Acta Neuropathol (2008) 115:461–470
DOI 10.1007/s00401-008-0352-8

ORIGINAL PAPER

Morphometry of the human substantia nigra in ageing and Parkinson's disease

Gay Rudow · Richard O'Brien · Alena V. Savonenko · Susan M. Resnick · Alan B. Zonderman · Olga Pletnikova · Laura Marsh · Ted M. Dawson · Barbara J. Crain · Mark J. West · Juan C. Troncoso

Received: 17 December 2007 / Revised: 12 February 2008 / Accepted: 13 February 2008 / Published online: 23 February 2008

Abstract To investigate the relation between the loss of substantia nigra (SN) neurons in normal ageing and Parkinson's disease (PD), we measured the total number and the cell body volume of pigmented (neuromelanin) neurons in the SN. We examined young ($n = 7$, mean age: 19.9), middle-aged ($n = 9$, mean age: 50.1), and older controls from the Baltimore Longitudinal Study of Aging ($n = 7$, mean age: 87.6), as well as PD cases ($n = 8$, mean age: 74.8). On random-systematically selected paraffin Nissl-stained sections, we used the Optical Fractionator to estimate the total number of neurons on one side of the SN. Using the Nucleator probe, we measured the volume of these neurons. In young and older controls, we also estimated the total number and volume of tyrosine hydroxylase (TH) positive (+) nigral neurons. We observed a significant loss of pigmented (-28.3%, $P < 0.01$) and TH (+) (−36.2%, $P < 0.001$) neurons in older controls compared with younger subjects. Analysis of the size distribution of pigmented and TH (+) neurons showed a significant hypertrophy in older controls compared to young controls ($P < 0.01$). In contrast, in PD we observed a significant atrophy of pigmented neurons compared to all control groups ($P < 0.01$). These data suggest that neuronal hypertrophy represents a compensatory mechanism within individual SN neurons that allows for normal motor function despite the loss of neurons in normal ageing. Presumably, this compensatory mechanism breaks down or is overwhelmed by the pathological events of PD leading to the onset of the characteristic motor disturbances.

Keywords Stereology · Neuronal volume · Alpha-synuclein · Neurodegenerative · Tyrosine hydroxylase

Introduction

Idiopathic Parkinson's disease (PD) is a common age-associated neurodegenerative disorder clinically characterized by

G. Rudow (✉) · O. Pletnikova · B. J. Crain · J. C. Troncoso
Neuropathology, Johns Hopkins School of Medicine,
Ross Research Building 555, 720 Rutland Avenue,
Baltimore, MD 21205, USA
e-mail: grudow1@jhmi.edu

J. C. Troncoso
e-mail: troncoso@jhmi.edu

R. O'Brien
Department of Neurology,
Johns Hopkins Bayview Medical Center,
B Building, Room 123, 4940 Eastern Avenue,
Baltimore, MD 21224, USA

A. V. Savonenko
Neuropathology, Johns Hopkins School of Medicine,
Ross Research Building 558, 720 Rutland Avenue,
Baltimore, MD 21205, USA

S. M. Resnick · A. B. Zonderman
Laboratory of Personality and Cognition,
Triad Technology Center, 4th Floor, 333 Cassell Drive,
Baltimore, MD 21224-6825, USA

L. Marsh
The Johns Hopkins Hospital, Phipps 300,
600 N. Wolfe Street, Baltimore, MD 21287, USA

T. M. Dawson
The Johns Hopkins Hospital,
The Institute for Cell Engineering, 733 North Broadway,
Suite 731, Baltimore, MD 21205, USA

M. J. West
Department of Neurobiology,
University of Aarhus, Building 1234 University Park,
8000 Aarhus, Denmark

bradykinesia, rigidity, resting tremor, and abnormal postural reflexes. Many PD patients eventually develop cognitive decline [1, 25] and, in some, the earlier clinical manifestations may include sleep disorders [10, 42]. The neuropathology of PD is characterized by degeneration of the substantia nigra (SN) with loss of neurons and the accumulation of α-synuclein aggregates within neurons and neurites, known as Lewy bodies and Lewy neurites, respectively [17, 23, 39]. PD is now considered to be part of the spectrum of Lewy body diseases [14]. Although the pathology of PD is not limited to the SN, as it also involves other brain stem nuclei, hippocampus, amygdala, neocortex, and olfactory bulb [3, 5, 12, 24], the degeneration of the SN pars compacta and its dopaminergic projection to the striatum is the most salient morphological feature of PD and plays a preeminent role in the motor manifestations that characterize the disease.

Since PD is an age-associated disorder, it is important to assess how the loss of neurons associated with normal ageing relates to the disease. Previous morphometric studies of the human SN, both non-stereological [15, 31, 32, 43, 44] and stereological [7, 29, 38, 41] agree that there is a loss of neuromelanin-containing (pigmented) SN neurons in normal ageing, but disagree on the rate of this loss. Some studies have estimated the loss as 4.3% per decade [7], whereas other studies have reported almost 10% [28]. There is no consensus either as to what happens with the size of SN neurons in normal ageing. Previous non-stereological studies have reported a decrease in the nuclear volume of SN neurons in ageing [16, 30] and enlargement of the cell bodies as demonstrated on Golgi stains [11]. Stereological studies have yielded divergent results. Although Ma et al. [28] reported smaller pigmented neurons in the SN of older subjects, Cabello et al. [7], using the rotator method, found an increase in the volume of these neurons. The variations in the outcomes of earlier studies may be attributed to the use of different morphometric techniques, variability in the demarcation of the SN, and to the use of samples of convenience with limited neuropathological characterization, because the majority of these studies were performed before α-synuclein immunostains became available for assessing the presence of PD or Lewy body disease. Because of the significant discrepancies of previous studies both on the rate of age-associated loss and the changes in size of SN neurons, we believe additional studies are warranted. In the present study, we used stereological approaches to examine the number and volume of neuromelanin-containing (pigmented) and tyrosine hydroxylase (TH) positive (+) neurons in the SN, including pars compacta and reticulata, in a large number of autopsy brains that included those from patients with PD as well as well-characterized clinically normal older controls from the Baltimore Longitudinal Study of Aging (BLSA). All specimens underwent a thorough neuropathological examination including α-synuclein and tau immunostains to identify and exclude cases of Lewy body disease or tauopathies, respectively. Our main finding is that the pigmented and TH (+) neurons of the SN decrease in number in the course of normal ageing, while at the same time their cell bodies grow larger. In contrast, SN neurons in PD are markedly reduced in number and show decreased neuronal volumes. In concert, these observations suggest that the increased neuronal volumes in the SN of older controls may represent a mechanism to compensate for the loss of neurons.

