

# Apoptotic-Like Changes in Lewy-Body-Associated Disorders and Normal Aging in Substantia Nigral Neurons

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***In Parkinson's disease and other Lewy-body-associated disorders, the substantia nigra pars compacta undergoes degeneration, but the mechanism of cell death has not been previously described. The substantia nigra of normal and Alzheimer's disease cases were compared with substantia nigra from patients with Lewy-body-associated disorders (Parkinson's disease, concomitant Alzheimer's/Parkinson's disease, and diffuse Lewy body disease) using in situ end labeling to detect fragmented DNA. In situ end-labeled neurons demonstrated changes resembling apoptosis: nuclear condensation, chromatin fragmentation, and formation of apoptotic-like bodies. Ultrastructural analysis confirmed nuclear condensation and formation of apoptotic-like bodies. Apoptotic-like changes were seen in the substantia nigra of both normal and diseased cases; concomitant Alzheimer's/Parkinson's disease and diffuse Lewy body disease cases had significantly higher amounts of apoptotic-like changes than normal controls or Alzheimer patients. The finding of neuronal death by apoptosis may have relevance for the development of new treatment strategies for Parkinson's disease and related disorders. (Am J Pathol 1997, 150:119-131)***

In Parkinson's disease (PD) and other Lewy-body-associated disorders there is neuronal loss, gliosis, and accumulation of intracellular Lewy bodies in the pigmented nuclei of the brainstem.<sup>1</sup> The expression of extrapyramidal signs (bradykinesia, rigidity, and tremor) in these diseases is believed to be a conse-

quence of the death of dopaminergic neurons in the substantia nigra pars compacta (SNpc).<sup>2</sup> The SNpc of neurologically normal individuals also undergoes neuronal death with advancing age, although the pattern of cell loss is different from that seen in PD.<sup>3</sup> Other Lewy-body-associated disorders, such as concomitant Alzheimer's disease/Parkinson's disease (AD/PD) and diffuse Lewy body disease (DLBD), have SNpc damage and may exhibit parkinsonian motor symptoms.<sup>4,5</sup> Pure AD is a progressive cortical dementia without significant SNpc damage or motor symptoms.<sup>6</sup> There appears to be a spectrum of Lewy-body-associated and Alzheimer-associated diseases, including AD/PD, DLBD, senile dementia of the Lewy body type,<sup>7-10</sup> and the Lewy body variant of AD<sup>11,12</sup>; some of these disease categories may overlap.<sup>13</sup> The mechanism of cell death, as well as the etiology of these disorders, is unknown.

Two general mechanisms of cell death prevail in the literature, necrosis and apoptosis,<sup>14</sup> although other types have been described.<sup>15</sup> Primary necrosis typically involves cytoplasmic and nuclear swelling, loss of plasma membrane integrity, and release of cellular contents into the extracellular medium, often precipitating a significant immune response. Apoptotic changes include nuclear and cytoplasmic condensation, with subsequent apoptotic body formation. Apoptotic bodies consist of membrane-bound organelles and condensed cytoplasm; they may contain chromatin fragments with a nuclear en-

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velope.<sup>16</sup> An immune response is not mounted during apoptosis; rather, apoptotic bodies are phagocytosed by macrophages and surrounding cells<sup>16</sup> or microglia.<sup>17</sup> Apoptotic bodies eventually undergo secondary necrosis, either free or within phagosomes.<sup>18</sup> Secondary necrosis occurs after cell death and can be found after primary necrosis (termed oncosis by Majno and Joris<sup>19</sup>) or apoptosis.

The endonucleolytic cleavage of DNA that occurs during apoptosis can be detected using *in situ* end labeling (ISEL).<sup>20,21</sup> Such techniques have recently been used to describe apoptosis in human neurodegenerative disorders such as AD,<sup>22-24</sup> Huntington's chorea,<sup>24,25</sup> and amyotrophic lateral sclerosis.<sup>26,27</sup> In addition to apoptosis, necrosis has been reported to occur in AD.<sup>23</sup> Furthermore, a recent electron microscopic study has shown apoptotic cell death to occur in Parkinson's disease.<sup>28</sup>

In this paper, we have examined the cell death mechanism of SNpc neurons in patients with Lewy-body-associated disorders. Human postmortem midbrain tissue was examined for *in situ* end-labeled chromatin and apoptotic morphology at the light microscopic and ultrastructural level. Our results demonstrate that a primary mechanism of cell death in the SNpc resembles apoptosis.

## Materials and Methods

### Human Tissue

Archival human autopsy tissue was obtained from the McLean Hospital Brain Tissue Resource Center at Harvard Medical School (Boston, MA). Diagnoses of AD and PD were confirmed at autopsy by standard neuropathological criteria.<sup>29-36</sup> Concomitant AD/PD cases met diagnostic criteria for both diseases and are likely to be classified as the Lewy body variant of AD by current standards at McLean Hospital.<sup>11,12,37</sup> DLBD cases did not meet consensus criteria for AD but demonstrated extensive cortical Lewy bodies and subcortical changes typical of PD.<sup>37-40</sup> The presence of neuritic changes in the CA2-3 region of the hippocampus also supports the diagnosis of DLBD.<sup>41</sup> Three neurologically normal controls, five cases of AD, four AD/PD cases, seven DLBD cases, and five cases of PD without dementia were examined. Analysis of variance between disease cases and controls was performed on those cases with a postmortem interval of less than 24 hours.

