Disentangling the Relationship between Lewy Bodies and Nigral Neuronal Loss in Parkinson's Disease

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Abstract

Progressive rostral spread of Lewy body (LB) pathology is thought to reflect the clinical course of Parkinson's disease (PD) although several studies have suggested that LBs are not the toxic species responsible for cell death. We investigated the relationship between nigral dopaminergic cell loss, distribution and density of α-synuclein-immunoreactive LBs and duration of motor symptoms in 97 patients with PD. Density of pigmented neurons was measured in a single section of one half of the substantia nigra (SN) with delineation of the dorsal and ventral tiers whereas the cortical and nigral LB densities were determined using a morphometric approach. The density of nigral neurons was estimated to decrease by 2% each year after confirmation of the clinical diagnosis of PD but showed marked heterogeneity with some PD patients with longer duration of illness still possessing a significant number of preserved pigmented nigral neurons at the time of death. An average 15% of surviving nigral neurones contained LBs and the age–adjusted proportion of LB-bearing neurons appeared relatively stable throughout the disease duration. No difference was observed in the age at death or duration of disease with respect to Braak PD stages. The nigral neuronal density was unrelated to either the Braak PD stage or to cortical LB densities. We conclude that nigral neuronal loss is slow and shows considerable variation in PD. Our data also provides no support for a primary pathogenic role of LBs as neither their distribution nor density was associated with the severity of nigral cell loss.

Keywords

Duration; Lewy bodies; neuronal loss; Parkinson's disease; substantia nigra; α-synuclein

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INTRODUCTION

The pathological findings in Parkinson's disease (PD) include a selective loss of dopamine-producing neurons in the pars compacta of the substantia nigra (SN) and the presence of intracytoplasmic aggregation of α-synuclein (αS) protein in the form of Lewy bodies (LBs) in surviving neurons. Because some of these αS-immunoreactive (IR) inclusions are found in the predilection sites of neuronal loss i.e., SN and locus coeruleus, it has been considered that their presence is deleterious to neuronal survival. In addition to strong genetic support for αS being crucial in the pathogenesis of PD, there are numerous *in vitro* and *in vivo* studies that have shown post-translational modifications and other properties of αS leading to increased protein aggregation to be intimately linked with neurotoxicity. However, it still remains unresolved which "species" of the αS aggregation process we should be targeting, and rather than the LBs, a number of "precursor LB" candidates have been proposed to be neurotoxic including the synaptic αS aggregates and earlier species of αS fibrillization (i.e., oligomeric or protofibrillar αS) [1-4]. Some authorities now consider LBs to be innocent bystanders or that they may represent a defence mechanism against the primary process underlying nerve cell death [5-7].

In PD, the loss of neurones in the ventrolateral tier of the pars compacta of the SN appears disproportionately high in comparison to the relatively small number of LBs and it seems unlikely that every dying neuron first goes through a stage of LB formation [8]. In patients with dementia with Lewy bodies (DLB) with widespread and extensive cortical LBs, no correlation has been found between the number of LBs and neuronal loss in the temporal cortex [9]. Large numbers of LBs are also found without substantial neuronal loss in some elderly people who have died without evidence of neurodegenerative disease [10-13]. Conversely, there are no LBs in the premotor cortex where significant neuronal loss selectively occurs in PD [14]. *In vitro* studies have suggested that over-expression of wild-type αS protects against apoptosis [15, 16] and that neuronal death may occur prior to LB formation in some neurons [17]. LB-containing neurons have been described to look morphologically healthier (cell and nucleolar size) than neighbouring non-LB-containing neurons [18] and it has been reported that majority of nigral neurons that die from apoptosis are those without inclusions and thus may be dying before LB formation occurs [19].

Despite evidence indicating that LBs *per se* may not be harmful, their topographical distribution is still considered to be an important determinant of PD-related clinical symptoms [20-22]. The progressive spread of LB pathology from the brainstem to neocortex has been suggested to reflect the clinical progression from prodromal to extrapyramidal symptoms and finally to dementia [20-22]. Furthermore, the finding of LBs in long surviving fetal mesencephalic neurons grafted into the striatum of PD patients [23] has led to the notion that a "prion-like" mechanism involving permissive templating and autophagy could be important in the pathogenesis of PD [24]. However, if the LB pathology spreads rostrally into the neocortex, one might expect that PD patients in the later Braak PD stages should be older than those in the preclinical stages. No such linear age dependence has yet been observed [25, 26]. In addition, PD patients with longer disease duration might be expected to have wider regional distribution and greater density of LB pathology, and some correlation with the degree of pars compacta nigral cell loss should be found.