Materials and methods

Subjects

Demographic and neuropathological information is summarized in Table 1.

Controls. *Young controls* ($n = 7$) were 18–21 years and had no histories of neurological disease. Autopsies showed no abnormalities of the nervous system and no α-synuclein or tau lesions. *Middle-aged controls* ($n = 9$) were 43–59 years with no history of neurological disease. Autopsies showed no abnormalities of the nervous system and no α-synuclein or tau lesions. *Older controls* ($n = 7$) were 76–96 years and were from the Baltimore Longitudinal Study of Aging (BLSA). They underwent annual evaluations that included a series of neuropsychological tests [26], neurological exam, interval medical history, medication review, and a structured subject and informant interview for clinical dementia rating (CDR) [21, 37]. In BLSA controls, the mean interval between the last clinical evaluation and death was 8.6 months. We excluded subjects with histories of PD, other movement disorders, cognitive decline, dementia, cerebrovascular disease or any neurological abnormalities. Autopsies revealed no α-synuclein lesions. CERAD neuritic plaque scores were 0 to A [36], and neurofibrillary Braak scores were II to III [4].

Parkinson's disease patients ($n = 8$) were 68–75 years. The majority were followed longitudinally at the Johns Hopkins University Morris Udall Parkinson's Disease Center, and the diagnosis of PD was confirmed at autopsy [6, 17, 20]. These individuals had histories of PD for 7–20 years, with or without dementia, and their brains had α-synuclein lesions in the SN and other brain regions [34]. There were no significant lesions indicative of other neurodegenerative disorders, i.e., tauopathies [13] or multiple system atrophy [18, 40].

Neuropathology

All autopsies were conducted by the Neuropathology Division of the Johns Hopkins University. Brains were fixed in

Table 1 Demographic and stereological data

Young controls	Age (years)	Sex	Brain weight (grams)	Number of pigmented neurons (10^3)	Volume of pigmented neurons (μm^3)	Number of TH (+) neurons (10^3)	Volume of TH (+) neurons (μm^3)
1	18	M	1,570	445,133	11,372	458,957	11,454
2	21	M	1,600	512,037	13,931	483,686	16,867
3	21	M	1,590	513,090	16,721	442,778	19,079
4	19	M	1,700	384,000	11,057	418,889	12,433
5	19	M	1,690	348,628	15,079	NA	NA
6	20	F	1,560	354,962	14,480	306,147	18,567
7	21	F	1,290	408,722	7,811	354,655	12,265
Mean (standard deviation)	19.9		1,571 (136.1)	423,796 (68,817)	12,922 (3015)	410,852 (67,563)	15,111 (3,447)
CV				0.16	0.23	0.16	0.23
Middle-aged controls	Age (years)	Sex	Brain weight (grams)	Number of pigmented neurons (10^3)	Volume of pigmented neurons (μm^3)		
8	48	F	1,210	352,475	16,577		
9	43	F	1,300	369,452	5,956		
10	47	F	1,380	386,743	10,345		
11	54	F	1,460	410,127	19,721		
12	59	F	1,330	360,345	12,483		
13	51	M	1,720	482,107	15,633		
14	54	M	1,380	253,045	10,079		
15	45	M	1,550	368,925	12,631		
16	50	M	1,540	421,595	12,772		
Mean (standard deviation)	50.1		1,430 (154.8)	378,313 (61,825)	12,911 (4,039)		
CV				0.16	0.31		
Older controls	Age (years)	Sex	Brain weight (grams)	Number of pigmented neurons (10^3)	Volume of pigmented neurons (μm^3)	Number of TH (+) neurons (10^3)	Volume of TH (+) neurons (μm^3)
17	96	M	1,290	263,578	11,734	262,963	10,965
18	91	M	1,500	351,817	19,383	266,240	20,124
19	78	M	1,210	252,548	22,492	290,106	27,067
20	90	M	1,430	374,447	15,277	261,515	18,018
21	76	M	1,300	256,234	22,666	250,105	22,580
22	95	M	1,250	400,794	13,638	332,859	9,701
23	86	M	1,281	232,360	20,694	171,330	22,061
Mean (standard deviation)	87.6		1,323 (103.5)	304,540 (68,692)	17,983 (4,413)	262,160 (48,635)	18,645 (6,320)
CV				0.22	0.24	0.18	0.34
Parkinson's disease cases	Age (years)	Sex	Brain weight (grams)	Number of pigmented neurons (10^3)	Volume of pigmented neurons (μm^3)	Duration of disease (years)	
24	68	F	1,180	60,665	11,007	NA	
25	69	M	1,294	77,019	11,901	8	
26	71	M	1,310	128,117	17,263	7	
27	82	F	1,310	74,196	20,010	13	
28	77	M	1,140	64,951	12,102	20	
29	73	F	1,300	40,229	5,921	18	
30	73	F	1,200	183,219	9,185	7	
31	85	F	1,260	36,864	7,562	13	
Mean (standard deviation)	74.8		1,249 (66.8)	83,158 (49,244)	11,869 (4,740)	12.29 (5.3)	
CV				0.59	0.39		

Summary of the subjects in each study group, demographic information, brain weights, and the total number and cell body volumes of pigmented (neuromelanin) and tyrosine hydroxylase TH (+) neurons in one side of the substantia nigra

NA not available, *CV* coefficient of variation, *F* female, *M* male

10% buffered formaldehyde for 2 weeks and examined grossly on coronal sections. For neuropathologic diagnosis, standard tissue blocks from cerebrum, brain stem, cerebellum, and spinal cord were dissected and processed for paraffin sections and hematoxylin-eosin staining. Selected sections were stained with silver (Hirano method) [50] and immunostained for α-synuclein (Synuclein-1, dilution 1:500 from Transduction Laboratories) and phosphorylated tau (PHF-1 antibody, dilution 1:100; a kind gift of Dr. P. Davies) as previously described [35]. The α-synuclein immunostains were used to rule out Lewy body disease and phosphorylated tau (PHF1) immunostains were used to exclude possible cases of Progressive Nuclear Palsy (PSP) or other tauopathies [13].