### In Situ End Labeling

Formalin-fixed, paraffin-embedded midbrains were sectioned at 10  $\mu\text{m}$  and mounted on gelatin- or silane-coated glass slides. Free 3'-hydroxyl ends of DNA were detected using the Apoptag kit (Oncor, Gaithersburg, MD), following the manufacturer's directions. Briefly, terminal transferase was used to add digoxigenin-labeled dUTP to free 3'-hydroxyl ends of DNA. After incubation with a fluorescein-tagged anti-digoxigenin antibody, sections were counterstained with propidium iodide (0.5  $\mu\text{g}/\text{ml}$ ), mounted in Vectashield (Vector Laboratories, Burlingame, CA), and viewed under ultraviolet illumination using a fluorescein/propidium iodide double filter on a Zeiss Axiophot microscope. Sections were examined using a 40 $\times$  objective lens.

Each experiment included a positive control for apoptosis (mouse intestine)<sup>20</sup> and a negative control (omission of terminal transferase enzyme). Additional controls included heat inactivation of the terminal transferase enzyme by the procedure of Wood et al<sup>42</sup> as a negative control and RNase treatment (DNase-free; 100  $\mu\text{g}/\text{ml}$  for 15 minutes at 37°C) to show specificity of the terminal transferase enzyme for DNA.

Only neuromelanin-containing neurons of the SNpc were considered. Due to the close proximity of the medially located paranigral nucleus and the difficulty of distinguishing it from the SNpc proper, the paranigral nucleus was included in the analysis. The paranigral nucleus is known to undergo degeneration and Lewy body accumulation similar to the SNpc.<sup>1</sup> Pigmented paranigral and SNpc neuronal nuclei were counted across an entire midbrain hemisection and categorized as normal, apoptotic, or necrotic. Identification of normal SNpc neurons was based on the presence of a large, euchromatic propidium-iodide-stained nucleus, often containing a prominent nucleolus.

Criteria for apoptotic-like changes were 1) ISEL of chromatin and 2) condensation of the labeled chromatin, with or without break-up into clumps and strands. These nuclear changes appeared in intact SNpc neurons as well as in accumulations of neuromelanin (apoptotic-like bodies). Therefore, apoptotic-like changes were further subdivided into early changes (intact cell) and late changes (apoptotic-like bodies). The average number of total nuclei in a section was  $435 \pm 79$  (control),  $281 \pm 55$  (AD),  $176 \pm 65$  (AD/PD),  $169 \pm 37$  (DLBD), and  $139 \pm 33$  (PD). The average percentage of apoptotic-like neurons undergoing early changes was 7.6% (control),

10.4% (AD), 6.1% (AD/PD), 7.5% (DLBD), and 30.5% (PD); the rest were undergoing late changes.

Swelling of *in situ* end-labeled chromatin, indicative of primary necrosis (oncosis) was not seen. Free ISEL<sup>+</sup> chromatin strands, often continuous with clumps of chromatin and neuromelanin, were seen in many cases and may represent necrosis secondary to apoptosis. Labeled chromatin strands that were not associated with neuromelanin were also seen.

### Fluorescent Double Labeling

Three cases of DLBD and one case of AD/PD with high levels of apoptotic-like changes (average, 11.09%) were *in situ* end labeled and stained for microglia using an anti-HLA antibody (LN3; Zymed Laboratories, South San Francisco, CA). Sections were deparaffinized, rehydrated, microwaved for 4 to 6 minutes in citrate buffer (0.01 mol/L, pH6.5),<sup>43</sup> and incubated in LN3 antibody (1:25) overnight at 4°C. The TdT reaction from the Apoptag kit was then carried out, followed by incubation in both fluorescent secondary antibodies: anti-digoxigenin-fluorescein isothiocyanate (FITC; Apoptag kit) and anti-mouse-Cy3 antibody (Jackson ImmunoResearch, West Grove, PA; 1:100).

To examine astroglia, one case each of control, AD, AD/PD, and DLBD midbrains were double labeled with anti-gial fibrillary acidic protein (Dako, Carpinteria, CA; 1:50) and the Apoptag kit as described above.

Two cases of DLBD and one case of PD were double labeled with anti-tyrosine hydroxylase (anti-TH; AB152, Chemicon, Temecula, CA; 1:30) and LN3 antibody. The sections were incubated in the two antibodies overnight at 4°C. A peroxidase-anti-peroxidase kit (Sternberger Monoclonals, Baltimore, MD) was used to amplify the LN3 antibody and was visualized with anti-horseradish peroxidase-FITC antibody (Jackson ImmunoResearch; 1:200). The anti-TH antibody was visualized with anti-rabbit-Cy3 antibody (Jackson ImmunoResearch; 1:100).

### Ultrastructural Analysis

Electron microscopy was performed on archival formalin-fixed SNpc from two DLBD cases. Briefly, the tissue was postfixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, followed by fixation in 1.0% osmium tetroxide in cacodylate buffer. The tissues were stained with 2.0% uranyl acetate in 10% ethanol *en bloc*, dehydrated, and embedded in Epon (25 g of Epon/15 g of Araldite/55 g of dodecylsuccinic anhydride/2.5 g of benzyldimethylamine). Thin sec-

tions (60 to 70 nm) were contrast enhanced by double staining with uranyl acetate and lead citrate. Sections were viewed on a Zeiss 902 transmission electron microscope at 80 kV.