In order to gain a clearer understanding of the progression of PD-related pathology, we have investigated the relationship between pars compacta nigral cell loss, regional distribution and density of αS-immunoreactive (IR) LB pathology and duration of PD in a large well-characterized group of patients with PD.

MATERIALS AND METHODS

Case selection and clinical assessment

Ninety-seven patients who fulfilled Queen Square Brain Bank (QSBB) criteria for the diagnosis of PD were identified from the records of donors to the QSBB for Neurological Disorders, all of whom had received a pathological confirmation of the diagnosis between 2000 and 2008. The London Multicentre Research Ethics Committee (reference 08/H0718/54) has approved procedures for the donation of brains to the QSBB as well as retention and access to clinical records.

We performed a systematic review of the case files. All patients had been regularly assessed by hospital specialists (neurologists or geriatricians with a special interest in PD). Cases were excluded if the medical records did not contain well-documented reports of the disease course or if the brain material for the neuropathological assessment was inadequate. All cases were genotyped and no monogenetic parkinsonism or LRRK2 and GBA mutation carriers were included in the study.

Neuropathological assessment

After fixation in 10% buffered formalin (i.e., 3.6% formaldehyde), the brains were examined by a neuropathologist and sampled in accordance with the standardised protocols of the QSBB [27]. In compliance with established criteria for the neuropathological diagnosis of PD [28], brain samples from selected regions were embedded in paraffin. For the αS immunohistochemistry, 8 μm-thick tissue sections were cut from three brainstem regions: medulla with dorsal motor nucleus of vagus, pons with locus coeruleus and midbrain with SN; from amygdala; and from five cortical regions: middle frontal gyrus, Brodmann's area (BA) 8/9; middle temporal gyrus, BA 21; inferior parietal lobule BA 40; entorhinal cortex BA 28; and anterior cingulate gyrus BA 24. These sections were deparaffinised, rehydrated and treated with formic acid for 15 min and pressure-cooked in citrate buffer at pH 6.0. Following epitope unmasking, monoclonal antibody to α-synuclein (clone KM51, dilution 1: 1000; Novocastra; Newcastle upon Tyne, UK) was applied and incubated overnight at +4°C. For detection, Histostain SP kit (Zymed, San Francisco, CA) was used with Romulin AEC chromogen (Biocare Medical, Walnut Creek, CA).

Each case was classified according to Braak PD stage (ranging from 0 to 6) depending on the topographic distribution of αS-IR inclusions seen in these 8μm-thick sections [20]. In addition, all compact intensely αS-IR LBs (not diffuse or punctate staining) were systematically counted by two investigators (LP and CC) within the total thickness of five cortical grey matter regions and in the SN and these counts were adjusted to the surface area (LB/mm^2) using Image-Pro Plus software package (MediaCybernetics, UK). Several LBs within one neuron were counted as a single inclusion. In each case, the "total cortical LB

density" was determined as the sum of counts in five cortical areas divided by a sum of respective surface areas. The proportion of SN neurons with LBs was calculated by dividing the total number of LBs by the total number of pigmented neurons in SN. All pathological analyses were done blinded to the clinical data.

For the nigral cell counts, a single, transverse 20 μm-thick section of the midbrain was cut at the level where the fascicles of the 3rd cranial nerve emerge from the midbrain and demarcate the ventral tegmental area from the SN and stained with the Luxol fast blue/cresyl violet method. This is an optimal section of midbrain as it permits the evaluation of pertinent nuclear groups at that level, [28] and thus, all those cases where this anatomical landmark (i.e., 3rd nerve) was not identified were excluded. Limits of each SN pars compacta were outlined manually and further divided into ventral and dorsal tiers (Fig. 1a) at ×20 magnification by using a Zeiss research microscope together with Image-Pro Plus software package (MediaCybernetics, UK) [29]. The Image-Pro Plus software automatically divides each manually outlined area into a number of non-overlapping counting squares of equal size of 300 μm ×300 μm where all neuromelanin-containing pigmented neurons with or without a nucleus were identified and labeled manually using magnification × 200. When the nucleus was not visible due to intense pigment deposition, neurons were identified on the basis of their size and morphological features. The pars lateralis was excluded from the analysis because in this region there is a considerable mixing of neuronal types. The total number of counting squares was used to determine the surface area (x × 90 000 μm^2 ~0.09 mm^2) and finally the neuronal density was expressed as neurons/mm^2. Our non-stereological study aimed to provide an estimate of nigral neuronal numbers and thus the Abercrombie's correction that requires the exact mean object height in the axis perpendicular to the section plane and z-axis collapse was not used. SN is affected only minimally by tissue shrinkage and the single section counting under proper conditions (i.e., well-defined cutting level, constant section thickness) has been demonstrated to be as reliable as the disector method in evaluating the neuronal loss from SN [30, 31]. The side of the brain selected for counting SN neuronal numbers was random and depended on which side had been embedded in paraffin (decision based on odd and even autopsy numbers).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, Ill). The number of nigral neurons was counted twice by a single investigator (LP) in one quarter of the cases (intra-rater reliability) and LB counts were assessed twice in all cases (inter-rater reliability) by 2 investigators (LP, CC). Intra- and inter-rater reliabilities were examined with intra-class correlation (ICC) coefficients that were >0.80 for all the ratings performed, thus reflecting high reliability. Only the primary counts were used for the analyses. Group comparisons were made using the two-tailed two-sample *t*-test, one-way analysis of variance (ANOVA) or Kruskal-Wallis test, as appropriate. Bivariate correlations were assessed by means of the Pearson (r) or Spearman non-parametric rank (rho) correlation coefficient. Generalized linear models that allow the adjustment for the different confounding variables were used to analyze the number of pigmented neurons and LBs. Since we were analysing count data adjusted for area (mm^2) and the variances of these variables were much larger than the corresponding means (i.e., overdispersed data), we fitted the regression model