Stereology

In all of the brains, we dissected 5 or 6 coronal tissue blocks of brain stem and diencepahalon that contained the entire SN. The span of the dissected tissues exceeded the rostral and caudal extents of the SN. The tissues were processed in an automated processor, dehydrated through alcohols, followed by sequential immersions in cedarwood oil, methyl salicylate, and xylene, embedded in paraffin and cut serially at 50 µm. This thickness was chosen because the sections undergo significant shrinkage during the staining processes. Then, we selected a set of sections that included every 20th section with a random start within the first 20 sections. This yielded ~12–14 sections, which were stained with cresyl violet (Nissl) for all four groups (Fig. 1). In addition, tissue sections sets from young and older control groups were immunostained with tyrosine hydroxylase (TH) polyclonal antibody (1:50 concentration, Pel-Freez) (Fig. 1). The borders of the SN were outlined using a 5× objective following the landmarks proposed by [45]. Neurons from one side of the SN, including pars compacta and pars reticulata, were counted using the optical fractionator probe [46, 47]. Stereological measurements were performed with a Zeiss light microscope equipped with a 100×, NA 1.30, oil Plan neofluor ∞/0.17 objective and interfaced with a Stereo-Investigator system (MBF Bioscience, Williston, VT, USA). The images were captured with a microFire video camera (Optronics, Goleta, CA, USA). The thickness of the sections was measured by focusing on the top of the section, setting the *Z*-axis to 0, and then refocusing to the bottom of the section and recording the actual thickness. The actual thickness of the histological sections was in the 30–44 µm range. The total number of SN neurons (pigmented) was estimated using the Stereo Investigator Optical Fractionator probe; the disector height was 25 µm. This height was chosen based on the actual thickness of the sections plus an allowance for top and bottom guards. The counting frame was 50 µm × 35 µm, and the sampling grid was 400 µm × 400 µm. Using the nucleator probe [19], the volume of pigmented neurons was estimated by placing six rays centered on the nucleolus and intersecting the cell membrane.

Statistical analyses

We compared the number of neurons in the four study groups by one-way ANOVA followed by Tukey's post-test using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

Since analysis of data distribution for neuronal volumes revealed significant departures from normal distribution with significant right skewness, we used parametric and non-parametric (Kruskal–Wallis) ANOVA to test the null hypothesis. With significant main effects, ANOVA was followed by post hoc tests (Kolmogorov–Smirnov) to analyze differences between particular groups. The Kolmogorov–Smirnov test was chosen because it is particularly sensitive to changes in the shapes of distribution. The results are represented as arbitrary categories of small (0–9,000 μm^3), medium (9,001–18,000 μm^3), large (18,001–27,000 μm^3), and very large (27,001–36,000 μm^3) neurons to simplify the description of changes in the shape of distributions. This range of neuronal sizes from small to very large neurons included more than 97% of all neurons and was divided into four equal groups to represent small, medium, large, and very large size neurons. Neurons with cell body volumes greater than 36,000 μm^3 were omitted from the figures representing the Kolmogorov–Smirnov test because of negligible percentages observed (Figs. 6, 7). Statistical analyses were also performed for the whole range of raw (not-clustered) data. These analyses were performed using Statistica 6.0, StatSoft, Inc. Tulsa, OK, USA.

Results

The results of the estimation of the total number of pigmented and TH (+) neurons in one side of the SN and their volumes are displayed in Table 1. Among controls, there is a significant age-associated loss of pigmented SN neurons (Figs. 2, 3a). This loss of neurons is 28.3% in older controls compared with younger controls. In cases of PD the loss of pigmented neurons is massive, reaching 80.4% compared with younger controls and 73% compared with older controls. We also found a 36.2% loss of TH (+) neurons in the older controls compared to younger controls ($P < 0.001$) (Fig. 3b). The regression analysis demonstrates a significant ($P < 0.05$) increase in the size of pigmented SN neurons in the course of normal ageing

