α-Synuclein redistributes to neuromelanin lipid in the substantia nigra early in Parkinson’s disease

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The distribution and tempo of neuronal loss in Parkinson’s disease correlates poorly with the characteristic and more widely spread intracellular changes associated with the disease process (Lewy bodies and Lewy neurites). To determine early intracellular changes in regions where cell loss is most marked (dopaminergic A9 substantia nigra) versus regions with Lewy bodies but where cell loss is limited, we assessed 13 patients with definite Parkinson’s disease at various disease stages in comparison with controls. Using immunohistochemistry for α-synuclein, we confirmed the concentration of this protein in the soma of normal A9 neurons and in Lewy body pathology in brainstem catecholamine neurons in Parkinson’s disease. Analysis of the degree of cell loss in brainstem catecholamine cell groups revealed that only the A9 substantia nigra had consistent significant cell loss early in the disease course with greater A9 cell loss correlating with increasing disease duration. To assess the earliest intracellular changes differentiating neurons more likely to degenerate, pigmented A9 and A10 neurons with and without obvious pathology were targeted, cell size and pigment density measured, and intracellular changes in α-synuclein location and lipid components analysed at both the light and electron microscope levels. There were no changes observed in healthy A10 neurons in Parkinson’s disease compared with controls. Pigmented A9 neurons in later stages of degeneration with obvious Lewy body formation had a significant reduction in intracellular pigment, as previously described. In contrast, A9 neurons of normal morphological appearance and no characteristic pathology in Parkinson’s disease exhibited significantly increased pigment density associated with a concentration of α-synuclein to the lipid component of the pigment and a loss of associated cholesterol. These changes in vulnerable but apparently healthy A9 neurons occurred without any change in cell size or in the amount of intracellular pigment compared with controls. The increase in pigment density is consistent with previously reported increases associated with oxidation and iron loading, reactions known to precipitate α-synuclein. The selectivity of the changes observed in A9 nigral neurons suggests that these early intracellular changes predispose these neurons to more rapid cell loss in Parkinson’s disease. The increased concentration of neuronal α-synuclein and pigment in normal A9 neurons may already predispose these neurons to precipitate α-synuclein around pigment-associated lipid under oxidative conditions. Overall, these changes may trigger a cascade of events leading to larger intracellular aggregates of α-synuclein and the dispersion of protective pigment to precipitate cell death in Parkinson’s disease.

Keywords: α-synuclein; lipid; neuromelanin; Parkinson’s disease

Abbreviations: ANOVA = analysis of variance; DMNX = dorsal motor nucleus of the vagus nerve; LC = locus coeruleus; NM = neuromelanin; SN = substantia nigra


Introduction

Parkinson’s disease is the most common neurodegenerative movement disorder characterized by a progressive cellular deposition of the synaptic protein α-synuclein in diverse brain regions (Braak et al., 2003). In many regions these abnormal depositions cause only limited neuron loss (e.g. amygdala, a site of early α-synuclein deposition (Harding
Dopamine neurons were first described in the brain by Dahlstrom and Fuxe who developed an anatomic technique for the anatomical identification of the different catecholamine cell groups (Dahlström and Fuxe, 1964). The most caudal group of catecholamine cells (lower ventrolateral medulla) is numbered A1, while the most rostral group (olfactory) is numbered A16 (see Pearson et al., 1990 for a review). The dopamine neurons of the SN pars compacta are designated A9 neurons. The dopamine neurons nearby in the ventromedial mesencephalic tegmentum are designated A10 neurons, while those in the more dorsal and caudal midbrain reticular formation are designated A8 neurons. Noradrenaline neurons in the locus coeruleus (LC) are designated A6 neurons, while noradrenaline neurons in the dorsal motor nucleus of the vagus nerve (DMNX) are designated A2 neurons. With the exception of adrenaline neurons, human catecholamine neurons contain neuromelanin (NM) pigment with nigral neurons containing the highest densities of NM (Hirsch et al., 1988).

Dopamine producing neurons in the SN are uniquely vulnerable to inflammatory attack and oxidative injury, as there is a naturally high density of inflammatory microglia in the SN (Lawson et al., 1990; Kim et al., 2000) and dopamine production and use depend on oxidative mechanisms (Jenner and Olanow, 1998; Faucheux et al., 2003). Oxidative stress can inhibit proteasome function (Ding and Keller, 2001) and the formation of intracellular protein aggregates is dependent on oxidative events (Demasi and Davies, 2003). However, α-synuclein does not form into Lewy bodies early in this region (Braak et al., 2003) and the cellular production of dopamine alone does not confer increased cellular vulnerability in Parkinson’s disease as nearby A8 and A10 dopamine neurons remain in situ abnormally accumulating α-synuclein (Hirsch et al., 1988; McRitchie et al., 1997). In humans, an obvious difference between A9 and other dopamine- and non-dopamine-containing neurons in the brain is the increased pigmentation found in this cell group (Mann and Yates, 1983; Hirsch et al., 1988; Gibb, 1992; Kastner et al., 1992). While the function of NM remains unclear, it has a complex structure associated with proteins, lipids, and oxidative metals such as iron (Doublet et al., 2002, 2003; Zecca et al., 2003). These unique properties may contribute to its obvious association with the early selective loss of A9 dopamine neurons in Parkinson’s disease.

