreviations: n/a, not available; M, male; F, female.

the brains of 9 demented patients with pathologically confirmed AD according to CERAD criteria (34) who died in the early (Mini-Mental State Examination [MMSE] >20 [35]) or advanced (MMSE <10) stage of AD.

### Conventional Neuropathology

Brains were immersion fixed in 10% buffered formaldehyde with postmortem intervals that ranged from 4 to 72 hours (Table 1). Tissue blocks were processed for paraffin embedding. Histological sections (10  $\mu$ m) were obtained from blocks of tissue that contained the hippocampal formation and adjacent ERC and inferior temporal cortex (ITC). Tissue sections were stained with hematoxylin & eosin and silver using the Hirano method (36) and were immunostained with an antibody for A $\beta$  (a gift of Athena Neurosciences, South San Francisco, California), using standard immunoperoxidase procedures (37).

### TUNEL

Histological sections were processed essentially according to Gavrieli et al (38). Briefly, 20- $\mu$ m sections were deparaffinized at 60°C for 30 minutes, washed two times in xylene for 5 minutes each, and then rehydrated in graded alcohols by two-minute incubations two times in 100%, 95%, and 70% ETOH

followed by water. Subsequently, sections were washed in 10 mM Tris-HCl (pH 8.0) for 5 minutes and digested with proteinase K (Boehringer-Mannheim, Indianapolis, Indiana), and 20  $\mu$ l/ml in 10 mM Tris-HCl (pH 8.0) for 15 minutes at room temperature. Following 2-minute washes 4 $\times$  in dH<sub>2</sub>O, inactivation of endogenous peroxidase activity with 3% H<sub>2</sub>O<sub>2</sub> for 5 minutes, and additional 2-minute rinses 4 $\times$  in dH<sub>2</sub>O, sections were preincubated in Tdt buffer (30 mM Tris base, pH 7.2, 140 mM sodium Ca cacodylate [Sigma, St. Louis, Missouri]), and 1 mM cobalt chloride (Sigma) for 10 minutes at room temperature. During preincubation, selected sections from some controls and AD cases were treated with DNase (Boehringer-Mannheim) (4  $\mu$ l/ml) to serve as positive controls. Following thorough rinses in dH<sub>2</sub>O, tissues were incubated in Tdt buffer containing 500 units/ml Tdt and 4  $\mu$ l/100 ml of biotinylated dUTP (Boehringer-Mannheim) for one hour at 37°C. The reaction was stopped by immersing slides in 2 $\times$  sodium citrate standard saline citrate (Sigma) for 15 minutes at room temperature. After a 5-minute wash in 1 M phosphate-buffered saline (PBS), pH 7.4, nonspecific binding sites were blocked with 5% BSA (fraction V, Sigma) in 1 $\times$  PBS for 10 minutes; sections were rinsed several times in dH<sub>2</sub>O and incubated in avidin-biotin solution in 1 $\times$  PBS (Vector, Burlingame, California) for

30 minutes at 37°C. Finally, after 3 washes in 1× PBS, sections were incubated in solution containing diaminobenzidine (Sigma) 0.025% in 1× PBS plus 0.03% H<sub>2</sub>O<sub>2</sub>, washed thoroughly, air dried, counterstained with cresyl violet, and coverslipped.

## RESULTS

### Neuropathology

Ratings of the densities of NFT and neuritic SP as sparse, moderate, or frequent follows recommendations made by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (34). Seven older control subjects had sparse or no A $\beta$  deposits or neuritic SP in the neocortex. However, 3 older individuals had moderate or abundant neuritic SP in the cerebral cortex, consistent with a diagnosis of possible AD, according to CERAD criteria (34). All 10 older controls showed sparse NFT in the ERC and hippocampus, but none in the neocortex. Younger controls were free of NFT and SP.

The brains of individuals with AD showed various degrees of atrophy, the severity increasing in advanced cases. Microscopic examination showed frequent neuritic SP and variable densities of NFT in neocortex, with frequent NFT in ERC and hippocampus. Advanced cases had larger numbers of NFT and SP in neocortex than early cases, but all fulfilled the histopathological criteria for a diagnosis of definite AD (34). The most severely affected cases showed marked rarefaction of superficial cortical layers and gliosis throughout the width of the neocortex.