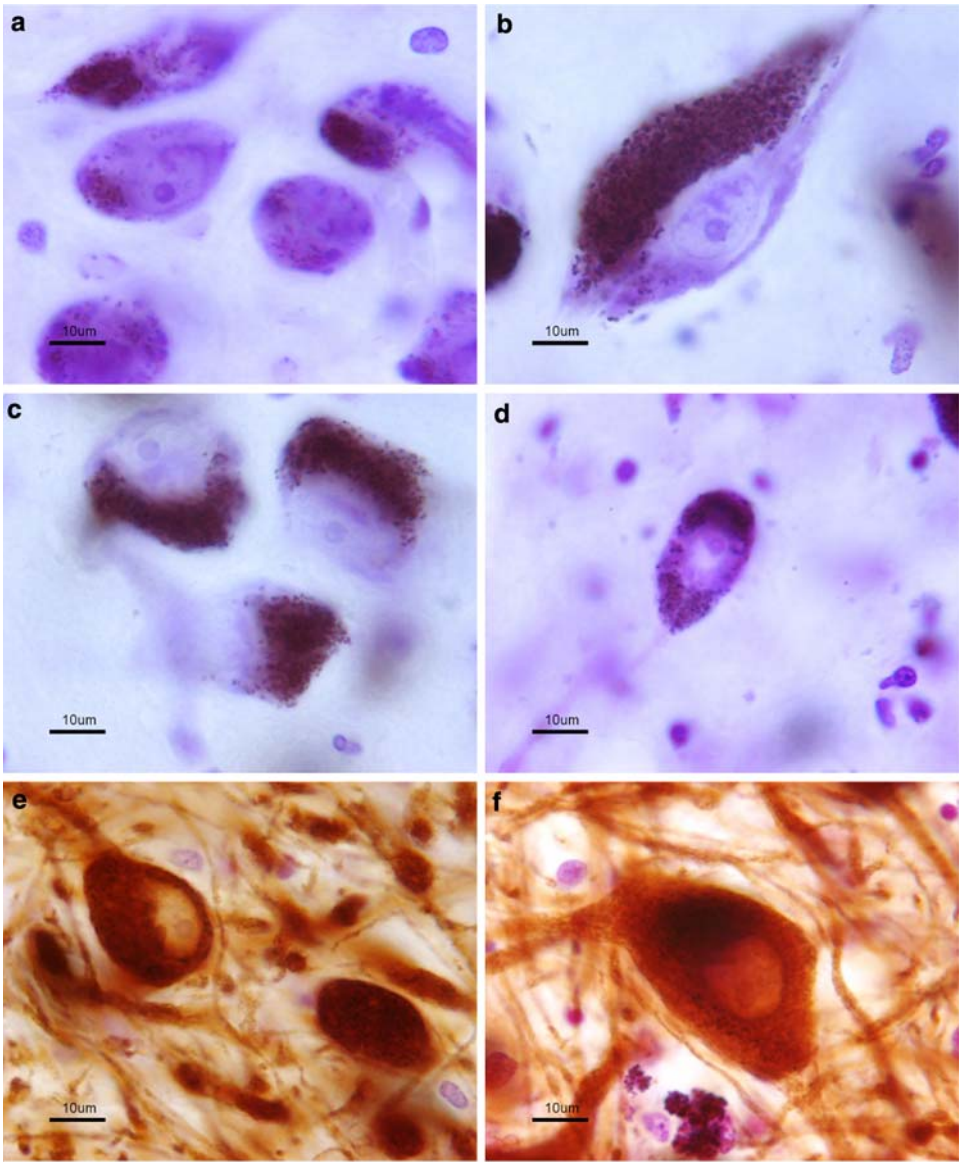

Fig. 1 This panel of photomicrographs compares the sizes of representative pigmented neurons of the SN in young controls (**a**), older controls (**b**), middle-aged controls (**c**), and PD cases (**d**). The lower figures display examples of TH (+) neurons in young controls (**e**) and older controls (**f**). All micrographs are at the same magnification (see *bars*) and show the hypertrophy of SN neurons in older controls (**b**, **f**) and the atrophy in PD (**d**). Paraffin sections were cut at 50 μm and stained with Cresyl Violet (Nissl method) (**a**–**d**) or immunostained for TH (**e**, **f**)

(Fig. 4). The mean volume of SN neurons appears 39% larger in older controls compared to younger controls (Fig. 5). Furthermore, the distribution analysis of cell body volume revealed significant (Kolmogorov–Smirnov test, $P < 0.01$) hypertrophy of pigmented neurons in older controls and atrophy in PD cases (Figs. 6, 7a). We also observed hypertrophy of TH (+) neurons in older controls compared to younger controls. The mean volume of TH (+) neurons was 23% larger in older controls. Although the difference of the means was not significant, the analysis of cell body volume distribution revealed a significant difference between the groups (Kolmogorov–Smirnov test, $P < 0.01$) (Fig. 7b), with the older controls having a larger proportion of large neurons. The hypertrophy of SN neurons in normal ageing and their atrophy in PD is illustrated in photomicrographs in Fig. 1.

Discussion

Our observations indicate that the human SN undergoes a significant loss of pigmented and TH (+) neurons from the pars compacta and reticulata during normal ageing (Figs. 2, 3). At the same time, there is a significant enlargement of those neurons (Figs. 4, 5, 6, 7). In PD, the loss of neurons from the SN is massive (Figs. 2, 3), and their cell body volumes are reduced (Figs. 6, 7).

Our measurements indicate that the human SN of young controls has a mean of 424,000 pigmented neurons (unilateral). As shown in Table 1 and Fig. 3a, however, we find a wide range of values from ~350,000 to ~500,000 neurons. This wide range in the total number of neurons is not unique to the SN as there is a similar variability in the number of neurons in hippocampus [46, 48] and entorhinal

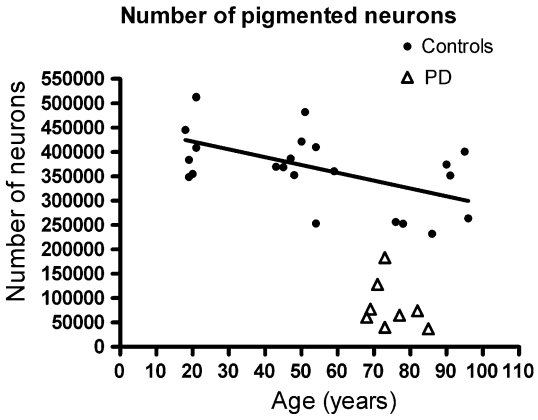

Fig. 2 Linear regression of the number of pigmented neurons (*closed circles*) versus age is significant ($P = 0.0060$) with $R^2 = 0.337$; CV = 0.20. The *open triangles* correspond to the mean number of pigmented neurons in the SN of Parkinson's disease cases

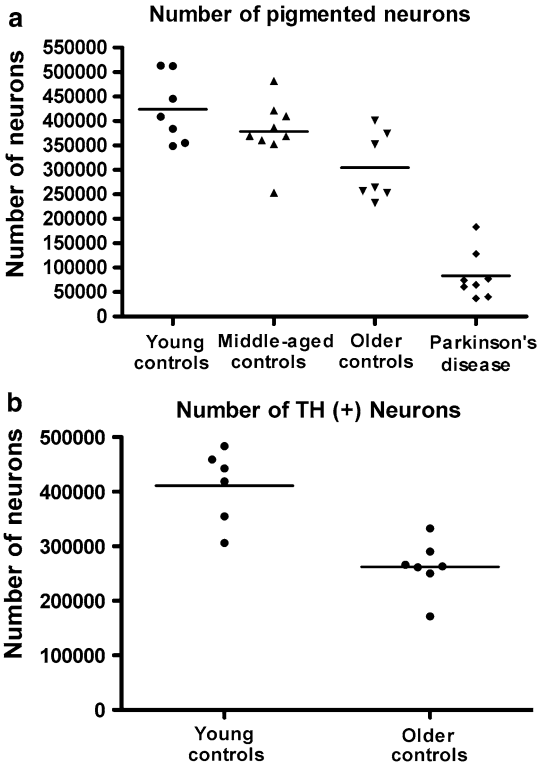

Fig. 3 These scatter plots show the total number of estimated pigmented neurons in one side of the substantia nigra for each study group (**a**) and for tyrosine hydroxylase (TH) (+) neurons in young and older controls (**b**). The *horizontal bars* indicate the mean values. One-way ANOVA revealed significant differences present between the number of pigmented neurons in younger and older controls ($P < 0.01$) and between each control group and the PD group ($P < 0.001$) (**a**). There is a 36.2% loss of TH (+) neurons in the older control group, also a significant difference ($P < 0.001$) (**b**)

cortex [49] of normal subjects. Our estimation of total number of pigmented neurons in younger subjects is higher than that of previous studies (adjusted for one side of the SN):