In order to test this theory, comparative human studies are necessary as NM is not found in laboratory animals (Barden and Levine, 1983; Hardman et al., 2002). Progressive changes in humans can be identified by studying patients at different time points and comparing Parkinson’s disease with non-diseased controls. This allows progressive changes to be observed. In addition, as the disease is very slowly progressive with cell loss occurring over decades, at any time point during the disease each Parkinson’s disease patient has A9 neurons at various stages (healthy, unwell, dying, dead). Characterization of the cellular changes over time in the most vulnerable neurons will identify important early neuronal changes in Parkinson’s disease. The aim of this study was to determine whether there are specific intracellular changes that identify vulnerable versus less-vulnerable cell groups in patients with definite Parkinson’s disease in comparison with controls.

Materials and methods

Cases

All cases were participants in the Parkinson’s New South Wales brain donor program at the Prince of Wales Medical Research Institute. The program is approved by the Human Ethics Committee of the University of New South Wales under the Human Tissue Act of the State of New South Wales and prospective consent for brain donation was obtained from all cases and their next of kin. The brain donor program prospectively follows patients (usually annually) using standardized recording procedures for medical, neurological and psychiatric symptoms. Control cases underwent the same clinical follow-up as the Parkinson’s disease cases with the same standardized recording procedures. Particulars of the extensive clinical and neuropathological data collection procedures have been published (Harding et al., 2002). Dementia is assessed using the Clinical Dementia Rating Scale (Morris et al., 1989) and the severity of Parkinson’s disease assessed using the Hoehn and Yahr scale (Hoehn and Yahr, 1967). Medications and dosages taken are recorded and updated at each assessment.

Cases with neurological and neuropathological diseases other than Parkinson’s disease were excluded. In particular, cases fulfilling diagnostic criteria for dementia were excluded. A total of 13 levodopa-responsive Parkinson’s disease cases (10 male and 3 female) were selected (Table 1) using clinical and neuropathological criteria (Gelb et al., 1999). All Parkinson’s disease cases had α-synuclein-positive Lewy bodies within some surviving NM-containing neurons and were chosen to fulfill pathological Lewy body Stage 4 (Braak et al., 2003). However, none had significant limbic or neocortical Lewy bodies or dementia. The mean age of the patients was 75 ± 8 years, with a mean post mortem delay of 14 ± 7 h. At the time of death, 4 patients had mild disease (Hoehn and Yahr Stages 2 and 3) and 9 patients had advanced disease (Hoehn and Yahr Stages 4 and 5). A total of 13 control cases (6 male and 7 female) with no neuropathology (pathological Lewy body Stage 0) or history of neurological or psychiatric symptoms were selected to age-match the Parkinson’s disease cases (Table 1). The mean age of the controls was 75 ± 9 years with a mean post mortem delay of 18 ± 12 h. There were no significant differences in the age at death (unpaired t-test; \( t_{23} = 0.16, P = 0.87 \)) or postmortem delay (\( t_{23} = 1.49, P = 0.15 \)) between groups.

Tissue preparation

Brains are fixed in 15% buffered formalin for 2 weeks. The brainstem is removed from the cerebrum and detached from the cerebellum, and the cerebrum and brainstem is embedded in 3% agar and
Table 1  Demographic and cellular variables in the different groups