### Statistical Analysis

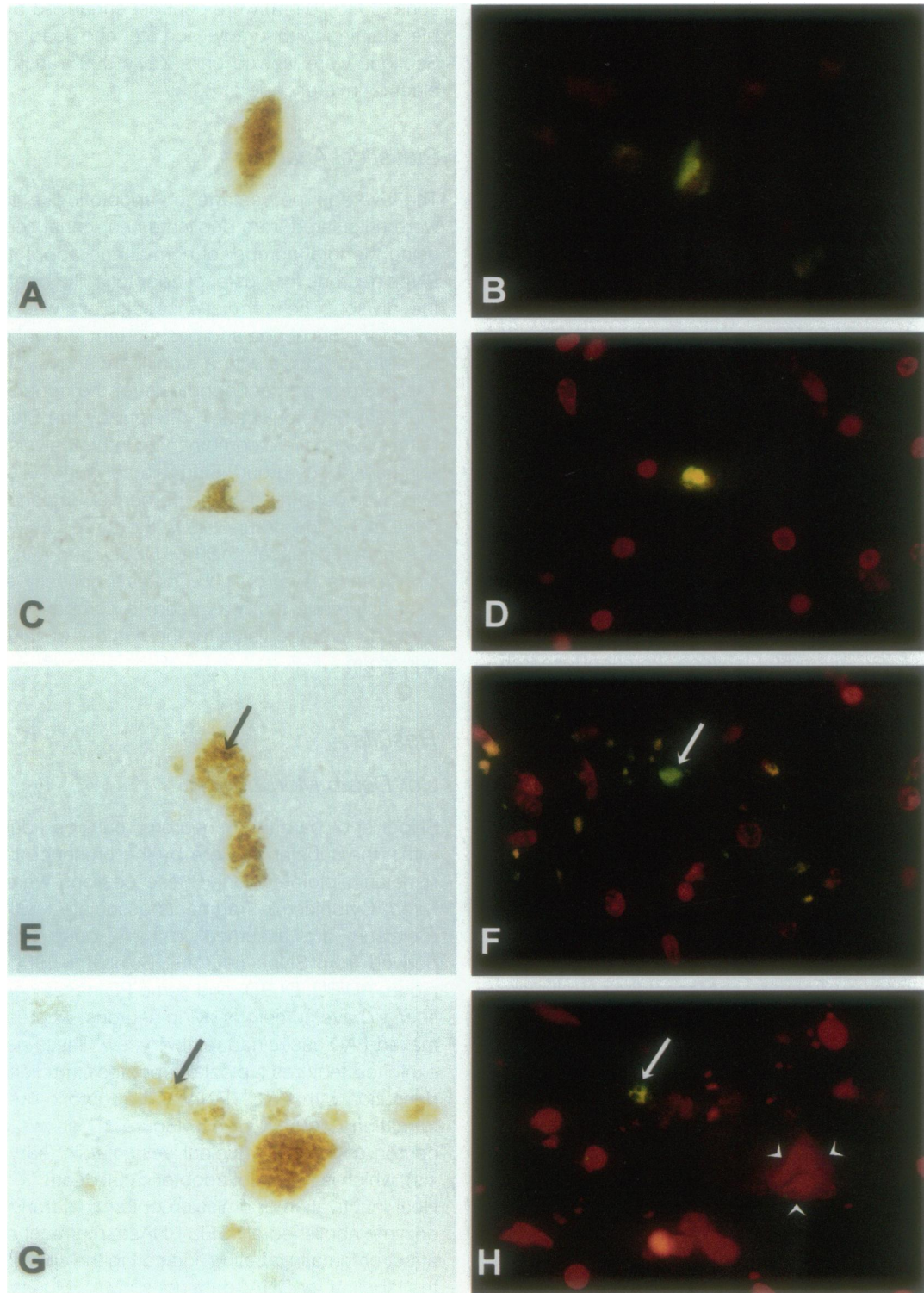
The average percentage of apoptotic-like figures were calculated from duplicate near-serial sections, using the total number of normal and apoptotic-like SNpc neurons (or cluster of apoptotic-like bodies) as the divisor. The correlation coefficient was calculated for plots of age and postmortem interval *versus* percent apoptosis with a significance level of 0.05. The average percent apoptosis for the controls and AD were not significantly different by the Student's *t*-statistic and were combined for later analysis into a non-Lewy-body group. Single-factor analysis of variance was performed on the percent apoptosis for the non-Lewy-body group *versus* the three Lewy-body-associated diseases (AD/PD, DLBD, and PD) with a significance level of 0.05. Tukey's honestly significant difference test was used to compare groups, using an average value for the number of values (n) per group.