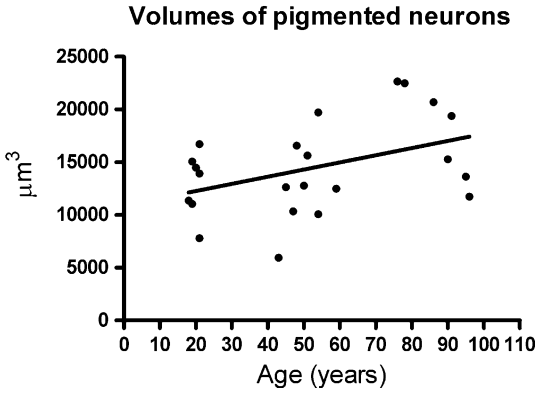

Fig. 4 Linear regression of the volumes of neurons of the three control groups (PD excluded) versus age is significant ($P = 0.046$) with $R^2 = 0.18$; CV = 0.31

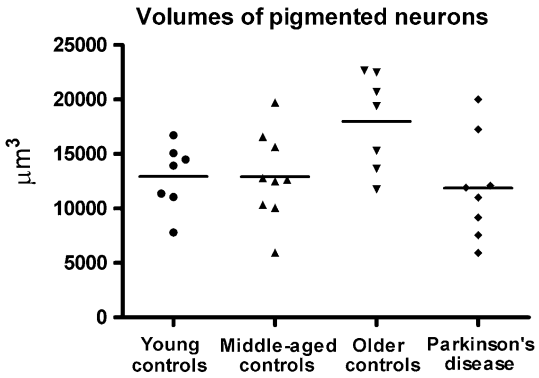

Fig. 5 Scatter plot comparing the mean volumes of the substantia nigra neurons among the four study groups. *Horizontal bars* indicate the means. Although the older controls show an increase in the mean value (+39%), this change is not statistically significant when the mean value is compared with any of the other groups

Ma et al. [29] ~150,000, and Cabello et al. [7] 189,000. These differences can be attributed to several factors including the use of different embedding media, histological stains, stereological methods, but more importantly the delineation of the boundaries of the SN region [41]. In the older controls, we estimated a mean of 305,000 pigmented neurons (unilateral), a number slightly higher than the 275,000 neurons (adjusted for one side of the SN) estimated by Pakkenberg et al. [38] in older controls (mean age: 81).

The mean number of pigmented neurons in older controls (mean age: 87.6) was 28.3% ($P < 0.01$) less than that of younger controls (mean age: 19.9). Distributed over the intervening 6.5 decades, this loss amounts to an average 4.35% per decade. Compared to non-stereological studies, this loss is less than the 48% loss by age 60 (~7% per decade) reported by McGeer et al. [32] and the 35% loss of neurons by age 90 observed by Mann et al. [31]. In a previous stereological study of the SN in ageing, Ma et al. [29] examined the brains of 26 normal subjects with an age

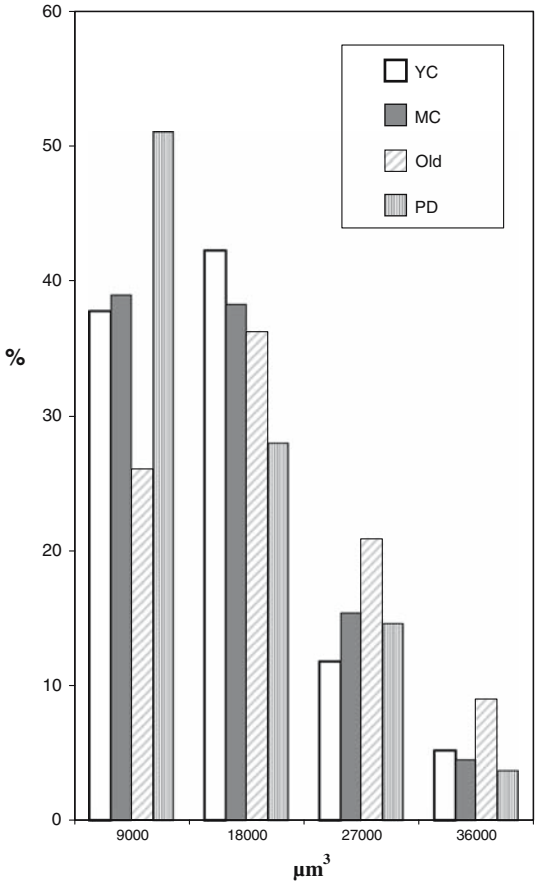

Fig. 6 Histogram of the frequency of small, medium, large, and very large pigmented SN neurons in young controls (YC), middle-aged controls (MC), Older Controls (OC), and Parkinson's cases (PD). Note the higher percentage of very large neurons in older controls and of small neurons in PD. The *Y*-axis corresponds to the frequency (%) of measured neurons in each size category. The *X*-axis represents the arbitrary neuronal volume bins in μm^3: small (0 to 9,000 μm^3), medium (9,001 to 18,000 μm^3), large (18,001 to 27,000 μm^3), and very large (27,001 to 36,000 μm^3)

range from 17 to 90 and demonstrated a significant loss of the total number of pigmented neurons in older subjects. Using paraffin sections and the physical disector method, that study estimated there to be ~300,000 neurons in the youngest subjects and ~100,000 neurons in the oldest subjects. The average decline in the number of pigmented neurons per decade was 9.8%. It is important to note that although the older subjects were clinically normal, the brains in that study were not examined for ubiquitin or α-synuclein lesions. In a subsequent stereological study, using plastic sections and the optical disector, Cabello et al. [7] examined the brains of 28 males aged 19–92. They observed a significant decrease in the total number of pigmented neurons as a function of age of approximately 4.3% per decade, an estimate identical to our study. Nevertheless, the difference between the 7 youngest (mean age: 27;