based on the negative binomial distribution. In these models, the dependent variable is either the number of pigmented neurons or LBs and the offset a natural logarithm (ln) of the area, whereas different confounding variables are included in the model as covariates. The estimated relative rate (ERR) was interpreted in a similar way to a relative risk and odds ratio (i.e., as the estimated count per unit area for a unit increase in the value of the covariate). The null hypothesis was rejected at the 0.05 significance level.

RESULTS

Study population

The 97 PD patients (63 male and 34 female) ranged in age from 40 to 92 years (mean 75.4, SD 9.0 years). The mean age of onset of PD (defined as the retrospective report of first symptoms) was 60.6 years (SD 11.1 years, range 36.5–84.2 years) and the mean duration of illness 15.2 years (SD 7.0 years, range 4.1–31.2 years). Patients were segregated into five groups depending on the duration of disease at death: 11 patients <5 years, 20 patients 6–10 years, 20 patients 11–15 years, 22 patients 16–20 years and 24 patients >20 years. There was no significant correlation between duration of PD and age at death ($r = -0.07$, $p = 0.51$), but there was significant negative correlation between age at onset and duration of illness ($r = -0.68$, $p < 0.001$) i.e., later onset, shorter survival.

Neuronal loss in SN and duration of PD

The mean neuronal counts, area measurements and densities are given in Table 1. The number and density of pigmented neurons correlated significantly with each other ($r = 0.75$, $p < 0.001$). There was a negative linear correlation between both number ($r = -0.31$, $p = 0.002$) and density ($r = -0.34$, $p = 0.001$) (Fig. 2a) of pigmented neurons in the SN and duration of PD. The fit of the linear model was similar that of the sigmoid ($r = -0.36$) and quadratic ($r = -0.35$) models. A slightly better fit for the neuronal loss ($r = -0.37$, $p < 0.001$) (Fig. 2b) was obtained with a negative exponential curve which was calculated with the following equation: Density of neurons in SN = $10^{(1.22-0.01\times\text{duration of PD in years})}$. This equation shows that the neuronal density in a given year is 0.98 times (i.e., $10^{-0.01}$) the neuronal density in the previous year. However, only 14% of the variation in the SN neuronal density could be explained by duration of PD. Some PD patients with longer >21-year duration of illness still possessed a fair number of pigmented nigral neurons (Fig. 1b, c). There was also a significant difference in both the mean number (ANOVA, $p = 0.02$, F = 3.14, df = 4) and the density (ANOVA, $p = 0.006$, F = 3.86, df = 4) of nigral neurons between the duration groups (Table 2).

Using univariable models, the duration of PD was negatively (ERR = 0.98, 95% CI = 0.97–0.99, $p < 0.001$) and the age at onset positively associated (ERR = 1.01, 95% CI = 1.00–1.02, $p = 0.003$) with the density of pigmented neurons in SN. Age at death ($p = 0.39$) and gender ($p = 0.79$) were not significantly associated with the nigral neuronal density. When the subregions of SN were analysed separately, the neuronal density decreased over time both in the dorsal ($p < 0.001$, ERR = 0.98, 95% CI = 0.95–0.99) and ventral tier ($p = 0.01$, ERR = 0.98, 95% CI = 0.96–0.99). The multivariable model showed that the duration of PD was negatively associated (ERR = 0.98, 95% CI = 0.97–1.00, $p = 0.03$) with the density of

nigral neurons after adjustment for age at onset. The age at onset-adjusted decrease in the density of pigmented neurons in time was found in the dorsal tier ($p = 0.03$, ERR = 0.98, 95% CI = 0.97–1.00) but not in the ventral tier ($p = 0.25$) of SN. In comparison to PD patients with <5 years of duration (reference group) the density of nigral neurons significantly decreased with 16–20 years (ERR = 0.72, 95% CI = 0.54–0.97, $p = 0.03$) and >20 years (ERR = 0.68, 95% CI = 0.50–0.94, $p = 0.02$) of duration (Table 3). The two earlier duration groups 5–10 years ($p = 0.56$) and 10–15 years ($p = 0.27$) showed no significant difference.