<table>
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<tr>
<th></th>
<th>Controls</th>
<th>PD Stage 2 and 3</th>
<th>PD Stage 4 and 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>13</td>
<td>4</td>
<td>9</td>
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<tr>
<td>Mean age of PD onset (y ± SD)</td>
<td>—</td>
<td>65 ± 16</td>
<td>60 ± 8</td>
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<td>PD duration (y ± SD)</td>
<td>—</td>
<td>7 ± 4*</td>
<td>17 ± 6.5*</td>
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<tr>
<td>Mean age at death</td>
<td>75 ± 9</td>
<td>72 ± 13</td>
<td>77 ± 5</td>
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<tr>
<td>Pathologic Lewy body stage</td>
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<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Severity of NM cell loss</td>
<td></td>
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<tr>
<td>A10 dopamine group</td>
<td>0</td>
<td>0</td>
<td>0—</td>
</tr>
<tr>
<td>A9 dopamine group</td>
<td>0</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>A6 noradrenergic group</td>
<td>0</td>
<td>0—</td>
<td>+++</td>
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<tr>
<td>A2 noradrenergic group</td>
<td>0</td>
<td>0—</td>
<td>+++</td>
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<tr>
<td>Mean % A9 cells remaining (±SD)</td>
<td>100 ± 20*</td>
<td>25 ± 3*</td>
<td>13 ± 4*</td>
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<tr>
<td>A9 neurons</td>
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<tr>
<td>Mean area (μm² ± SD)</td>
<td>604 ± 16</td>
<td>630 ± 12</td>
<td>612 ± 16</td>
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<tr>
<td>% NM area (∆±SD)</td>
<td>47 ± 13</td>
<td>46 ± 13</td>
<td>48 ± 15</td>
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<td>NM density (∆±SD)</td>
<td>0.30 ± 0.11</td>
<td>0.52 ± 0.13</td>
<td>0.48 ± 0.14b</td>
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<td>% NM lipid (∆±SD)</td>
<td>35 ± 14</td>
<td>36 ± 13</td>
<td>31 ± 11</td>
</tr>
<tr>
<td>A10 neurons</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean area (μm² ± SD)</td>
<td>517 ± 19</td>
<td>514 ± 16</td>
<td>488 ± 17</td>
</tr>
<tr>
<td>% NM area (∆±SD)</td>
<td>45 ± 13</td>
<td>41 ± 9</td>
<td>48 ± 11</td>
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<tr>
<td>NM density (∆±SD)</td>
<td>0.31 ± 0.13</td>
<td>0.32 ± 0.11</td>
<td>0.30 ± 0.13</td>
</tr>
<tr>
<td>3rd nerve neurons</td>
<td></td>
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<td></td>
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<tr>
<td>Mean area (μm² ± SD)</td>
<td>542 ± 17</td>
<td>531 ± 10</td>
<td>530 ± 21</td>
</tr>
</tbody>
</table>

+, sparse cell loss; ++, moderate cell loss; ++++, very severe cell loss; PD, Parkinson’s disease. *Different from other groups P < 0.05; bDifferent from controls P < 0.05.

Table 2  Synuclein-specific antibodies

<table>
<thead>
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<th>Antibody</th>
<th>Epitope detection</th>
<th>Intrasomal or dendritic staining</th>
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</thead>
<tbody>
<tr>
<td>BSY-I</td>
<td>Affinity rabbit IgG raised against synthetic peptide corresponding to residues 99–111 in human β-synuclein (Li et al., 2002)</td>
<td>No</td>
</tr>
<tr>
<td>ASY-I</td>
<td>Affinity rabbit IgG raised against purified human recombinant α-synuclein (Jensen et al., 1999)</td>
<td>✓</td>
</tr>
<tr>
<td>#610787 (BD Transduction Laboratories)</td>
<td>Affinity rabbit IgG raised against synthetic peptide corresponding to residues 15–123 in human α-synuclein</td>
<td>✓</td>
</tr>
<tr>
<td>N-terminal α-synuclein</td>
<td>Affinity rabbit IgG raised against synthetic peptide corresponding to residues 11–26 in human α-synuclein (Li et al., 2002)</td>
<td>✓</td>
</tr>
<tr>
<td>LB509 (Zymed Laboratories Inc)</td>
<td>α-synuclein specific mouse monoclonal antibody aa115–122 (Jakes et al., 1999)</td>
<td>✓</td>
</tr>
<tr>
<td>ASY-4</td>
<td>Affinity rabbit IgG raised against synthetic peptide corresponding to residues 116–131 in human α-synuclein (Li et al., 2002)</td>
<td>Only with antigen retrieval methods</td>
</tr>
</tbody>
</table>