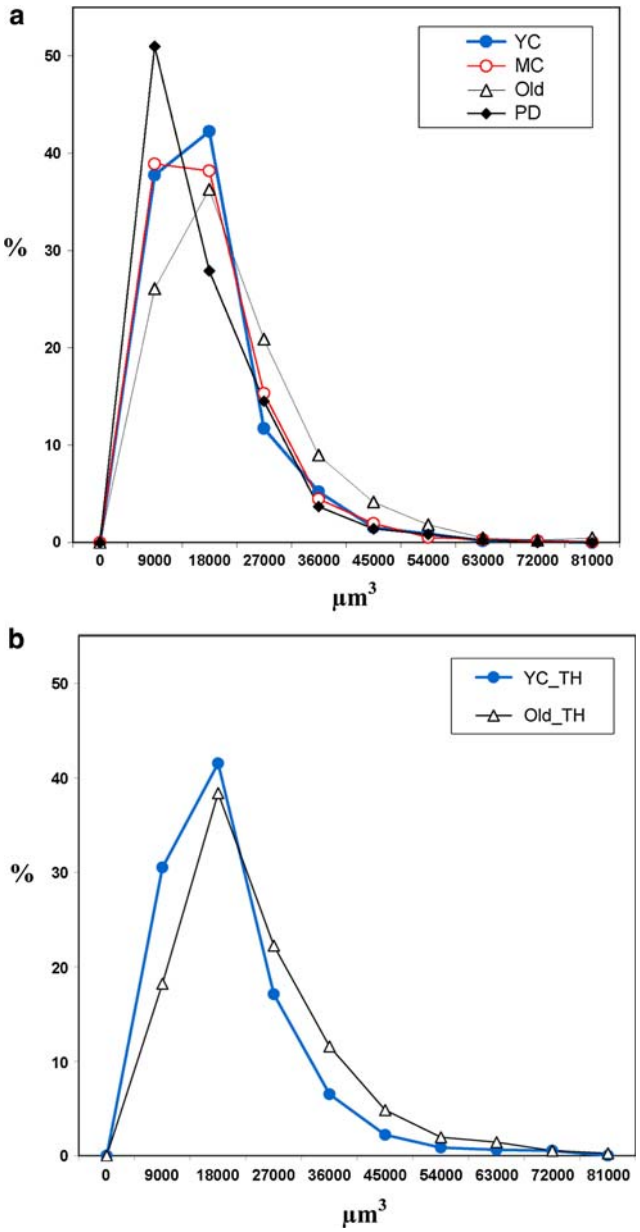

Fig. 7 These graphs represent the distribution of cell bodies of SN neurons according to their volumes. **a** Shows the distribution of pigmented SN neurons in young controls (YC), middle-aged controls (MC), Older Controls (Old), and Parkinson's cases (PD). Comparison of the curves shows neuronal hypertrophy in older controls and atrophy in PD. Statistical analysis (Kolmogorov–Smirnov Test) demonstrated significant differences ($P < 0.01$) when comparing older controls to either middle-aged or young controls, and when comparing PD to each control group. Note the displacement of the older controls curve to the *right* indicating a higher proportion of larger neurons compared to all other groups. In contrast, the curve of PD cases is shifted to the *left* due to the larger proportion of smaller neurons. **b** Displays the distribution of TH (+) neurons in young controls (YC) versus older controls (Old). Statistical analysis (Kolmogorov–Smirnov Test) demonstrated significant increase in the proportion of larger neurons in older controls compared to young controls ($P < 0.01$), as revealed by the displacement of the older controls curve to the *right*

378,000 neurons) and the 7 oldest (mean age: 82; 256,000) subjects was not significant. Although the absolute numbers of pigmented neurons in the SN in young subjects in our series are different from those reported in two previous stereological studies [7, 29], our results are consistent with an age-associated loss of pigmented neurons in the SN [7, 29, 41]. Although it should be noted that other stereological studies have found no age-related losses of pigmented SN neurons, those same studies have reported marked decreases in the number of neurons expressing specific phenotypic markers [8,9].

There are few morphometric studies of TH (+) neurons of the SN in normal ageing. Kubis et al. [27] did not find a significant loss of TH (+) in the SN, nor in other midbrain regions, in a series of 21 control subjects who died at ages 44–110. In contrast, we have shown a 36% ($P < 0.001$) loss of TH (+) SN neurons in older controls compared to younger subjects. This loss is slightly higher than the 28.3% observed for pigmented neurons. Our observations are consistent with the observation that in normal ageing there is a marked loss of dopamine transporter-immunoreactive neurons in the human SN [28], and that all dopaminergic neurons that express DAT mRNA are also neuromelanin positive [2].

As discussed in the Introduction, there is no consensus with regard to the changes in the volume of SN neurons in normal ageing. Non-stereological studies have reported a decrease in the nuclear volume of SN neurons in ageing [16, 30] and enlargement of the cell bodies as demonstrated on Golgi stains [11]. Stereological studies have yielded divergent results. Although Ma et al. [28, 29] reported smaller pigmented neurons in the SN of older subjects, Cabello et al. [7], using the rotator method, found an increase in the volume of these neurons. Our measurements, using the nucleator [19], a different stereological probe than that used by Cabello et al. [7], are consistent with the latter assessment. Indeed, our observations indicate that there is an increase in the volume of SN in older subjects and that although the comparison by ANOVA of the mean neuronal volumes in younger versus older controls does not reach statistical significance, the analysis of frequency distributions of neuronal volumes does show a significant difference between younger and older subjects (Figs. 6, 7). Importantly, examination of the frequency distribution histograms reveals that the increase in the mean volume of SN in older subjects is not the result of selective loss of small size neurons, but rather a genuine hypertrophy of the cell body of pigmented nerve cells.

A previous morphological study of the TH (+) neurons in the human SN found no correlation of cell body size with age [27]. Although stereological studies of the SN have not examined the volume of TH (+) neurons in normal ageing, there is evidence for a close correspondence between pigmented and TH (+) neurons [2, 7]. Based on this notion, one would expect a hypertrophy of TH (+) neurons in normal ageing. Indeed, we found that TH (+) neurons become larger in normal ageing, a change similar to that observed in pigmented neurons. The mechanism responsible for this neuronal hypertrophy is not directly addressed in the present study; nevertheless, some theoretical possibilities can be entertained. Progressive accumulation of neuromelanin and other pigments could account for the neuronal enlargement. However, this does not explain why the neuronal hypertrophy is present in older subjects, whereas no difference is detected when we compare young and middle-aged subjects. A second possibility is that the increased neuronal size is a response to injury [33]. A third possibility, and the one we favor, is that as SN neurons are lost with age, the remaining neurons take over and reinnervate deafferented targets, predominantly in the striatum. This compensatory hypothesis, also proposed by Cabello et al. [7], is supported by the observation that the total volume of SN pigmented neurons, i.e., volume × number of neurons, stays stable across the age groups. A similar mechanism has been proposed for the hypertrophy of cortical neurons in asymptomatic Alzheimer's disease (D. Iacono, personal communication). Age-associated hypertrophy has been reported in the pigmented neurons of the human locus coeruleus by Iwanaga et al. [22], who attributed the expansion of the somatic cytoplasm to the enveloping of synaptic terminals.