### TUNEL

For descriptive purposes, we divided subjects into 5 groups based on their clinical and neuropathological evaluations: young controls; older controls without significant neocortical lesions; older controls with frequent neocortical neuritic SP; early AD; and advanced AD (Table 1). We considered neurons to be TUNEL-positive or labeled when immunoreactivity covered at least 80% of the cross-sectional area of the nucleus in cells >20  $\mu$ m independent of nuclear morphology or chromatin condensation (Fig. 1B, 2A, B). The number of neurons labeled was rated as follows: absent; sparse (+) (<5 neurons/mm<sup>2</sup>); moderate (++) (6–20 neurons/mm<sup>2</sup>); or frequent (+++) (>20 neurons/mm<sup>2</sup>). We followed the neuroanatomical nomenclature of Amaral and Insausti (39). The ERC was identified by islands of neurons in layer II; the trans-entorhinal cortex (trans-ERC) corresponded to the border between ERC and area 35 (40) in the medial bank of the collateral sulcus; and the ITC corresponded to the neocortex in the lateral bank of the collateral sulcus and beyond.

No TUNEL was observed in the neocortex, ERC, or hippocampal formation of young controls. Among the 7 older controls without neocortical SP (Fig. 1A), only one (#862) showed TUNEL of neurons, but the remaining 6

did not. In case #862 (not shown in Fig. 1), neuronal labeling was most prominent (++) and had a distinct laminar pattern in layer III of the ITC. In addition, labeling was noted in neurons of layer V of the ITC (+), in the trans-ERC (+), and ERC (+) and in hippocampal CA4 (+), CA1 (+), and subiculum (+). In contrast to the previous group, the brains of all 3 older controls with abundant neocortical SP revealed TUNEL in lamina III of the ITC (++) (Fig. 1B), throughout all layers of ERC (++) and in hippocampal CA1 (+) and CA2 (+).

TUNEL was present and conspicuous in all AD brains (Fig. 2) with the exception of a very advanced case (#1024). In the early stages of AD, we observed a distinct laminar pattern of labeling involving lamina III of the ITC (+++) and extending to the trans-ERC (+), ERC (++) and hippocampus involving predominantly CA4 (+ or ++) (Fig. 3). Although neuronal labeling predominated, we also detected occasional immunoreactivity in glial elements. In advanced AD, TUNEL involved neurons and glia throughout all laminae of the ITC (+++) that did not appear to correlate to the distribution of NFT or SP. In these advanced cases, labeling occurred in the trans-ERC (+) and ERC (+) and in hippocampal CA4 (++) and CA1 (+/++), and dentate (+/+++). In general, labeling of hippocampal neurons in AD was robust in CA4 and CA1 but sparse in the dentate gyrus and virtually absent in CA2-3.

Although TUNEL of neurons in early cases of AD had a distinct laminar pattern, A $\beta$  deposits and SP appeared distributed throughout the thickness of the cortex. Thus, we were unable to delineate a correlation between TUNEL-labeled cells and A $\beta$  deposits or SP. Furthermore, we observed no labeling of neurons in layer II of the ERC that showed abundant NFT in silver sections.

In addition to TUNEL of neurons, we observed labeling of glial and microglial cells in some cases of AD, as described by previous investigations (30–32). These cells were identified by their conventional morphologies. Because the focus of this investigation was the pattern of neuronal degeneration, no attempt was made to identify glial and microglial cells by double immunolabeling. TUNEL of glia or microglia was not present in controls. Treatment of selected histological sections with DNase prior to TUNEL led to labeling of all nuclei in tissues from controls and cases of AD.

## DISCUSSION

Our observations provide direct evidence of degenerating neocortical and ERC neurons in AD and in a subset of older subjects with normal cognition. Several points are relevant to the interpretation of our observations. TUNEL of cells was accepted originally as a manifestation of apoptosis (38, 41); however, the possibility that it may be associated with nonapoptotic cell death has also