Proportion of nigral neurons with LBs and duration of PD

The estimated proportion of nigral neurons with LBs was 14.9 ± 1.3% (mean ± S.E.M), ranging from 0.3% to 68%. The proportion of LB-bearing neurons was <30% in all cases except in 10 "outliers": in 3 of these (2 with duration of PD <5 years) the proportions were >50% (68%, 61%, 53%) due to relatively high LB and low nigral neuronal numbers. There was a negative correlation between proportion of LB-bearing nigral neurons ($\rho = -0.31$, $p = 0.002$) and duration of PD (Fig 2C). No significant difference was seen in the median proportion of LB-bearing neurons between the duration groups (Kruskal-Wallis; $p = 0.05$) (Table 2). The median proportion of nigral neurons with LBs was twice as high in the first 5 years and then remained relatively stable.

Univariable models showed that duration of PD (ERR = 0.97, 95% CI = 0.95–0.99, $p = 0.02$) and the age at death (ERR = 0.98, 95% CI = 0.96–1.00, $p = 0.02$) were significantly negatively associated with the proportion of LB-bearing neurons. Age at onset ($p = 0.78$) and gender ($p = 0.61$) were not significantly associated with the proportion of nigral neurons with LBs. Age at death-adjusted duration of PD remained significantly negatively associated (ERR = 0.97, 95% CI = 0.95–0.99, $p = 0.002$) with the proportion of LB-bearing neurons. The proportion of LB-bearing neurons in PD patients decreased by ~50% within the first 5-year duration of illness and then seemed to plateau (Table 3). However, when we excluded the 3 "outliers" with >50% of LB-bearing neurons, the observed significant associations were lost (except when comparing >20-year to <5-year duration) and every age group appeared to have a similar proportion of nigral neurons with LBs.

LBs and their relationship to duration of PD and neuronal loss in SN

There was a significant negative association between duration of PD and age-adjusted LB densities in the temporal (ERR = 0.91, 95% CI = 0.86–0.96, $p < 0.001$), entorhinal (ERR = 0.93, 95% CI = 0.90–0.97, $p < 0.001$) and total cortices (ERR = 0.95, 95% CI = 0.91–0.98, $p = 0.005$) as well as in the SN (ERR = 0.94, 95% CI = 0.92–0.96, $p < 0.001$) (Table 4). There was also a significant difference in the median cortical LB density between the different duration groups of PD (Kruskal-Wallis; $p = 0.04$) (Table 2). However, we found no difference in the mean duration of PD between Braak PD stages (ANOVA, $p = 0.29$ F = 1.26, df = 2). The mean duration of illness was 17.2 years in 4 patients with Braak PD stage 3, 16.4 years in 37 patients with stage 5 and 14.2 years in 56 cases with stage 6. In addition, mean age at death did not differ between Braak PD stages (ANOVA, $p = 0.07$, F = 3.86, df = 2).

There was no significant correlation between total cortical LB density and the density of nigral neurons ($\rho = -0.03$, $p = 0.78$). A positive correlation was found between the number of pigmented neurons in the SN ($\rho = 0.23$, $p = 0.03$) and the total count of LBs within this nucleus (Fig. 2D), i.e., more neurons, more LB-bearing neurons can be seen. Multivariable model showed no association between total cortical LB ($p = 0.94$) and nigral neuronal densities but a significant positive association was seen between nigral LB (ERR = 1.10, 95% CI = 1.05–1.15. $p < 0.001$) and nigral neuronal densities after adjustment for the age at death (Table 4). We found no difference in either mean SN neuronal number (Kruskal-Wallis, $p = 0.36$) or density (Kruskal-Wallis, $p = 0.32$) between Braak PD stages.