As expected, the loss of SN neurons in PD cases is massive (Figs. 2, 3a) and the remaining neurons are atrophic (Figs. 6, 7). Most of the cases that we examined were in the end stage of the disease. The numbers of remaining pigmented neurons in the SN were in the 20–30% range of a healthy SN, a number consistent with Pakkenberg et al. [38]. Notably, the two PD cases with the higher number of neurons in the SN were those with the shortest duration of disease. Inspection of scatter plots of the mean volume of SN neurons in PD and controls (Fig. 5) shows that the volume of the few remaining SN-pigmented neurons in PD was not significantly different from that of middle-aged subjects. However, further analysis of the volume frequency distribution (Figs. 6, 7a) reveals that the SN of PD cases has a larger proportion of small neurons compared with all of the control groups. This atrophy is opposite to the neuronal hypertrophy of normal ageing and supports the notion that the changes of the SN in PD are not a manifestation of accelerated ageing, but a specific pathologic disorder.

As a result of the neuronal atrophy and the massive loss of neurons, the total volume, (i.e., the product of number and volume) of SN-pigmented neurons in PD was drastically reduced to ~20% compared with controls. This observation suggests that the normal functioning of the nigrostriatal system requires a certain total volume, not number, of SN-pigmented neurons below which motor impairment begins to manifest.

In conclusion, our observations indicate that in the course of normal ageing, neuromelanin-containing and TH (+) neurons of the SN decrease in number, but at the same time their cell bodies enlarge. This finding suggests the presence of a compensatory mechanism within individual SN neurons that allows for normal motor function despite the loss of neurons in normal ageing. Presumably, this compensatory mechanism breaks down or is overwhelmed by the pathological events of PD leading to the onset of the characteristic motor disturbances.

Acknowledgments We are grateful to the BLSA participants and staff. Ms. Karen Wall is recognized for editorial assistance and Dr. An Yang for his advice on statistics. This work was supported by the Johns Hopkins University Morris K. Udall Parkinson's Disease Research Center of Excellence (P50 NS38377) and Alzheimer's Disease Research Center (P50 AG05146) and by the Intramural Research Program of the National Institute on Aging, NIH.