been raised (42). As in a previous study (31), we observed that the majority of neurons with nuclear labeling did not show the chromatin condensation characteristic of apoptosis, suggesting that these neurons were dying by necrosis or a nonapoptotic PCD mechanism (43). Thus, in the present study, we interpret TUNEL as a marker of cell injury and/or degeneration, independent of the mechanism of these processes. Because TUNEL identifies dying cells during a narrow time window, labeled neurons represent only a subset of all dying neurons. For reasons of probability, labeled cells most likely belong to neuronal populations with the highest rates of degeneration. The large proportion of TUNEL-positive neurons involved in some cases of AD (Fig. 2) was surprising and raises concerns of whether this massive neuronal degeneration can be attributed exclusively to AD. We cannot rule out that immediate premortem circumstances may have contributed to the death of neurons that were already vulnerable because of AD (31). This massive TUNEL labeling has also been reported in previous studies (31, 33). The influence of postmortem delay on TUNEL is also an important consideration. However, a comparison of postmortem delays and the presence or absence of TUNEL did not reveal any significant correlation. This observation is consistent with previous reports of the absence of correlation in human brains between the number of labeled cells and the amount of postmortem delay (28, 31) and with an experiment demonstrating that varying the postmortem exposure of rat brains to room temperature fails to increase the number of neurons staining with TUNEL; the maximum postmortem time in that experiment was 6 hours (29). A recent study (33) showed increased TUNEL labeling in the brains of cases of AD with postmortem intervals greater than 6.5 hours. In that investigation, cells labeled with prolonged postmortem delays were distributed throughout all cortical layers but were never confined to neurons of layer III, as observed in our cases. Previous studies have demonstrated that sensitivity of TUNEL can be increased by microwave treatment prior to protease K digestion (44). It is possible that moderate postmortem autolysis results in a similar increase in sensitivity. Thus, a

short postmortem interval may also result in decreased sensitivity to TUNEL and perhaps account for the lack of labeling in some cases of advanced AD with short postmortem intervals (i.e. #1024).

The cause of death (Table 1) and the length of agonal period may also influence TUNEL. However, we observed no correlations of labeling with these variables. For example, among TUNEL(-) controls, some died suddenly (i.e. aortic dissection, pulmonary embolus), whereas others have had prolonged agonal periods and hypoxia (pneumonia and sepsis). Among subjects with TUNEL(+), there were instances of presumably sudden death as well as protracted agonal periods.

The laminar pattern of TUNEL-positive neocortical neurons observed in early cases of AD is consistent with previous descriptions of laminar loss of neurons, atrophy, and development of NFT in AD (16, 17, 45). The specificity of this laminar pattern of TUNEL to AD is supported by the absence of staining in age-matched controls who were free of neuropathology and contrasts with TUNEL of neurons in most frontal cortical layers recently reported in cases of Huntington's disease (29) or attributed to prolonged postmortem interval in cases of AD (33). Moreover, the same pattern of labeling limited to layer III was present in brains with a wide range of postmortem intervals (11 to 72 hours).