sectioned at 3 mm in the coronal and transverse planes. Two 3 mm transverse tissue blocks containing the SN (at the level of the exiting oculomotor nerve) were sampled in all cases, one for paraffin embedding and sectioning at 10 μm and the other for thick frozen serial sectioning. The tissue block for frozen sectioning was cryoprotected in 30% buffered sucrose for 2 days after which 20 μm thick serial sections were cut on a cryostat and collected onto gelatinized slides. The next rostral transverse tissue block containing the SN was serially sectioned at 3 mm and 10 μm. Immunohistochemistry for α-synuclein was performed on brainstem 10 μm paraffin sections using peroxidase visualization and counterstained with cresyl violet, as previously described (Broe et al., 2001), and on midbrain 20 μm frozen sections following antigen retrieval in 99% formic acid for 3 min using Nova Red chromagen (Vector Laboratories, Burlingame, CA). Numerous specific antibodies against synuclein were used (for antibody details see Table 2) at similar final concentrations of 27 μg/ml with and without a pre-treatment protocol (99% formic acid for 5 min). The sensitivity of ASY-1 and the rabbit affinity purified antibodies (Table 2) for α-synuclein were confirmed by dot-blot analysis. Briefly, recombinant α-synuclein was blotted against nitrocellulose membrane at concentrations of 500, 250, 100, 50, 25, 5, 1 and 0.5 ng. Membranes were then incubated with antibodies at concentrations that were equivalent to those used for immunohistochemistry (27 μg/ml). Chemiluminescence was used for detection, and blots were exposed in second increments to establish antibody affinity.
All antibodies detected α-synuclein with a similar affinity (protein dots appeared after 2 s exposures) at protein concentrations of ≥100 ng. Pre-treated Parkinson’s disease tissue was used as a positive control for the immunohistochemical reactions and the specificity of the reaction tested by omitting the primary antibody. As expected, no peroxidase reaction was observed in the negative test sections, while in the pre-treated sections Lewy bodies and Lewy neurites were strongly immunoreactive for all antibodies against α-synuclein. The location of synuclein proteins within tissue elements and cellular compartments was assessed for each case. Experiments were also performed to establish the α-synuclein antibody titre (N-terminus, Table 2) at which normal nigral neuron staining was no longer observed.

**Quantification of brainstem cell loss**

The degree of NM cell degeneration in the A2, A6, A9 and A10 cell groups was evaluated using semiquantitative visual grading (none, sparse, moderate and severe degeneration) at ×100 magnification consistent with standard neuropathological evaluations. The loss of NM-containing A9 neurons was also calculated using a BH-2 Olympus microscope and a previously published areal fraction method (Halliday et al., 1996). Briefly, the area of the SN was measured and the areal fraction occupied by NM-containing neurons determined for each case by point counting using a 11 × 11 eyepiece grid on ×400 magnification. The area was multiplied by the fraction to determine the cell number of the SN occupied by NM-containing cells and the data expressed as a percentage of mean control values (Table 1). Repeated measurements by the same or different investigators gave an average variance of 3–7%. Spearman rank correlations were used to determine any relationship between cell loss and disease duration in the Parkinson’s disease group. A P-value <0.05 was taken as significant in this and subsequent statistics.

**Quantification of cellular variables in pigmented midbrain neurons**

The cell size, cell area occupied by NM and NM density were measured in unstained (for NM density) and 0.5% aqueous cresyl violet stained sections of the midbrain using a BH-2 Olympus microscope fitted with a Spot Digital Cooled Camera (Diagnostic Instruments, MI) linked to a computer using Image Pro Plus software (Media Cybernetics, MD). Captured images were analysed using computer drawing tools that allow manual definition of regions of interest within the photomicrograph and calculate the area in μm² and optical density compared to a standard (nearby neuropil). For the analysis, large remaining NM-containing neurons were targeted to evaluate the earliest neuronal changes. Pilot studies in three different cases determined that 20 neurons per region per slide were needed to maintain the coefficient of error in the measurements at ≤0.10. Average intra- and inter-rater variance was 4% for cell area and 3% for optical density. All four major cell clusters of the A9 group (McRitchie et al., 1996)—the most dorsal A10 cell cluster (McRitchie et al., 1996) and the oculomotor nucleus (Fig. 1A and B)—were sampled in both Nissl-stained and unstained sections (240 neurons per person for a total of 6240 sampled neurons). The total neuronal area and the area within the neuron occupied by NM were measured in non-degenerating neurons (nucleolated neurons of normal morphology in Nissl-stained section) and the density of unchanged NM determined in large NM-containing neurons (unstained section). The average coefficient of error for the neuronal area was 0.070 ± 0.017 for controls (0.032–0.089 range) and 0.080 ± 0.019 for the Parkinson’s disease cases (0.044–0.100 range). Analysis of variance (ANOVA) was used to control measures of cell size, area occupied by NM and optical density to determine differences between anatomical regions. Two-way ANOVA (diagnosis and regions analysed) determined any specific changes that may have occurred due to Parkinson’s disease. When necessary, post hoc Bonferroni tests were used to determine which regions and groups differed.