References

1. Aarsland D, Andersen K, Larsen JP, Lolk A, Nielsen H, Kragh-Sorensen P (2001) Risk of dementia in Parkinson's disease: a community-based, prospective study. Neurology 56:730–736
2. Bannon MJ, Whitty CJ (1997) Age-related and regional differences in dopamine transporter mRNA expression in human midbrain. Neurology 48:969–977
3. Braak E, Sandmann-Keil D, Rub U, Gai WP, de Vos RA, Steur EN, Arai K, Braak H (2001) alpha-synuclein immunopositive Parkinson's disease-related inclusion bodies in lower brain stem nuclei. Acta Neuropathol 101:195–201
4. Braak H, Braak E (1991) Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol 82:239–259
5. Braak H, Braak E, Yilmazer D, de Vos RA, Jansen EN, Bohl J (1996) Pattern of brain destruction in Parkinson's and Alzheimer's diseases. J Neural Transm 103:455–490
6. Braak H, Ghebremedhin E, Rub U, Bratzke H, Del Tredici K (2004) Stages in the development of Parkinson's disease-related pathology. Cell Tissue Res 318:121–134
7. Cabello CR, Thune JJ, Pakkenberg H, Pakkenberg B (2002) Ageing of substantia nigra in humans: cell loss may be compensated by hypertrophy. Neuropathol Appl Neurobiol 28:283–291
8. Chen EY, Kallwitz E, Leff SE, Cochran EJ, Mufson EJ, Kordower JH, Mandel RJ (2000) Age-related decreases in GTP-cyclohydrolase-I immunoreactive neurons in the monkey and human substantia nigra. J Comp Neurol 426:534–548
9. Chu Y, Kompoliti K, Cochran EJ, Mufson EJ, Kordower JH (2002) Age-related decreases in Nurr1 immunoreactivity in the human substantia nigra. J Comp Neurol 450:203–214
10. Comella CL (2007) REM sleep disorders and parkinsonism. J Neurol 254(Suppl 5):56–60
11. Cruz-Sanchez FF, Cardozo A, Tolosa E (1995) Neuronal changes in the substantia nigra with aging: a Golgi study. J Neuropathol Exp Neurol 54:74–81
12. Del Tredici K, Rub U, De Vos RA, Bohl JR, Braak H (2002) Where does parkinson disease pathology begin in the brain? J Neuropathol Exp Neurol 61:413–426
13. Dickson DW (1999) Neuropathologic differentiation of progressive supranuclear palsy and corticobasal degeneration. J Neurol 246(Suppl 2):II6–II15
14. Dickson DW (2001) Alpha-synuclein and the Lewy body disorders. Curr Opin Neurol 14:423–432
15. Fearnley JM, Lees AJ (1991) Ageing and Parkinson's disease: substantia nigra regional selectivity. Brain 114(Pt 5):2283–2301
16. Finch CE (1993) Neuron atrophy during aging: programmed or sporadic? Trends Neurosci 16:104–110
17. Forno LS (1996) Neuropathology of Parkinson's disease. J Neuropathol Exp Neurol 55:259–272
18. Gilman S, Low PA, Quinn N, Albanese A, Ben-Shlomo Y, Fowler CJ, Kaufmann H, Klockgether T, Lang AE, Lantos PL, Litvan I, Mathias CJ, Oliver E, Robertson D, Schatz I, Wenning GK (1999) Consensus statement on the diagnosis of multiple system atrophy. J Neurol Sci 163:94–98
19. Gundersen HJ (1988) The nucleator. J Microsc 151:3–21
20. Hughes AJ, Daniel SE, Blankson S, Lees AJ (1993) A clinicopathologic study of 100 cases of Parkinson's disease. Arch Neurol 50:140–148
21. Hughes CP, Berg L, Danziger WL, Coben LA, Martin RL (1982) A new clinical scale for the staging of dementia. Br J Psychiatry 140:566–572
22. Iwanaga K, Yamada M, Wakabayashi K, Ikuta F, Takahashi H (1996) A newly discovered age-related synaptic change in the human locus ceruleus: morphometric and ultrastructural studies. Acta Neuropathol 91:337–342
23. Jellinger KA (2001) The pathology of Parkinson's disease. Adv Neurol 86:55–72
24. Jellinger KA (2002) Alzheimer disease and cerebrovascular pathology: an update. J Neural Transm 109:813–836
25. Jellinger KA (2006) The morphological basis of mental dysfunction in Parkinson's disease. J Neurol Sci 248:167–172
26. Kawas C, Gray S, Brookmeyer R, Fozard J, Zonderman A (2000) Age-specific incidence rates of Alzheimer's disease: the Baltimore Longitudinal Study of Aging. Neurology 54:2072–2077
27. Kubis N, Faucheux BA, Ransmayr G, Damier P, Duyckaerts C, Henin D, Forette B, Le Charpentier Y, Hauw JJ, Agid Y, Hirsch EC (2000) Preservation of midbrain catecholaminergic neurons in very old human subjects. Brain 123(Pt 2):366–373
28. Ma SY, Ciliax BJ, Stebbins G, Jaffar S, Joyce JN, Cochran EJ, Kordower JH, Mash DC, Levey AI, Mufson EJ (1999) Dopamine transporter-immunoreactive neurons decrease with age in the human substantia nigra. J Comp Neurol 409:25–37
29. Ma SY, Roytt M, Collan Y, Rinne JO (1999) Unbiased morphometrical measurements show loss of pigmented nigral neurones with ageing. Neuropathol Appl Neurobiol 25:394–399
30. Mann DM, Yates PO (1979) The effects of ageing on the pigmented nerve cells of the human locus caeruleous and substantia nigra. Acta Neuropathol 47:93–97
31. Mann DM, Yates PO, Marcyniuk B (1984) Monoaminergic neurotransmitter systems in presenile Alzheimer's disease and in senile dementia of Alzheimer type. Clin Neuropathol 3:199–205
32. McGeer PL, McGeer EG, Suzuki JS (1977) Aging and extrapyramidal function. Arch Neurol 34:33–35
33. McIlwain DL, Hoke VB (2005) The role of the cytoskeleton in cell body enlargement, increased nuclear eccentricity and chromatolysis in axotomized spinal motor neurons. BMC Neurosci 6:19
34. McKeith IG, Ballard CG, Perry RH, Ince PG, O'Brien JT, Neill D, Lowery K, Jaros E, Barber R, Thompson P, Swann A, Fairbairn AF, Perry EK (2000) Prospective validation of consensus criteria for the diagnosis of dementia with Lewy bodies. Neurology 54:1050–1058
35. Mikolaenko I, Pletnikova O, Kawas CH, O'Brien R, Resnick SM, Crain B, Troncoso JC (2005) Alpha-synuclein lesions in normal aging, Parkinson disease, and Alzheimer disease: evidence from the Baltimore Longitudinal Study of Aging (BLSA). J Neuropathol Exp Neurol 64:156–162
36. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L (1991) The Consortium to Establish a Registry for Alzheimer's Disease

(CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 41:479–486
37. Morris JC (1997) Clinical dementia rating: a reliable and valid diagnostic and staging measure for dementia of the Alzheimer type. Int Psychogeriatr 9(Suppl 1):173–176 (discussion 177–178)
38. Pakkenberg B, Moller A, Gundersen HJ, Mouritzen Dam A, Pakkenberg H (1991) The absolute number of nerve cells in substantia nigra in normal subjects and in patients with Parkinson's disease estimated with an unbiased stereological method. J Neurol Neurosurg Psychiatry 54:30–33
39. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M (1997) Alpha-synuclein in Lewy bodies. Nature 388:839–840
40. Spillantini MG, Crowther RA, Jakes R, Cairns NJ, Lantos PL, Goedert M (1998) Filamentous alpha-synuclein inclusions link multiple system atrophy with Parkinson's disease and dementia with Lewy bodies. Neurosci Lett 251:205–208
41. Stark AK, Pakkenberg B (2004) Histological changes of the dopaminergic nigrostriatal system in aging. Cell Tissue Res 318:81–92
42. Takeda A, Kikuchi A, Matsuzaki-Kobayashi M, Sugeno N, Itoyama Y (2007) Olfactory dysfunction in Parkinson's disease. J Neurol 254(Suppl 4):IV2–IV7
43. Thiessen B, Rajput AH, Laverty W, Desai H (1990) Age, environments, and the number of substantia nigra neurons. Adv Neurol 53:201–206
44. Tooyama I, McGeer EG, Kawamata T, Kimura H, McGeer PL (1994) Retention of basic fibroblast growth factor immunoreactivity in dopaminergic neurons of the substantia nigra during normal aging in humans contrasts with loss in Parkinson's disease. Brain Res 656:165–168
45. van Domburg PHMF, ten Donkelaar HJ (1992) The human substantia nigra and ventral tegmental area: a neuroanatomical study with notes on aging and aging disease. Adv Anat Embryol Cell Biol 121:32–69
46. West MJ, Gundersen HJ (1990) Unbiased stereological estimation of the number of neurons in the human hippocampus. J Comp Neurol 296:1–22
47. West MJ, Slomianka L, Gundersen HJ (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. Anat Rec 231:482–497
48. West MJ, Coleman PD, Flood DG, Troncoso JC (1994) Differences in the pattern of hippocampal neuronal loss in normal ageing and Alzheimer's disease. Lancet 344:769–772
49. West MJ, Slomianka L (1998) Total number of neurons in the layers of the human entorhinal cortex. Hippocampus 8:69–82
50. Yamamoto T, Hirano A (1986) A comparative study of modified Bielschowsky, Bodian and thioflavin S stains on Alzheimer's neurofibrillary tangles. Neuropathol Appl Neurobiol 12:3–9