## Results

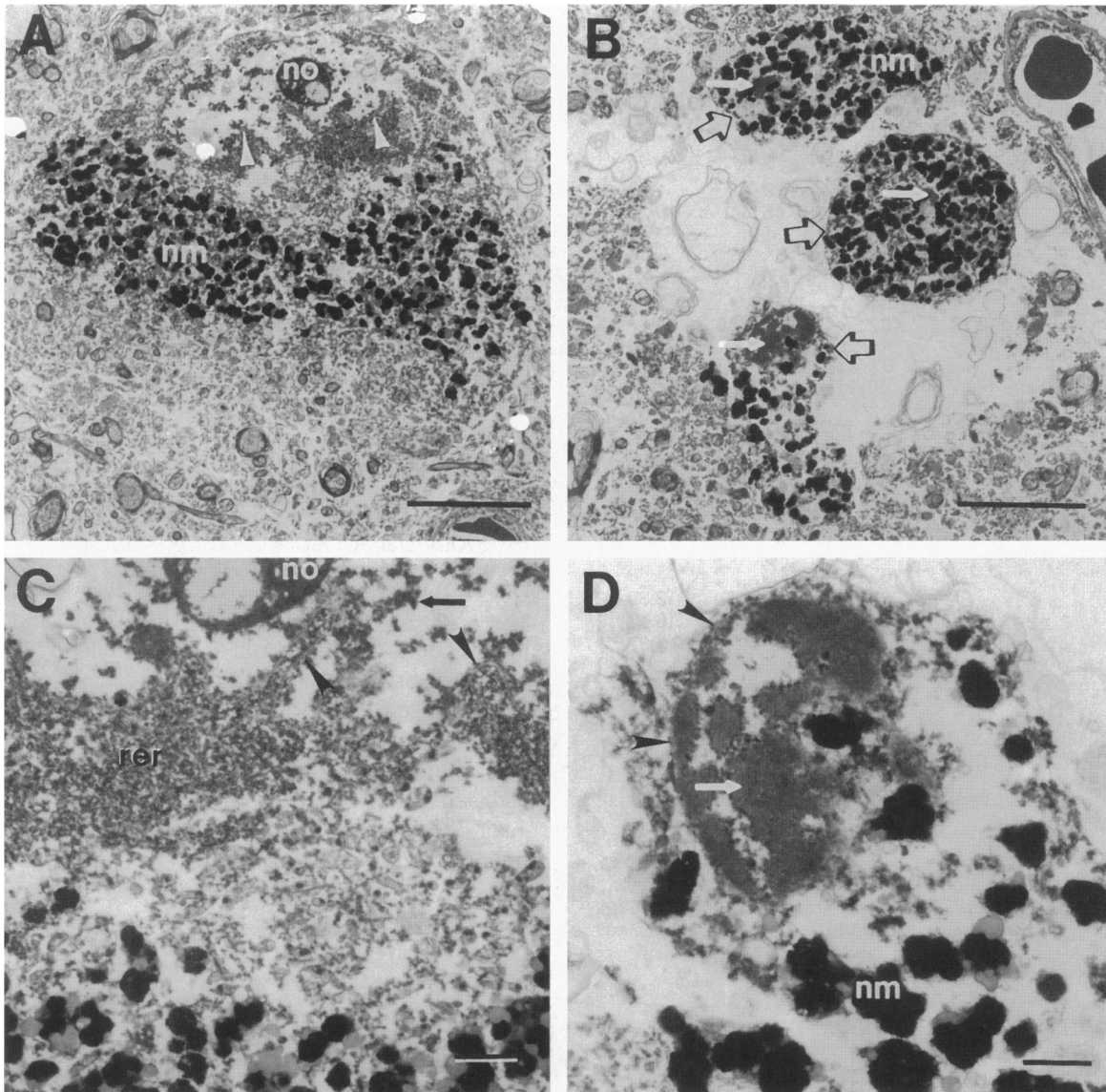
### Cell Death Morphology

SNpc and paranigral neurons can be identified within the substantia nigra by the presence of neuromelanin pigment; only these neurons were analyzed. Cytoplasmic fragments associated with neuromelanin are assumed to have been originally derived from SNpc neurons. Using the ISEL technique, AD/PD, DLBD, and some PD cases could be seen to have numerous dying neurons, whereas normal and AD cases had relatively few. These neurons exhibited features typical of apoptosis: nuclear condensation with ISEL<sup>+</sup> DNA and apoptotic-like body formation (Figure 1, A-H). Figure 1D shows a rare neuron undergoing nuclear vesiculation (karyorrhexis), which is typical of apoptotic cell death.<sup>16,19,44,45</sup> Heat inactivation or omission of terminal transferase enzyme abolished all ISEL. RNase treatment did not affect chromatin labeling, indicating the specificity of the labeling for DNA (data not shown). Mouse intestine was included in each experiment as a positive control for apoptosis.

Figures consistent with early apoptotic changes, in which nuclear changes were apparent but the cell remained intact, were distinguished from those consistent with later apoptotic changes, in which apop-



**Figure 1.** In situ end labeling of chromatin in SNpc neurons demonstrating apoptotic-like changes and possible secondary necrosis. A, C, E, and G: Bright field. B, D, F, and H: Fluorescein/propidium iodide channel. In situ end-labeled chromatin shows green (fluorescein) labeling, propidium iodide stains chromatin red, and lipofuscin autofluorescence is yellow. A to D: Early apoptotic-like changes: condensation of chromatin with normal cytoplasm. Chromatin is partially condensed in B and fully condensed with nuclear vesiculation (karyorrhexis) in D. E to H: Late apoptotic-like changes: formation of apoptotic-like bodies, some of which contain condensed chromatin (arrows). ISEL<sup>+</sup> condensed chromatin in H is partially covered by granules of neuromelanin. Normal SNpc nucleus with prominent nucleolus is also present (arrowheads). Magnification,  $\times 14,000$ .



**Figure 2.** Ultrastructure of apoptotic-like changes in SNpc. **A:** Normal SNpc neuron with euchromatic chromatin and nuclear envelope (arrowheads). **B:** Apoptotic-like bodies of SNpc origin (open arrows) containing condensed chromatin (arrows). **C:** Enlargement of section adjacent to **A** showing the nuclear envelope (arrowheads), euchromatic chromatin (arrow), and rough endoplasmic reticulum. **D:** Enlargement of apoptotic-like body in **B** showing abnormally condensed chromatin (arrow) and nuclear envelope (arrowheads). This may represent a microglial cell with unusually condensed chromatin after phagocytosis of neuronal material. Scale bars, 10  $\mu\text{m}$  (**A** and **B**) and 1  $\mu\text{m}$  (**C** and **D**). nm, neuromelanin; no, nucleolus; rer, rough endoplasmic reticulum.

otic-like bodies were formed. Most figures seen were consistent with late apoptotic changes (90 to 94% for control, AD, AD/PD, and DLBD; 70% for PD).

Primary necrotic changes can also be labeled with ISEL,<sup>46-48</sup> necessitating the use of morphology to distinguish between apoptosis and necrosis. Although chromatin and cytoplasmic condensation are more typical of apoptosis, they may occur in some later stages of necrosis. Apoptotic body formation, on the other hand, appears to be unique to apoptosis.<sup>15</sup> We therefore used ultrastructural analysis to identify apoptotic-like bodies.

Ultrastructural analysis demonstrated apoptotic-like changes including nuclear condensation and apoptotic-like body formation (Figure 2). These apoptotic-like bodies had condensed cytoplasm and frequently contained fragments of condensed chromatin. Some may be located within microglial phagosomes (Figure 2D). Although normal SNpc neurons had extensive rough endoplasmic reticulum, the apoptotic-like bodies did not (Figure 2, B-D). Signs of primary necrosis, including nuclear swelling, cytoplasmic swelling, blebs devoid of cytoplasm, and large vacuoles were not seen.