**Electron microscopy of pigmented nigral neurons**

Midbrain tissue from three cases with Stages 2 and 3 Parkinson’s disease, three cases with Stages 4 and 5 Parkinson’s disease and three controls were used for the final analysis. These cases were selected because they had the shortest postmortem delays. The SN was cut into small samples and washed in 0.1 M cacodylate buffer, pH 7.4 (2 days followed by 10 min on the day of processing), 2% osmium tetroxide (4 h), 2% sodium acetate (5 min) and finally 2% uranyl acetate (1 h). The tissue was then dehydrated through graded alcohols to acetone, incubated in two acetone/resin mixes (1:1, 1 h; 1:2, overnight), placed in pure resin (Procure 812 Epon resin, Proscitech, Qld, Australia; 4 h), and embedded and cured at 70°C for 48 h. Ultrathin sections were cut at 90 nm and mounted on formvar-coated nickel 200 mesh grids. Half of the grids were used for immunocytochemistry with the N-terminal α-synuclein antibody (Table 2) following etching (Stirling and Graff, 1995) with sodium metaperiodate (100 mg/ml MilliQ water) at 50°C (5 min) and 0.01 M sodium citrate buffer at 95°C (pH 6.0, 30 min). All grids were counterstained in alcoholic uranyl acetate and Reynolds lead citrate (10 min each) and examined in a Zeiss transmission electron microscope.

**Assessment of the lipid component of NM**

Filipin fluorescence (0.05 mg/ml for 2 h, #P9765 Sigma, St Louis, USA) was used to investigate the presence of cholesterol in NM and the amount of lipid within NM was analysed on electron micrographs using the areal fraction method. Ten NM-containing nucleolated neurons per case were photographed on the electron microscope and printed at ×13 500 magnification. A grid of 4 × 4 mm was used to assess the proportion of lipid in the NM granules. Repeated counts of NM in five neurons in two cases gave ≤5% intra- and inter-rater variance. ANOVA was used to determine any changes between the control and Parkinson’s disease group.

**Results**

All NM-containing cell groups abnormally deposit α-synuclein in Parkinson’s disease

Consistent with previous studies (Jakes et al., 1999; Braak et al., 2000; Mori et al., 2002), all antibodies against α-synuclein strongly immunolabelled synapses in the neuropil as punctate structures. Most of the cytoplasm of the brainstem catecholaminergic neurons in humans is obscured by NM (Figs 1 and 2). Despite this, the thin tissue sections of control SN revealed cytoplasmic α-synuclein immunolabelling within NM-containing neurons extending into the primary dendrites. This staining pattern occurred without tissue pre-treatment procedures (Fig. 1C) and was visible with all but one of the α-synuclein antibodies (Table 2). Following pretreatment, this antibody (Table 2) also visualized dendritic and somal
Fig. 1 NM-containing neurons in the substantia nigra (SN), locus coeruleus (LC) and dorsal motor nucleus of the vagus nerve (DMNX) in representative control (A, C, E and G) and Parkinson’s disease (B, D, F and H) cases. Scale in (B) equivalent for (A). Scale in (C) equivalent for (D). Scale in (H) equivalent for (E–H). (A and B) Low power photomicrographs of midbrain cresyl violet stained section from a representative control (A) and Parkinson’s disease (B) case. Medial, lateral, dorsal (SNd) and ventral (SNv) A9 cell groups are indicated. Arrows in (B) indicate the ventral region of greatest cell loss in the Parkinson’s disease cases analysed. 3 = oculomotor nucleus, 3n = exiting oculomotor nerve, A10d = dorsal A10 cell region, R = red nucleus. (C and D) α-Synuclein-immunoreactive A9 NM-containing neurons (arrows) from the SN of a representative control (C) and Parkinson’s disease (D) case. In sections without pre-treatment, the cytoplasm contained abundant α-synuclein in controls (C) with a concentration into Lewy bodies (asterisk) observed in some of the remaining neurons in Parkinson’s disease (D). (E and F) A6 NM-containing neurons from the LC in a representative control (E) and Parkinson’s disease (F) case. In sections without pre-treatment, the cytoplasm was largely devoid of α-synuclein in controls (E) with immunopositive Lewy bodies (asterisk) observed in some of the remaining neurons in Parkinson’s disease (F). (G and H) A2 NM-containing neurons from the DMNX in a representative control (G) and Parkinson’s disease (H) case. In sections without pre-treatment, the cytoplasm was largely devoid of α-synuclein in controls (G) with immunopositive staining and Lewy bodies observed in some of the remaining neurons in Parkinson’s disease (H).
a-synuclein, suggesting that this portion of the protein is most likely to be masked due to fixation. Similarly, pretreatment was required for the somal and dendritic localization of a-synuclein in other brainstem catecholamine cell groups (Fig. 1E and G). In contrast, even without pretreatment, a-synuclein concentrated in intracellular deposits in patients with Parkinson’s disease with a loss of dendritic staining in these cell types (Fig. 1D, F and H), as described previously (Mori et al., 2002; Braak et al., 2003).