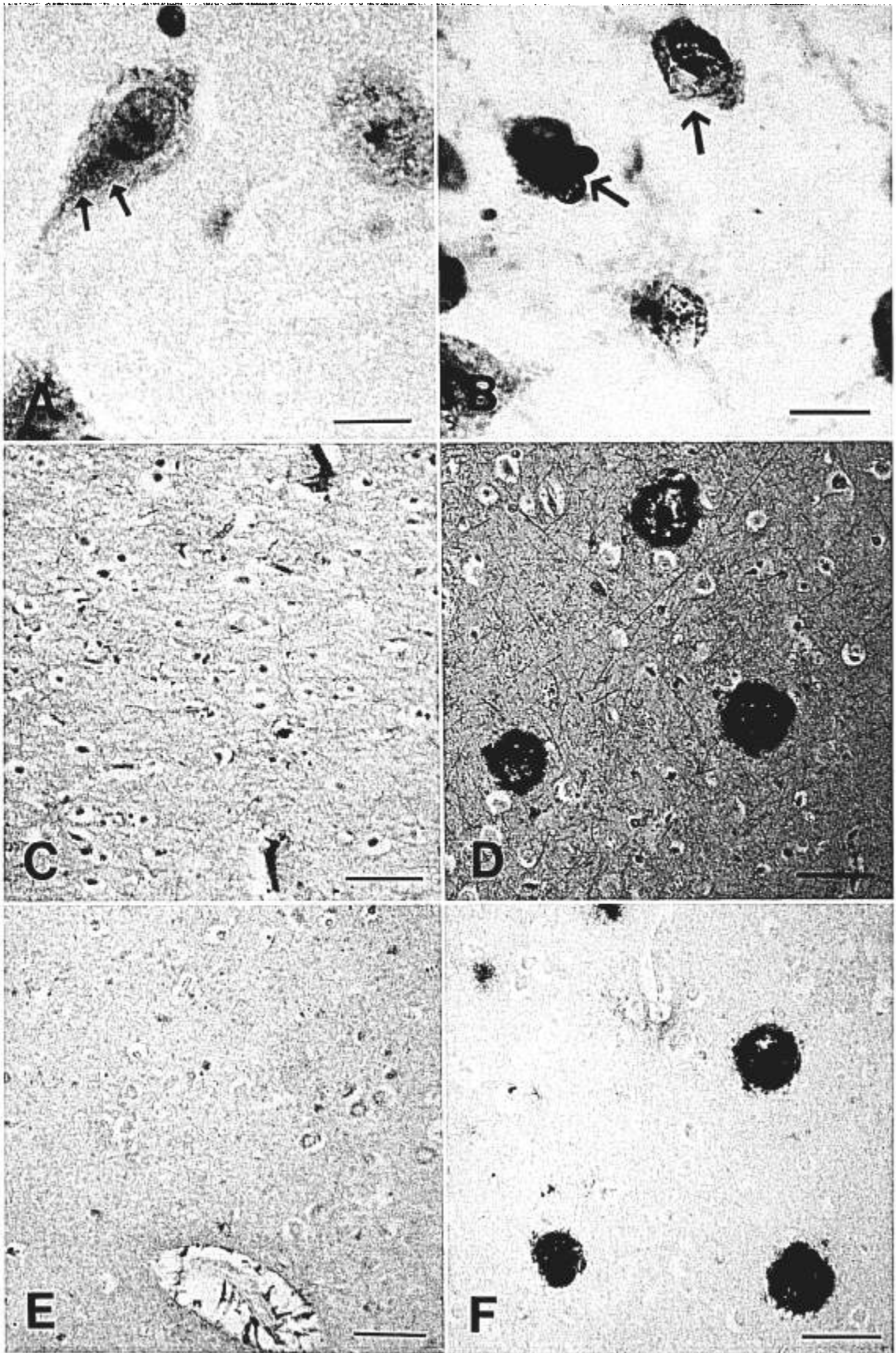
Pyramidal neurons of lamina III give origin to corticocortical connections. Thus, degeneration of these neurons, as demonstrated by TUNEL, should result in multifocal disruption of neocortical circuitry and the destruction of parallel distributed cortical networks that underlie cognition (46). Our observations underscore the belief that AD is a laminar and regional disorder involving specific neural systems (45).

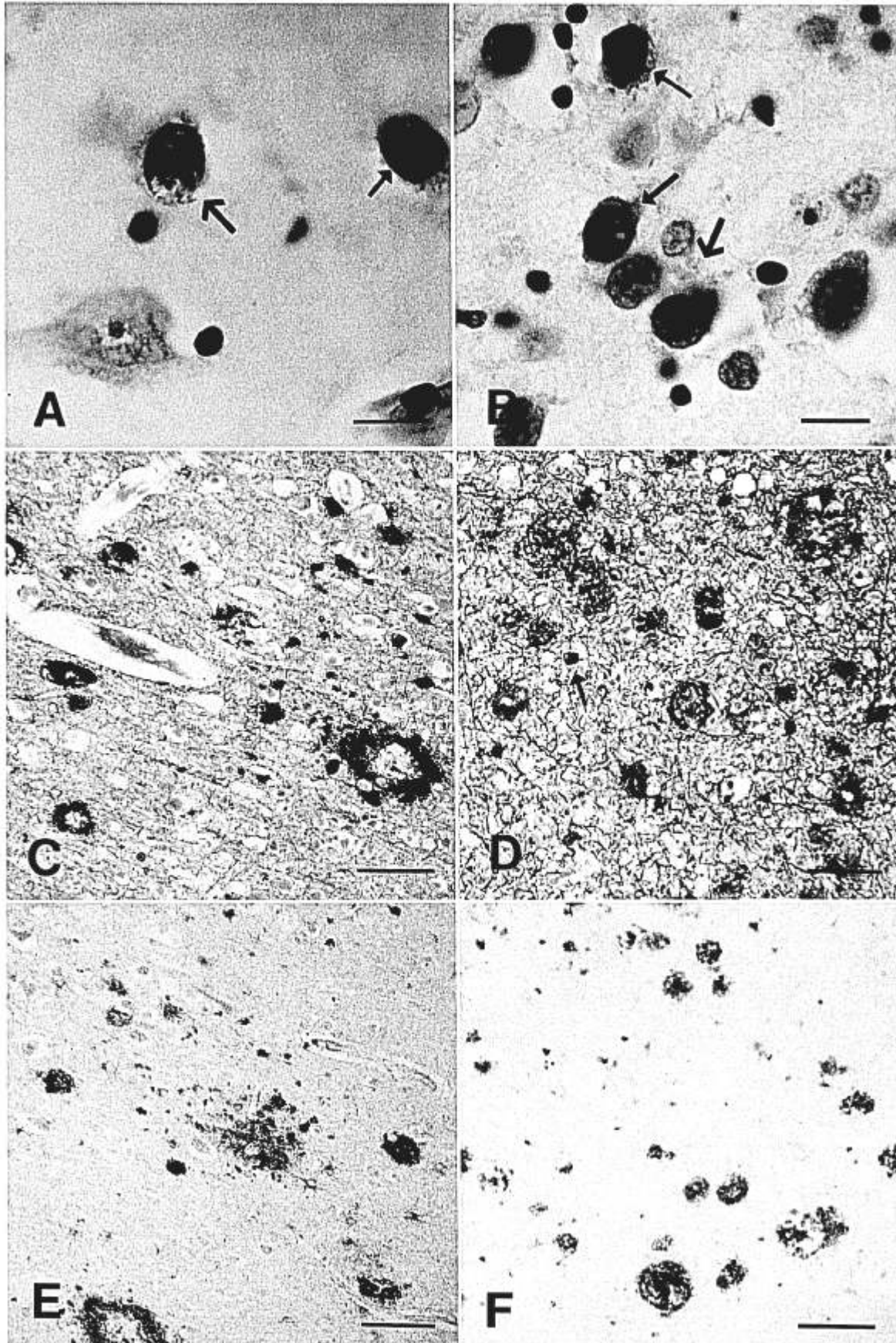
TUNEL of cortical neurons was observed in all cases of early AD and in most cases of advanced AD; however, we observed no labeling in one subject in the late stage of the disorder. This finding suggests that, in this late stage, either the rate of neuronal death is very low or the majority of vulnerable neocortical neurons has already degenerated. A similar observation has been made in the