**Table 1.** *Apoptotic-Like Changes in Lewy Body Diseases*

	Age (years)	Postmortem interval (hours)	% apoptotic-like changes*
Control	86	3.0	0.73
	72	7.9	1.82
	75	3.5	0.23
Alzheimer's disease	80	12.0	0.56
	84	10.8	2.55
	62	14.6	0.17
	81	14.4	1.68
	84	16.2	3.74
Alzheimer/ Parkinson's disease	80	5.5	13.73
	77	12.4	9.74
	93	20.3	5.06
	79	12.6	2.72
Diffuse Lewy body disease	83	34.5	14.58
	87	15.9	12.39
	64	16.0	16.13
	69	6.5	7.87
	79	2.5	9.61
	81	11.7	13.91
	75	3.3	8.87
Parkinson's disease	86	8.7	6.07
	91	1.7	2.91
	63	2.7	10.13
	89	31	5.53
	66	11.5	0.38

\*Percentage of apoptotic-like SNpc neurons/total SNpc neurons (apoptotic plus nonapoptotic); average of duplicate sections.

### *Apoptosis in Lewy Body Disease*

The percentage of apoptotic-like figures was compared with the age and postmortem interval of each case (Table 1 and Figure 3). Non-Lewy-body cases (control and AD) and Lewy-body-associated disease cases (AD/PD, DLBD, and PD) were grouped separately and the correlation coefficients (*r*) were calculated (Figure 3). Neither age nor postmortem interval was significantly correlated with the percentage of apoptotic-like changes seen. The percentage of SNpc apoptotic-like changes for each case is shown in Table 1 and Figure 4. Two cases listed in Table 1 were omitted from further analysis due to high postmortem intervals (>24 hours). The control and AD (non-Lewy body) cases were not significantly different and were grouped together for comparison with the Lewy-body-associated disorders (Table 2). Analysis of variance showed a significant difference ( $F = 11.61$ ;  $P = 0.0001$ ); specifically, the AD/PD and DLBD groups, but not the PD group, had signifi-

cantly more apoptotic-like changes than the control/AD group. The PD cases had a higher average percentage of apoptotic-like changes compared with AD and controls (Figure 2), but the variance was too large to demonstrate significance. The DLBD group also had significantly more apoptotic-like changes than the PD group.

*In situ* end-labeled chromatin strands associated with neuromelanin granules, which did not appear to be membrane bound, were often seen in the neuropil of diseased cases (data not shown). Due to the lack of evidence for primary necrosis, we interpreted this finding as necrosis secondary to apoptosis. *In situ* end-labeled chromatin strands not associated with neuromelanin were also seen in the neuropil (data not shown).

### *Phagocytosis of Apoptotic-Like Bodies*

Cases of DLBD and AD/PD with high levels of apoptosis were examined for microglial phagocytic activity by double labeling with anti-HLA antibody and ISEL. We observed numerous neuromelanin-containing phagosomes (previously described by McGeer et al<sup>53</sup>) which contained ISEL<sup>+</sup> DNA (Figure 5, A–D). The neuromelanin in these phagosomes appeared as discrete clusters of neuromelanin smaller than an intact SNpc neuron. This fits our description of late apoptotic-like changes (formation of apoptotic-like bodies). Other apoptotic-like bodies were not within HLA-DR<sup>+</sup> microglia (data not shown). Also, some neuromelanin-containing phagosomes did not have ISEL<sup>+</sup> DNA (data not shown); these were not counted as apoptotic-like. Many neuromelanin-containing phagosomes were labeled with anti-TH antibody, confirming the identity of phagosomal contents as SNpc in origin (Figure 6, E–G). Therefore, our counts of apoptotic-like changes include structures resembling apoptotic bodies with ISEL<sup>+</sup> condensed DNA within phagosomes. Apoptotic-like bodies with ISEL<sup>+</sup> DNA were not seen associated with glial fibrillary acidic protein-positive astrocytes (data not shown).

## *Discussion*

### *Morphology of Apoptotic-Like Changes*

We found that morphological characteristics of apoptosis are present in normal human substantia nigra, with significantly greater apoptotic-like changes in cases of Lewy body disease. Apoptosis is best described using morphological criteria at the light microscopic and ultrastructural level.<sup>49</sup> ISEL enhances