A9 dopamine neurons are selectively targeted for degeneration early in Parkinson’s disease

As expected, patients with Parkinson’s disease had a consistent loss of A9 dopamine neurons with a relative preservation of A10 dopamine neurons and a variable loss of A6 noradrenaline neurons in the LC and A2 noradrenaline neurons in the DMNX (Table 1). Parkinson’s disease cases at earlier disease stages had less cell loss than those with end stage disease (Table 1, P = 0.005) with greater A9 cell loss correlating with increasing disease duration (R = 0.76, P = 0.002).

Direct comparison between A9 and A10 dopamine neurons

In controls, the size of A9 neurons differed depending on their location (ANOVA, P < 0.0001; Figs 1A and 2A and B). NM-containing neurons in the dorsal, ventral and lateral SN were significantly larger than NM-containing neurons in the medial SN and dorsal A10 region (Fig. 2A and B; Table 1; post hoc P-values < 0.003). The size of the medial A9 and A10 neurons was similar to those in the oculomotor nucleus (Table 1, post hoc P-values > 0.5). Only A9 neurons in the pars lateralis differed in the proportion of cytoplasm occupied by NM (ANOVA, P = 0.004). An average 5% more of the cytoplasm of neurons in pars lateralis contained NM (53 ± 2%) compared with the other dopamine cells (48 ± 1%, post hoc P-values <0.005). The optical density of NM did not differ between the A9 and A10 regions (Table 1; ANOVA diagnosis = 0.02, P = 0.88). A substantial increase in the optical density of the A9 NM-containing neurons was observed in these intact neurons in the Parkinson’s disease cases (Table 1; Fig. 2C and D). This unexpected increase in the density of pigment occurred without a reduction in the size or amount of NM and suggests a fundamental

Fig. 2 Comparison between the A9 and A10 dopamine neurons and between A9 neurons in a representative control and Parkinson’s disease case. Scale in (D) equivalent for (A–D). Scale in (F) equivalent for (E). (A and B) High power photomicrographs of cresyl violet (CV) stained A9 neuron in the ventral tier of the SN (A) versus a dorsal A10 neuron (B). (C and D) High power photomicrographs of unstained NM-containing A9 neurons in a control (C) and Parkinson’s disease (D) case showing an increased density of pigment in Parkinson’s disease. (E and F) Electron microscopy of NM-containing A9 neurons in a control (E) and Parkinson’s disease (F) case showing the similar structural characteristics of NM, including the lipid droplets.
Identification of A9 NM changes in Parkinson’s disease

Ultrastructurally, NM is not a uniform structure but has three components of different electron density (Fig. 2E and F)—an electron dense component, a component of intermediate electron density and an electron lucent component. These electron lucent components are not oxidized with osmium (lucent not electron dense) and are round resembling a lipid droplet (Fig. 2E and F). These characteristics are similar to those of lipid storage droplets in adipose tissue (Okuda et al., 1983; Ito et al., 1991) and differ from the other oxidizable lipid components found in neural cellular membranes and other neuronal compartments (Peters et al., 1976). These electron-lucent lipid droplets made up ~35% of the NM with no difference between the size of this lipid-associated NM component in Parkinson’s disease versus control cases (Table 1).

As previous studies identified cholesterol within NM (Zecca et al., 2000), NM was stained with filipin. In control A9 (Fig. 3A–C) and A10 neurons and in Parkinson’s disease A10 neurons, the NM granules contained filipin-positive cholesterol and did not appear to be α-synuclein-positive when optimally titred to see the NM—a finding confirmed with electron microscopy (Fig. 3I). In Parkinson’s disease cases intraneuronal α-synuclein-immunoreactive Lewy bodies were observed in all types of NM-containing neurons (Fig. 3G and H), as expected. In addition, NM in the A9 neurons of the Parkinson’s disease cases had reduced filipin-positive cholesterol staining (Fig. 3D and E) and concentrated α-synuclein-immunoreactivity (Fig. 3F). Under the electron microscope the α-synuclein-immunoreactivity concentrated around the lipid droplets (Fig. 3J).