DISCUSSION

We have confirmed that both neuronal number and density in the SN in PD decrease over time [32, 33] and that the best fit for nigral neuronal loss is a negative exponential curve. Our neuronal counts in SN, especially in the dorsal tier, were higher than those reported in the study by Fearnley and Lees carried out almost 20 years ago in the QSBB, despite similar age and duration of PD [32]. This is likely to be due to differences in the technique of cell counting and sub-division of SN. Furhermore, the sections were three times thicker in the present study which is probably the single most important difference. Marked heterogeneity was observed with some PD patients with longer duration of illness still possessing a fair number of pigmented nigral neurons. One explanation could be that these individuals may have had a higher number of nigral neurons to start with but it is perhaps more plausible to suggest that the rate of nigral neuronal loss differs between patients. We have recently found that nigral cell loss is less in benign tremulous Parkinsonism than in patients with PD (Selikhova et. al., unpublished data). It is also possible that preterminal synaptic failure may be the more critical pathogenic factor for bradykinesia rather than the neuronal loss *per se* [34, 35].

Age at death appeared not to be associated with the nigral neuronal density, even in the dorsal tier, which also contrasts with previous work [32, 36]. A lower age at onset and longer duration of PD were both found to be associated with decreased neuronal numbers in SN. We also observed an age of onset-adjusted association suggesting an average 2% loss in the density of pigmented neurons in SN each year once clinical symptoms had appeared. In comparison to the PD patients with up to 5-year duration of illness, the density of nigral neurons in PD patients with 15–20 and >20 years of duration decreased by 28% and 33%, respectively. The age at onset-adjusted negative association between nigral neuronal number and duration of PD was restricted to the dorsal tier further emphasizing the possibility of different time-dependent mechanisms of sub-regional nigral cell loss.

We found an average of 15% of nigral neurons to contain αS-IR LBs, a somewhat higher figure than previous reports [37, 38]. In agreement with the findings of Greffard and colleagues, we found that the proportion of LB-containing neurons remained relatively stable throughout the course of the disease [37]. This could be explained by a passive "one-pass" phenomenon whereby the LBs appear at the beginning of the disease and then decrease at the same rate as nigral neurons are lost or alternatively that a dynamic "turn-over" occurs with LBs continuously produced and destroyed at the same rate [37]. If the

number of LB-containing neurons dying is balanced by the number of newly-created LB-containing neurons, further studies are now needed to determine what happens to the LBs when the host-neuron dies? What proportion of them remains lying free in the neuropil analogous to "ghost tangles" (i.e., extracellular neurofibrillary tangles, NFT) that are seen in Alzheimer's disease [39] and how quickly are they degraded? αS-IR LBs have been reported to be rather insoluble [40], albeit not as insoluble as tau-IR NFTs, so they may be more quickly removed compared to NFTs that can survive in hippocampal neurons for 20 years [41]. In addition, if LBs do turn around, it needs to be established whether this only occurs in the SN or also in other brain regions.

Greffard and colleagues estimated LBs to have a six-month life span (extending to 16 months for any type of αS-IR inclusions) based on the fact that they found twice as many neurons dying each year compared to the proportion of LB-bearing neurons [37]. If we apply the same mathematical modelling to our observations, we would conclude that LBs survive for 7.5 years (15% of LB-bearing neurons/2% neuronal death per a year). It must be remembered however that this calculation is based on the unproven assumption that neuronal death only occurs in the LB-bearing neurons.

In common with some previous studies, our early (i.e., lower brainstem) versus advanced (i.e., neocortical involvement) Braak PD stage cases showed no age differential [25, 26]. Furthermore, we found a decrease in the density of LBs in most regions over time suggesting that cases of PD are unlikely to progress at the same rate [42]. The patients with a shorter disease duration and an older age at onset may have a more malignant form of the disease, with much faster progression as has been suggested recently [43, 44]. Recent work has suggested that concomitant amyloid and tau pathologies are also important in determining severity and time to cognitive deficit in PD (i.e., the rate of disease progression) in addition to cortical LB burden [44].

The PD patients with a widespread distribution of αS pathology (i.e., higher Braak PD stages) or higher cortical LB densities in our study did not show a decreased neuronal density in SN in keeping with the reported weak link between the burden of αS-IR LB pathology and clinical symptoms [10-13]. The only area where density of LBs positively associated with cell loss was in the SN itself most likely indicating that the more neurons there are the more LBs can be seen inside them. Greffard and colleagues have shown that the number of nigral LBs was not correlated with nigral neuronal density, rigidity, akinesia, UPDRS or duration of the disease [37]. We also failed to detect an inverted u-shape distribution over time where the number of inclusions increases with the progression of the disease until the neurons start to die further suggesting that these two phenomena are unlikely to be linked in a direct causative chain.

The strict inclusion of a large number of PD patients for which clinical information was available together with uniform and detailed counts of pigmented neurons at a well-defined level of SN and comprehensive assessment of LB pathology throughout the brain represent strengths of the present study. The retrospective nature of the study, the lack of a control group or individuals with modest pathological burden (i.e., incidental Lewy body disease) and the quantification of nigral cell numbers from only on one side of the SN are

shortcomings of our study. In addition, as LB-containing neurons in most cases represent a small fraction of the total neuronal count in the SN, there is almost certainly more variability from slide to slide in their number than anticipated.