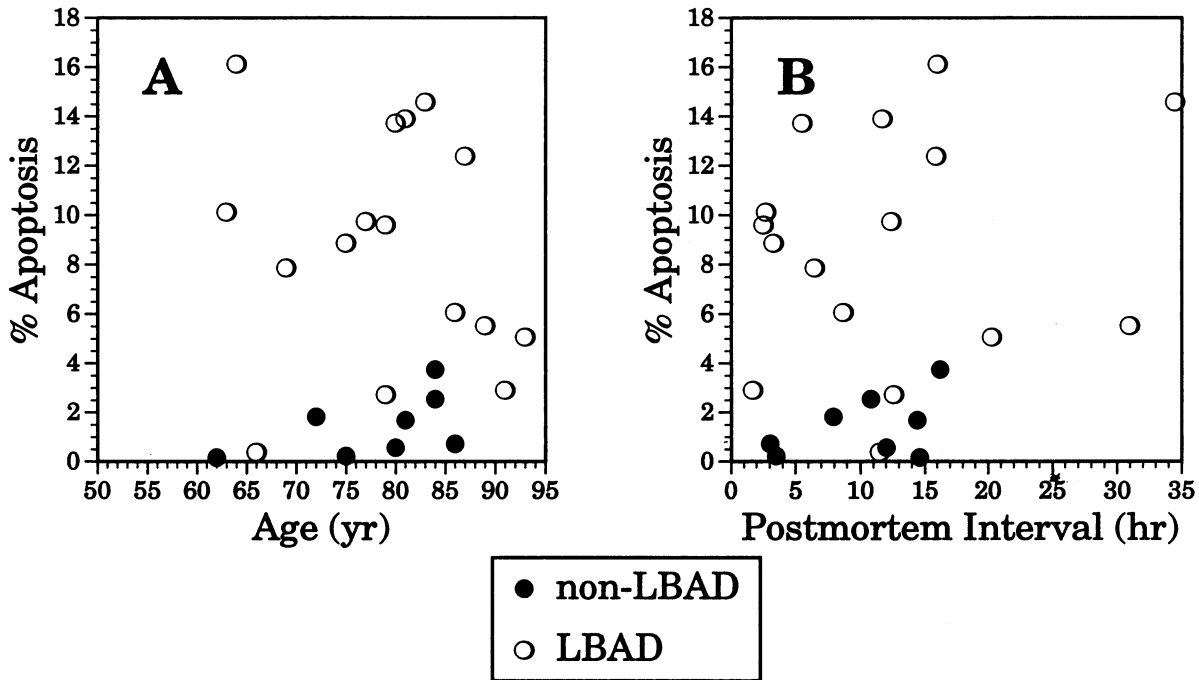


Figure 3. Age and postmortem interval versus percent apoptotic-like changes of SNpc neurons. A: Age versus percent apoptotic-like changes of SNpc neurons. Non-LBAD  $r$  value = 0.502 (not significant); LBAD  $r$  value = -0.184; (not significant). B: Postmortem interval versus percent apoptotic-like changes. Non-LBAD  $r$  value = 0.464 (not significant); LBAD  $r$  value = 0.150 (not significant). LBAD, Lewy-body-associated disorder. Non-LBAD included control and AD cases, LBAD included AD/PD, DLBD, and PD cases.

the ability to discover these apoptotic profiles but needs to be united with a morphological description to allow a distinction to be made between apoptosis and primary necrosis.<sup>46</sup> Early apoptotic changes are characteristically harder to locate, presumably due to their transient nature.<sup>16</sup> Consistent with this, 6 to 10% (control, AD, AD/PD, and DLBD) or 30% (PD) of the apoptotic-like figures seen were classified as early. The fact that the SNpc neurons are melanized allows a distinct advantage in tracing the cytoplasmic remnants of this population of neurons after cell death. Apoptotic-like bodies were recognized by the

break-up of the neuromelanin into clumps closely associated with *in situ* end-labeled chromatin. Ultrastructural analysis confirmed the presence of chromatin condensation and apoptotic-like bodies. The absence of rough endoplasmic reticulum that we noted in apoptotic-like bodies has been documented in several cell types.<sup>50-52</sup>

SNpc-derived neuromelanin remnants have previously been described within microglia.<sup>53,54</sup> Our immunohistochemistry results show the presence of *in situ* end-labeled DNA and neuromelanin within reactive microglia. Some neuromelanin-containing