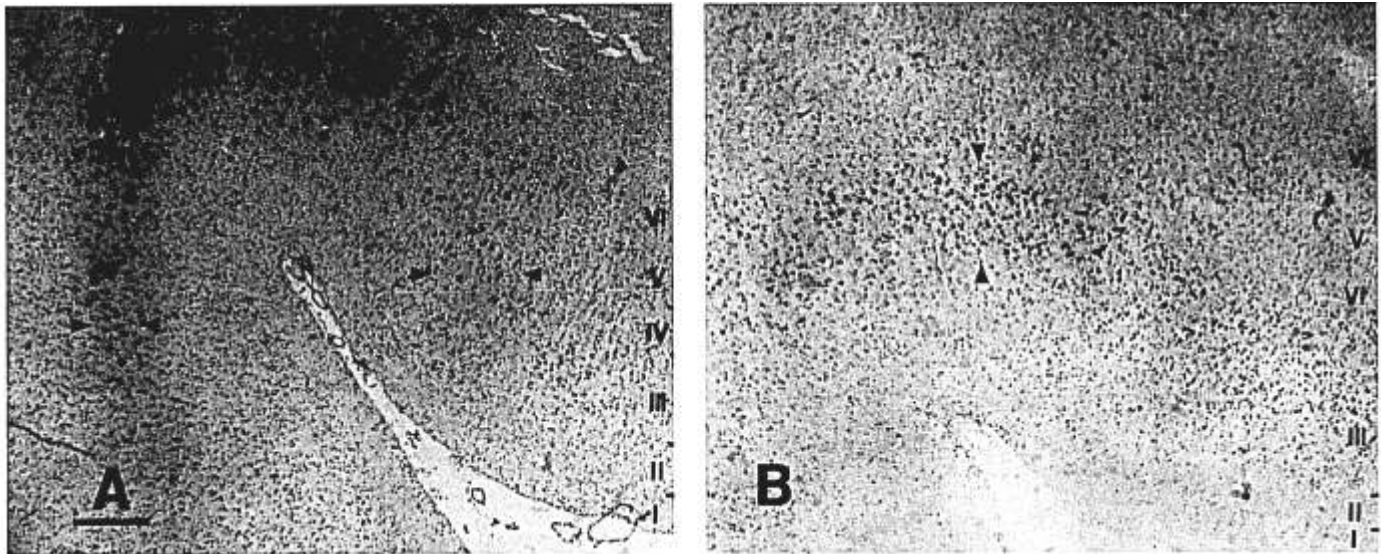
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**Fig. 1.** Representative tissue sections from the inferior temporal cortex in older controls without (left) and with (right) senile plaques. Sections were processed for TUNEL (top), Hirano silver staining (middle), and A $\beta$  immunoreactivity (bottom). In the subject without SP, there is no TUNEL staining of the neuronal nucleus (A), and the faint immunoreactivity present in the perikaryon corresponds to lipofuscin (arrows). The cortex of the subject with SP, demonstrated by silver (D) and A $\beta$  immunostaining (F), shows intense TUNEL staining of neuronal nuclei (B) (arrows). Scale bars: A, B, 25  $\mu$ m; C-F, 50  $\mu$ m.