Despite the reasonably close correlation between neuronal density in SN and severity of bradykinesia and rigidity in PD [33], our results suggest that nigral cell loss is gradual and there is considerable variability which may explain the clinical heterogeneity. The observed stability in the proportion of LB-containing nigral neurons throughout the disease duration can be explained by either "one-pass" or "turn over" models. Neither of these paradigms however unambiguously approve that LBs are noxious to their host cells. In our view, the fact that neither the widespread regional distribution of LBs (i.e., Braak PD stages) nor increased cortical LB densities were found directly linked with pars compacta nigral cell loss lends support to the view that they are not the primary cause of the pathological process leading to cell death in vulnerable regions in the brain in PD. Further studies focusing to how Lewy neurites or presynaptic aggregates which make up the majority of αS pathology relate to nigral cell and synaptic loss are therefore warranted.

Acknowledgments

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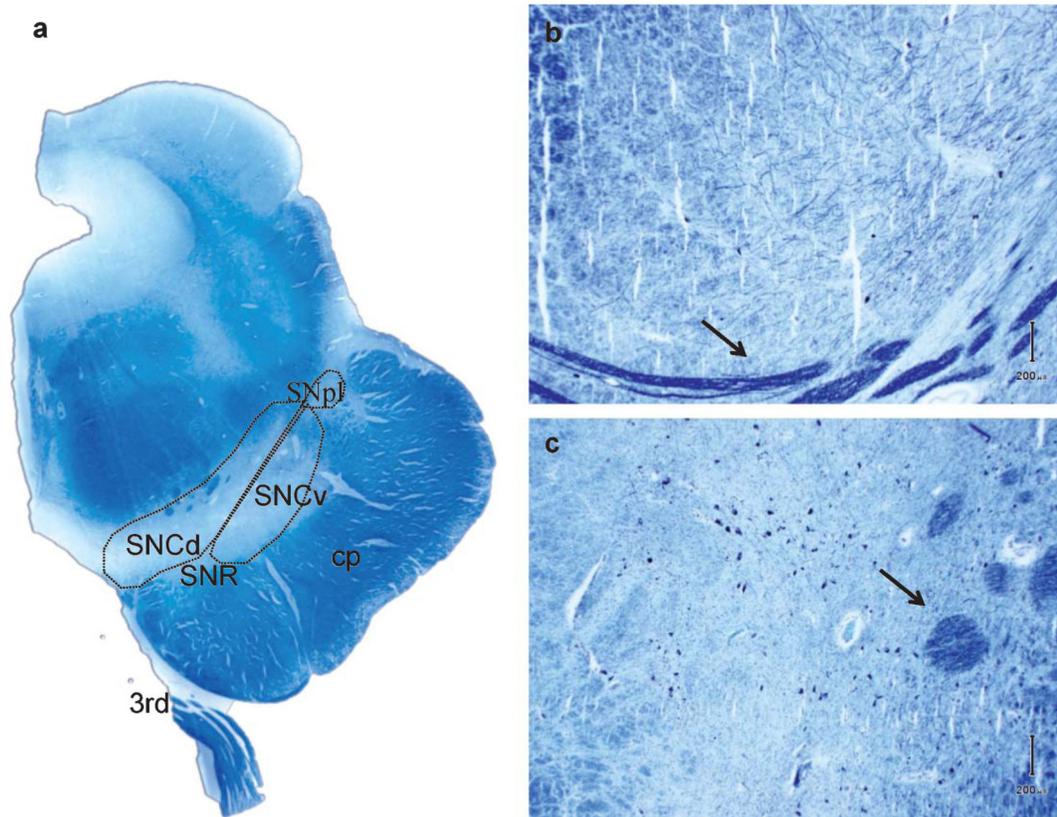
Fig. 1.
(a) Transverse level of the midbrain at the level of the 3rd cranial nerve showing how the two tiers of substantia nigra pars compacta (dorsal, SNCd and ventral, SNCv) were depicted (adapted from Halliday 