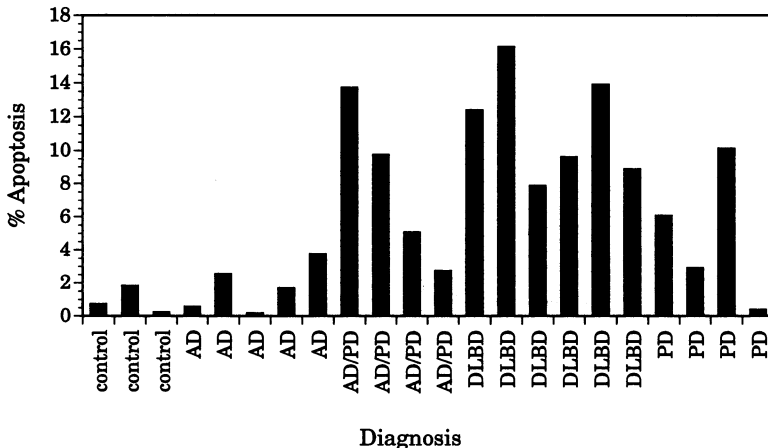


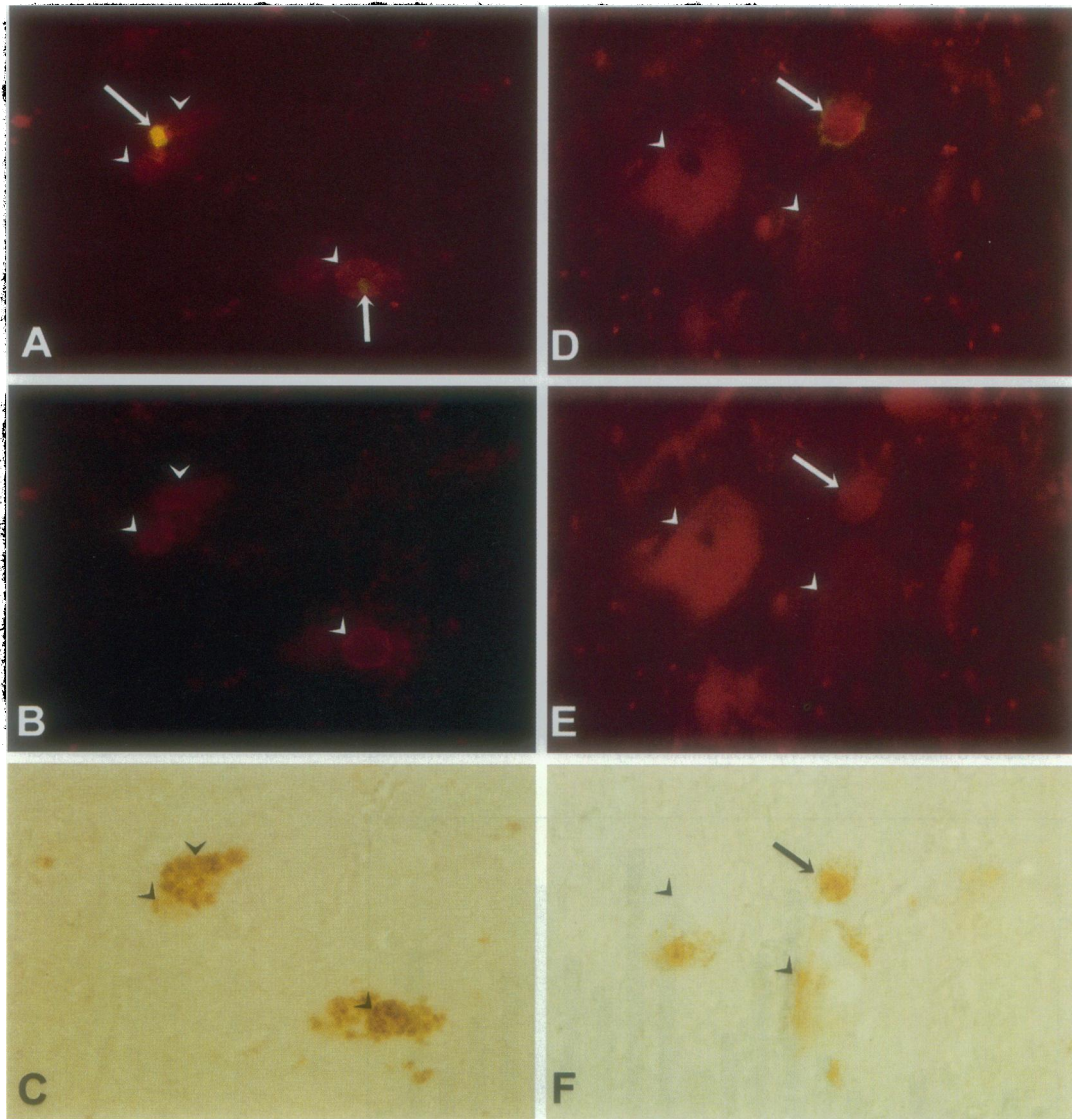
Figure 4. % Apoptotic-like changes of SNpc neurons versus diagnosis. Results are shown as the percentage of apoptotic-like figures/total SNpc neurons (apoptotic plus nonapoptotic).

**Table 2.** Average Percentage of Apoptotic-Like Changes of SNpc Neurons

Diagnosis	n	% apoptosis*
Control	3	0.93 ± 0.47
Alzheimer's disease	5	1.74 ± 0.65
Alzheimer's/Parkinson's disease	4	7.81 ± 2.45
Diffuse Lewy body disease	6	11.46 ± 1.31
Parkinson's disease	4	4.87 ± 2.10

\*Percentage of apoptotic-like SNpc neurons/total SNpc neurons; average of duplicate sections.

phagosomes stain positive for tyrosine hydroxylase, confirming the SNpc origin of the phagosomal contents and possibly suggesting they were derived from intact membrane-bound structures. Phagocytosis of apoptotic bodies by surrounding cells is a basic tenant of the original description of apoptosis.<sup>16</sup> Our results indicate that apoptotic-like bodies are undergoing phagocytosis by microglia. We did not find evidence of glial apoptosis, although this may be occurring. Remnants of end-labeled DNA and neuromelanin were also seen that did not appear to be contained within apoptotic-like bodies or



**Figure 5.** Microglial phagocytosis of apoptotic-like bodies. A to C: Double labeling of AD/PD SNpc with ISEL (FITC) and anti-HLA (LN3) antibody (Cy3). Two cells with broken-up neuromelanin (C) and condensed ISEL<sup>+</sup> chromatin (A; arrows) are encased within microglia (A and B; arrowheads). D to F: Double labeling of DLBD SNpc with anti-tyrosine hydroxylase (Cy3) and anti-HLA (FITC). An apoptotic-like body contained within a microglial phagosome (D) is TH<sup>+</sup> (D and E; arrows); SNpc neurons with and without TH labeling are also seen (arrowheads). A and D: FITC/propidium iodide channel. B and E: Rhodamine channel. C and F: Bright field. Magnification, ×960 (A and B), ×1000 (C), and ×1500 (D to F).



microglia. These remnants may have been the result of secondary necrosis of apoptotic-like bodies or primary necrosis of SNpc neurons or even of microglia.<sup>19</sup>

Primary necrosis as the major form of cell death in Lewy body disease seems unlikely. The type of inflammatory necrosis that has been described for encephalitic infections of the substantia nigra<sup>55</sup> has not been described in idiopathic PD, nor was there evidence of inflammation or lymphocytic infiltration in our cases. The range of morphology we have described covers the likely progression seen in apoptosis, and the formation of apoptotic-like bodies particularly points to apoptosis rather than other types of cell death.<sup>15</sup> Finally, the ultrastructural data do not show the nuclear and cytoplasmic swelling or vacuole formation typical of primary necrosis. However, we cannot rule out the possibility that some primary necrosis has taken place in addition to apoptosis, as has been reported for AD.<sup>23</sup>

The substantia nigra normally undergoes cell death during aging<sup>3</sup>; the data presented here suggests that this sporadic neuronal loss may be due to apoptosis. Descriptions of SNpc cell death in normal aging are strongly reminiscent of apoptosis, ie, nuclear and cytoplasmic shrinkage and aggregation of neuromelanin, which we interpret as apoptotic-like bodies.<sup>56</sup>

In a previous report, apoptosis was not detected in parkinsonian SNpc probed with ISEL.<sup>24</sup> There may have been technical reasons for this; the authors did not get labeling with DNase treatment of the SNpc as a positive control, and they used diaminobenzidine, which produces a brown reaction product that may have been difficult to distinguish from neuromelanin. To avoid this potential difficulty, a fluorescent label was used in the current study. Apoptotic SNpc neurons were identified in a recent electron microscopic study of cell death in PD.<sup>28</sup> SNpc neurons were also observed undergoing autolytic cell death. We were not able to rule out the possibility that some of our early apoptotic-like cell death was due to autolysis, as chromatin condensation may occur during autolytic cell death.<sup>15</sup> However, as autolytic cell death does not involve formation of apoptotic bodies, the late apoptotic-like changes we observed were mostly not the result of autolytic cell death. Thus, the majority of apoptotic-like changes we observed were not due to autolysis.