Discussion

The present study shows that early brainstem cell loss in Parkinson’s disease is mainly confined to the A9 neurons in the SN and associated with more widespread Lewy body formation, consistent with current diagnostic criteria for Parkinson’s disease (Geb et al., 1999). In this region of greatest vulnerability to Parkinson’s disease, intraneuronal lipid changes occur prior to evidence of Lewy body formation indicating an early pathogenic event. In the remaining healthy A9 neurons (no change in their size or NM amount), unstained intracellular NM has an increased optical density associated with a loss of cholesterol and an aggregation of α-synuclein to NM lipid. Of course a proportion of A9 neurons are more affected with large α-synuclein accumulations in Lewy bodies. At early disease stages Lewy bodies are not confined to the A9 cell group with substantial cell loss, but also occur in a number of reasonably preserved brain regions, including the amygdala affected early in Parkinson’s disease (Harding et al., 2002; Braak et al., 2003). While the formation of Lewy bodies in non-nigral brain regions disturbs cell function, these regions do not require cell replacement therapies (due to limited cell loss); it is, therefore, important to determine the more selective changes associated with the loss of A9 neurons—the population most vulnerable to degeneration in Parkinson’s disease. We have shown in the present study that NM-containing brainstem neurons (A6 and A2) are relatively preserved in early Parkinson’s disease and do not exhibit the same intracellular changes seen in the A9 cells. The selectivity of the early changes described suggests that the NM lipid changes in A9 neurons are likely to be related to their early vulnerability in Parkinson’s disease.

NM changes have been previously observed in Parkinson’s disease, but have not been well studied due to the reduced quantities or complete absence of this substance in the catecholamine neurons of all laboratory animals (Cowen, 1986; Hardman et al., 2002). Human NM has a complex structure with a core insoluble polymer (Double et al., 2002; Zecca et al., 2003). Its synthesis is poorly understood, with many considering NM to be an inert cellular by-product of dopamine-synthesis produced via simple autoxidation (Double et al., 2002; Zecca et al., 2003). However, there are substantially fewer NM-containing neurons compared with tyrosine hydroxylase-immunoreactive neurons, with many dopamine and noradrenaline neurons completely lacking NM (Saper and Petito, 1982; Gaspar et al., 1983; Halliday et al., 1988; Hirsch et al., 1988; McRitchie et al., 1996). In particular, central adrenergic neurons do not produce NM although dopamine is a necessary precursor (Halliday et al., 1988). Additionally, if NM is produced as an oxidative by-product of dopamine, patients treated with levodopa should exhibit increased pigmentation in all NM-containing neurons (and possibly even in those that normally would not make NM)—a finding we and others (Mann and Yates, 1983) cannot substantiate. We observe no change in NM in A10 neurons in Parkinson’s disease patients treated for their disease, suggesting that the NM changes we have observed are unrelated to levodopa medication. Also, it is difficult to think that simple autoxidation could produce the complex structure of the NM granules, including the non-oxidized lipid droplets that we and others (Foley and Baxter, 1958; Moses et al., 1966) have observed. It would seem that more complex intraneuronal mechanisms for the normal production of NM exist, possibly similar to the multiple enzymatic reactions responsible for all other melanin pigments (Mishima, 1994; Benedito et al., 1997).

Previous studies have combined optical density measurements of NM in A9 and A10 neurons and report either a small (6%) reduction (Kastner et al., 1992) or no change (Mann and Yates, 1983) in the optical density of NM in Parkinson’s disease compared with controls; and this is consistent with the overall loss of A9 neurons (Hirsch et al., 1988). When NM aggregates in the A9 region alone are sampled, but both
Fig. 3 NM changes and Lewy bodies (asterisks) in Parkinson’s disease. Scale in (E) equivalent for (A–E). Scale in (G) equivalent for (H). Scale in (J) equivalent for (I). (A–C) High power photomicrographs of NM in a control A9 neuron (outlined) stained with filipin for cholesterol. Note the absence of specific fluorescence for filipin in (C). (D and E) High power photomicrographs of NM in Parkinson’s disease A9 neuron (outlined) stained with filipin for cholesterol. There is a reduction in the amount of cholesterol associated with NM compared with controls (A). (F and G) High power photomicrographs of A9 neurons in Parkinson’s disease with α-synuclein-immunoreactive Lewy bodies [asterisk in (G)] and NM granules [arrows in (F and G)]. In non-counterstained sections, α-synuclein-immunoreactivity can be visualized aggregating on the NM granules prior to overt abnormalities being detectable (F). (H–J) Immunogold detection of α-synuclein within the cytoplasm of a control A9 neuron [arrowhead indicates 10 nm gold particles, (I)] and associated with the electron lucent lipid droplets [arrowheads indicate 10 nm gold particles, (J)] and Lewy body filaments [15 nm gold particles, (H)] in Parkinson’s disease A9 neurons.
healthy and degenerating neurons are included, an average 40\% decrease in the optical density of NM is reported (Faucheux et al., 2003), consistent with the disper-son of NM in degenerating neurons in this region. Ours is the first study to investigate the earliest intracellular changes by restricting analyses to the remaining healthy A9 neurons in Parkinson’s disease. Our data demonstrates a noticeable increase in the optical density of NM in these neurons which is not observed in the nearby but less vulnerable A10 neurons. Interestingly, the change in NM density we observed is comparable to the increase in NM density reported following its oxidation with H_2O_2 and its association with increased Fe^{3+} in Parkinson’s disease (Faucheux et al., 2003), suggesting increased cellular oxidation and the saturation of NM’s metal-chelating properties in Parkinson’s disease (Jellinger et al., 1992; Kienzl et al., 1999; Doublet et al., 2002; Faucheux et al., 2003; Zecca et al., 2003).