2004). More ventrally positioned substantia nigra reticulata (SNR) and dorsolaterally located pars lateralis (SNpl) are also indicated. (b, c) Photomicrographs depicting the heterogeneity in the number of pigmented nigral neurons between two PD patients with similar disease duration. (b) PD patient with 27.5 year duration; nigral cell count 7.0/mm^2, (c) PD patient with 27.0 year duration, nigral cell count 21.8/mm^2. Arrows point to the 3rd nerve fascicles. Luxol fast blue/cresyl violet. Scale bars: 200 μm.
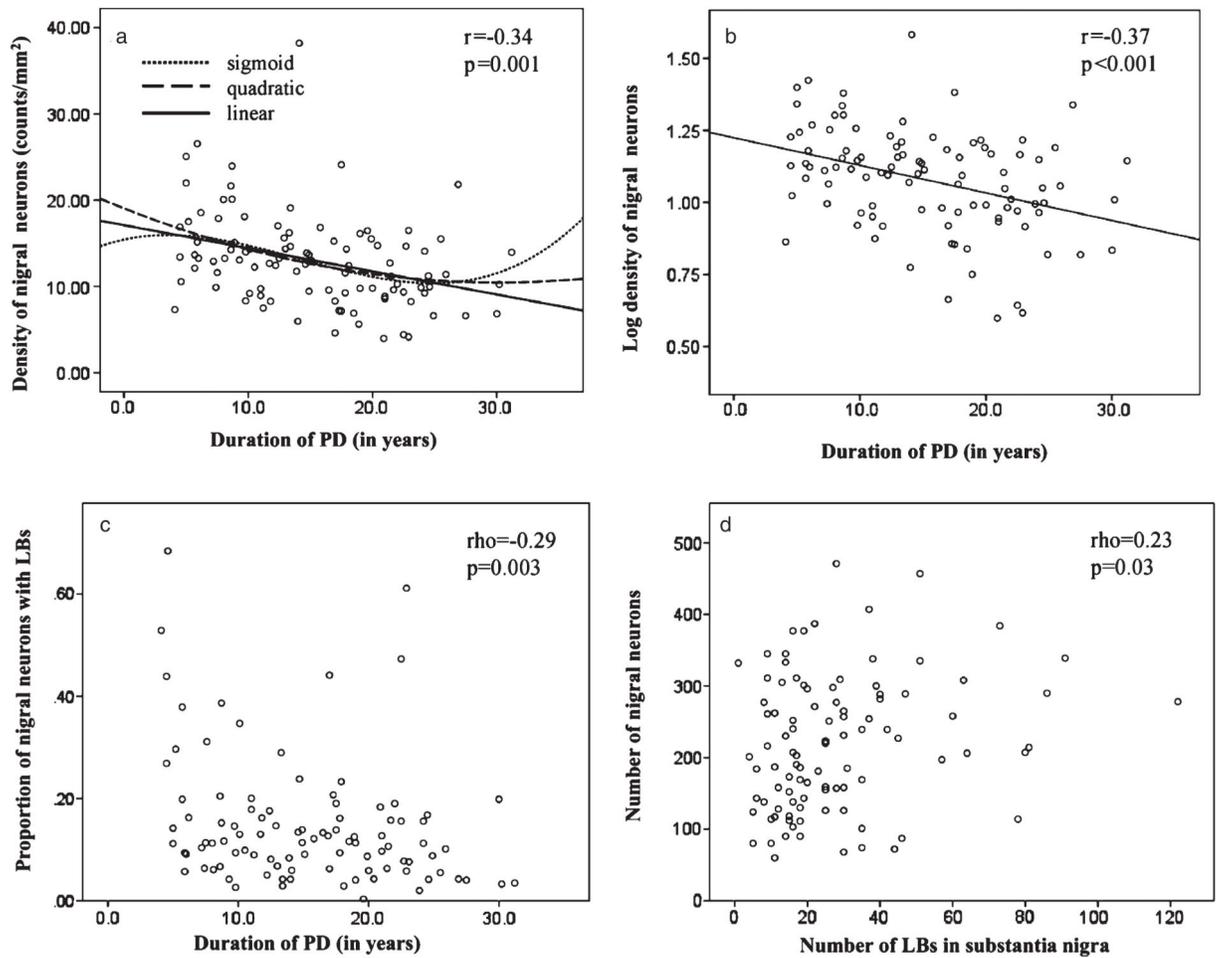
Fig. 2.
Graphs depicting correlations between duration of Parkinson's disease and (a) the density and (b) log of the density of nigral pigmented neurons and (c) proportion of nigral neurons with Lewy bodies. D shows correlation between the number of Lewy bodies and the number of neurons in the substantia nigra.

Table 1

Mean neuronal counts, area measurements and densities in total, ventral and dorsal tiers substantia nigra

	Mean ± SD pigmented neuronal count (range)	Mean ± SD area in mm^2 (range)	Mean ± SD pigmented neuronal density (count per mm^2) (range)
Total SN	220 ± 94 (60–471)	17.2 ± 4.8 (8.4–30.7)	13.0 ± 5.4 (4.0–38.2)
Dorsal tier of SN	161 ± 70 (57–364)	11.0 ± 3.0 (5.0–18.4)	14.9 ± 6.3 (5.6–46.0)
Ventral tier of SN	60 ± 39 (0–210)	6.3 ± 2.2 (1.5–12.3)	9.7 ± 5.8 (0–28.5)

SN = substantia nigra; SD = standard deviation.