### *Time Course of Apoptotic-Like Changes*

The level of apoptotic-like changes seen in the AD/PD and DLBD cases appeared to be much too

high for a progressive neurodegenerative disorder. Apoptosis takes only a few hours *in vitro*.<sup>16,45,57,58</sup> Therefore, one would expect the SNpc of cases with 8 to 11% apoptotic-like changes to be depleted of neurons in months, whereas Lewy-body-associated disorders may progress over 10 to 15 years. However, although early stages of apoptosis proceed quickly, apoptotic bodies (free or within phagosomes) may be detected for some time after apoptosis has been initiated. Examples are the Councilman or acidophilic bodies of the liver, Civette bodies in lichen planus, and sunburn cells of the epidermis.<sup>16</sup> The AD/PD and DLBD cases we examined had 0.4 to 0.9% of the total number of SNpc cells undergoing early apoptotic-like changes, whereas 8 to 11% of total SNpc neurons showed late apoptotic-like changes. Furthermore, in Lewy-body-associated disorders, it is possible that the phagocytic system is overwhelmed when large numbers of cells are dying by an apoptotic-like mechanism or that SNpc glia as well as neurons are affected by the degenerative process. Apoptotic bodies that are not phagocytosed may persist for an extended period prior to undergoing secondary necrosis due to breakdown of the surrounding membrane.<sup>16</sup> In a recent study, human SNpc neuromelanin was injected into rat mid-brain.<sup>59</sup> The rat microglia responded slowly to this insult; some neuromelanin remained in the neuropil and within phagosomes even 6 months after injection. The authors concluded that microglia could not easily digest neuromelanin, supporting the theory that remnants of apoptotic-like bodies may remain long after cell death has taken place.

### *Apoptotic-Like Changes in Lewy Body Disease*

The PD cases we examined had large amounts of SNpc cell loss and some apoptotic-like changes, although there was great variability among cases. In contrast, the DLBD and AD/PD cases had less SNpc neuronal loss than the PD cases and more apoptotic-like changes. This suggests that the rate of cell death may vary during Lewy-body-associated SNpc degeneration. It has been reported that PD patients have a 45% cell loss in the first decade after the onset of symptoms, which tapers off as the illness progresses.<sup>3</sup> As the DLBD and AD/PD cases had more SNpc neurons, they may be at an earlier stage of degeneration than the PD cases. Furthermore, several DLBD and AD/PD cases had no evidence of parkinsonian symptoms or levodopa therapy. These cases, diagnosed with SNpc damage at autopsy,

appear to have preclinical parkinsonism with dementia either of the Alzheimer type (AD/PD) or the Lewy body type (DLBD). The fact that higher amounts of apoptotic-like changes were seen in the DLBD and AD/PD brains than in the PD brains supports the hypothesis that there is a high level of cell death in earlier stages of Lewy body disease, which tapers off in later stages.<sup>2,54</sup> This tapering off may be because the PD cases have few remaining SNpc neurons, and the surviving neurons may be less vulnerable to the disease process. Alternatively, the disease process involving cortical dementia (AD/PD and DLBD) may be different from that of pure parkinsonism (PD).

Our study as well as others have shown that post-mortem time does not appear to affect the amount of ISEL.<sup>23,24,60</sup> However, premortem influences may be a factor. Lassmann and colleagues have suggested that the primary pathology of AD may require other precipitating events to initiate apoptosis, such as hypoxia or vascular changes, just before the death of the patient.<sup>23</sup> The cause of death of the patient may therefore affect the amount of apoptosis seen in the brain. We could not address this issue due to lack of information about the immediate cause of death and agonal state for many of the cases in this study.

Diverse pathogenic factors may contribute to SNpc neuronal death in PD and related disorders.<sup>61-64</sup> Several of these factors have been shown to cause apoptosis of cultured cells, such as dopamine toxicity,<sup>65</sup> inhibition of cytochrome P450<sup>66</sup> or mitochondrial activity,<sup>58,67</sup> and induction of oxidative stress.<sup>68</sup> We also cannot rule out direct DNA damage including ISEL<sup>+</sup> fragmentation as a result of oxidative stress. This might represent a nonapoptotic form of cell death that shares some of the features of apoptosis.

It has been shown in a number of systems that cell death *via* apoptosis can be reduced or prevented. For example, apoptosis can be inhibited by overexpression of *bcl-2* in some instances,<sup>69-71</sup> the addition of growth factors has been shown to rescue some neurons from developmental or induced apoptosis,<sup>45,72-74</sup> and withdrawal of glial cell-line-derived neurotrophic factor (GDNF) from cultured dopaminergic neurons, a model for SNpc neurons, causes cell death.<sup>75</sup> In addition, GDNF treatment rescues dopaminergic neurons *in vivo* from cell death due to axotomy<sup>76</sup> or induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).<sup>77</sup> The current study provides evidence that suggests that SNpc neurons may be dying *via* apoptosis in Lewy-body-associated disorders. If so, then it provides a rational basis on which to explore ways to prevent cell death in these disorders by growth factor treat-

ment or targeted gene therapy. Interfering with the apoptotic pathway in these neurodegenerative disorders may be valuable as it has the potential to rescue neurons even in the presence of the ongoing disease process.

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