The addition of Fe^{2+} to soluble α-synuclein generates H_2O_2 causing iron oxidation and α-synuclein fibril formation (Turnbull et al., 2001; Uversky et al., 2001)—a potential mechanism for the changes we have observed. In particular, we found the increase in NM density occurred in association with α-synuclein accumulations around the NM granules. Extracted nigral NM from Parkinson’s disease brains is composed of more highly cross-linked, protease-resistant, lipoprotein material compared to normal NM (Aime et al., 2000) with more recent studies identifying α-synuclein in NM in Parkinson’s disease (Fasano et al., 2003), consistent with the changes we have observed in Parkinson’s disease. The amount of α-synuclein aggregation is dependent on the amount or type of soluble α-synuclein (Osterrova-Golts et al., 2000) and we and others have observed high levels of α-synuclein in A9 neurons (Li et al., 2002; Mori et al., 2002; Andringa et al., 2003). The concentration of α-synuclein within nigral neurons is developmentally regulated, with somatic staining observed at 15 weeks of gestation and a decrease in somatic and increase in synaptic staining at 18 weeks of gestation (Galvin et al., 2001). Over-expression of α-synuclein is now known to precipitate Parkinson’s disease (Kirik et al., 2003; Lauwers et al., 2003; Singleton et al., 2003; Farrer et al., 2004), although there is no increase in α-synuclein transcription in this region in Parkinson’s disease (Kingsbury et al., 2004). Further, acidic lipids enhance metal-catalyzed α-synuclein aggregation (Lee et al., 2003; Sharon et al., 2003a, b) and thus changes in the NM lipid pool may contribute to intracellular conditions favoring neurodegeneration in Parkinson’s disease.

In addition to the deposition of α-synuclein on NM, the lipid composition of NM also changes. Approximately 20\% of the mass of A9 NM consists of a novel class of polyunsaturated lipids with high molecular mass, low volatility and low oxygen content (Zecca et al., 2000), consistent with the large volume of non-oxidized lipid (not reactive to osmium) we observed in NM. This large unusual lipid component of NM has not been well recognized previously and these lipid droplets appear unique in central nervous system neurons. While this unusual characteristic of NM requires further study, our data confirm that cholesterol is a component of NM lipid (Zecca et al., 2000) and that this lipid component changes its configuration in most A9 neurons in Parkinson’s disease cases. In melanocytes and melanoma cells, fatty acids regulate pigmentation via proteosomal degradation of tyrosinase (Ando et al., 2004), and the changes in NM lipid may occur because of proteosome dysfunction in Parkinson’s disease (McNaught et al., 2001; Moore et al., 2003). Proteosome dysfunction may also lead to an increase in α-synuclein with in vitro studies showing that α-synuclein preferentially accumulates on phospholipid monolayers surrounding triglyceride-rich lipid droplets protecting the stored triglycerides from hydrolysis (Cole et al., 2002). Patients with Parkinson’s disease have more active phospholipid biosynthetic enzymes in the SN (Ross et al., 2001) and an increase in phospholipids and α-synuclein may occur in Parkinson’s disease NM granules. The importance of early intracellular lipid changes in the neurodegeneration of Parkinson’s disease has been demonstrated recently in the drosophila model of the disease (Scherzer et al., 2003), in the identification of mutations in the glucocerebrosidase gene (Aharon-Peretz et al., 2004) and with new therapeutics targeting various lipids showing long-term beneficial effects (Beal and Shults, 2003; Muller et al., 2003; Sharma et al., 2004; Shults et al., 1999, 2002, 2004).

Changes in intracellular mechanisms are considered critical to the neurodegeneration observed in Parkinson’s disease. Our data suggest an integral change in NM as a prelude to Parkinson’s disease neurodegeneration in vulnerable A9 neurons. The increased concentration of neuronal α-synuclein and NM in A9 neurons may predispose these neurons to precipitate α-synuclein around NM lipid under oxidative conditions. These changes may trigger a cascade of events leading to larger intracellular aggregates of α-synuclein and the dispersement of NM within the cell. A potential key component to these changes may be the observed increase in iron in NM in Parkinson’s disease which is thought to increase oxidative load within the microenvironment of the pigment granule (Doublet et al., 2002; Faucheux et al., 2003; Zecca et al., 2003).

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Early neuromelanin changes in Parkinson's disease


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