Table 2

Mean nigral neuronal counts and densities, and median proportion of nigral neurons with LBs and cortical LB densities within duration groups

Duration group	N	Mean ± SD nigral neuronal count (range)	Mean ± SD nigral neuronal density (count/mm^2) (range)	Median proportion of nigral neurons with LBs (%) (range)[*]	Median cortical LB density (LB/mm^2) (range)
<5 years	11	271 ± 109 (87–457)	16.4 ± 6.0 (7.3–26.5)	26.8 (6.0–68.0)	1.4 (0.1–6.6)
5–10 years	20	252 ± 75 (101–407)	15.0 ± 4.3 (8.3–24.0)	11.3 (3.0–39.0)	0.3 (0–5.1)
11–15 years	20	234 ± 99 (90–471)	13.8 ± 6.7 (6.0–38.2)	12.2 (3.0–29.0)	0.2 (0–1.3)
16–20 years	22	194 ± 95 (60–384)	11.5 ± 4.9 (4.0–24.1)	11.9 (0–44.0)	0.4 (0–5.2)
>20 years	24	182 ± 79 (72–377)	10.6 ± 4.0(4.1–21.8)	9.2 (2.0–61.0)	0.2 (0–1.9)
p value		0.02 [†]	0.006 [†]	0.05 [‡]	0.04 [‡]

LB=Lewy body; SD = standard deviation.

[*] Calculated by dividing the total number of Lewy bodies by the total number of pigmented neurons in substantia nigra.

[†] Group comparisons were made using ANOVA

[‡] Group comparisons were made using Kruskall-Wallis.

Table 3

Association of nigral neuronal density and proportion of nigral neurons with LBs with duration of PD

Duration of PD	Nigral neuronal count/mm^{2†}		Proportion of nigral neurons with LBs‡	
	ERR (95% CI)	p value	ERR (95% CI)	p value
<5 years	1$^{\#}$	–	1$^{\#}$	–
6–10 years	0.92 (0.69–1.22)	0.56	0.53 (0.31–0.90)	0.02
			0.68 (0.39–1.17)*	0.16*
11–15 years	0.85 (0.64–1.13)	0.27	0.46 (0.27–0.79)	0.005
			0.59 (034–1.02)*	0.06*
16–20 years	0.72 (0.54–0.97)	0.03	0.46 (0.27–0.79)	0.005
			0.60 (0.35–1.03)*	0.06*
>20 years	0.68 (0.50–0.94)	0.02	0.45 (0.27–0.75)	0.003
			0.51 (0.29–0.87)*	0.01*

†Multivariable model adjusted for age at onset

‡Multivariable model adjusted for age at death

$^{\#}$different categories of duration are compared to the reference category of 1–5 years

*results when 3 outliers with >50% proportion of LB-bearing neurons are excluded from the analysis. CI = confidence interval; ERR = estimated relative rate; LB = Lewy body; PD = Parkinson's disease.

Table 4

LB densities and their age-adjusted association to duration of PD and nigral neuronal density

Brain region	Mean ± SD LB density (count per mm²) (range)	Duration of PD[*]		Nigral neuronal density[*]	
		ERR (95% CI)	p value	ERR (95% CI)	p value
Parietal cortex	0.1 ± 0.3 (0–2.4)	0.97 (0.91–1.04)	0.39	0.93 (0.83–1.05)	0.26
Frontal cortex	0.3 ± 0.8 (0–4.6)	0.95 (0.90–1.00)	0.05	1.02 (0.91–1.14)	0.67
Temporal cortex	0.7 ± 2.0 (0–13.5)	0.91 (0.86–0.96)	<0.001	0.90 (0.90–1.09)	0.87
Cingulate gyrus	1.4 ± 2.2 (0–13.3)	0.97 (0.93–1.00)	0.08	0.99 (0.94–1.05)	0.81
Entorhinal cortex	1.6 ± 2.7 (0–18.6)	0.93 (0.90–0.97)	<0.001	1.02 (0.96–1.09)	0.53
Total cortex	0.7 ± 1.2 (0–6.6)	0.95 (0.91–0.98)	0.005	1.00 (0.94–1.06)	0.94
Substantia nigra	1.8 ± 1.6 (0.1–9.3)	0.94 (0.92–0.96)	<0.001	1.10 (1.05–1.15)	<0.001

[*] Multivariable model adjusted for age at death. CI = confidence interval; ERR = estimated relative rate; LB=Lewy body; PD = Parkinson's disease; SD = standard deviation.