**Fig. 2.** Representative tissue sections from the inferior temporal cortex in cases of early (left) and advanced (right) AD. Sections were processed for TUNEL (top), Hirano silver staining (middle) and A $\beta$  immunostaining (bottom). Abundant SP and amyloid deposits are present in both stages of the disease (C-F), but some NFT (arrow) are present in advanced AD (D). TUNEL is abundant in nerve cells of both groups (A, B). In A, 2 neurons with reduced size show intense TUNEL staining of nuclei (arrows). A healthy neuron (bottom left) is unlabeled. In B, several neurons from an advanced case of AD appear atrophic and exhibit intense labeling (arrows). There is also labeling of smaller cells, probably glia. Scale bars: A, B, 25  $\mu$ m; C-F, 50  $\mu$ m.







**Fig. 3.** TUNEL counterstained with cresyl violet. TUNEL-positive neurons (arrowheads) in the inferior temporal cortex (ITC) (A) and entorhinal cortex (ERC) (B) of an early case of AD (#976). The ITC shows a distinct laminar labeling pattern limited almost exclusively to lamina III, whereas the ERC reveals more widespread neuronal labeling. Scale bar: 800  $\mu$ m.

striatum of subjects with advanced (Vonsattel grade 4) Huntington's disease (29) and end-stage AD (33).

Our observations may provide insight into the natural history of AD. The observations of TUNEL-positive cortical neurons in subjects with normal cognitive evaluations and abundant neocortical neuritic SP are of particular importance. This category of individuals has been described by several investigators (47–51) and has been classified as "pathological aging" (49) or "possible AD" in the CERAD classification (34), but their relationship to AD has remained controversial. TUNEL of neurons in lamina III of the neocortex indicates that the brains of these subjects are undergoing neuronal degeneration with a distribution similar to that of demented patients in the early stages of AD. This observation supports the idea that subjects who are cognitively intact but have abundant neuritic SP in the brain are in a preclinical stage of AD (47, 51). Furthermore, we observed TUNEL of neurons in an older control (#862) who had no A $\beta$  deposits or SP in neocortex, suggesting that, in some older subjects, neurons may degenerate independently of A $\beta$  deposition. This subject had her last clinical evaluation three years before death and was classified as normal on the basis of a Blessed Information-Memory-Concentration score (52) of 2. However, she had an abnormal MMSE (score of 25) that was difficult to interpret because of the subject's poor vision. Furthermore, it is possible that she had declined cognitively during the intervening period.

TUNEL of cortical neurons and other cells in AD may provide clues as to the pathogenetic mechanisms associated with the disorder. Although the relationship of TUNEL to apoptosis is debatable, neuronal labeling in

AD raises the possibility that genes that regulate PCD (53, 54) play a role in the neuronal degeneration associated with AD. This possibility warrants further exploration including ultrastructural and gene expression studies (55) beyond the scope of the present investigation.

In conclusion, our observations of TUNEL of neurons in AD constitute a direct demonstration of neuronal degeneration in hippocampus, ERC, and ITC and highlight the laminar pattern of this process in neocortex. These observations are consistent with earlier predictions based on morphological/morphometric observations and with the clinical presentation of the disease. More importantly, this method can reveal, because of its high sensitivity and resolution at the cellular level, age-associated changes in the brain that have been heretofore undetectable. Finally, in the present study, the discovery of dying hippocampal, ERC, and neocortical neurons in cognitively intact individuals supports the notion that AD can have a preclinical stage characterized by abundant neuritic SP and neuronal degeneration in the hippocampus and cerebral cortex.

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