Objective: To explore the relationship between α-synuclein pathology and mitochondrial respiratory chain protein levels within single substantia nigra neurons.

Design: We examined α-synuclein and mitochondrial protein expression in substantia nigra neurons of 8 patients with dementia with Lewy bodies, 5 patients with Parkinson disease, and 8 control subjects. Protein expression was determined using immunocytochemistry followed by densometric analysis.

Patients: We examined single substantia nigra neurons from 5 patients with idiopathic Parkinson disease (mean age, 81.2 years), 8 patients with dementia with Lewy bodies (mean age, 75 years), and 8 neurologically and pathologically normal control subjects (mean age, 74.5 years). The control cases showed minimal Lewy body pathology and cell loss. Patients with dementia with Lewy bodies and idiopathic Parkinson disease fulfilled the clinical and neuropathologic criteria for these diseases.

Results: Our results showed that mitochondrial density is the same in nigral neurons with and without α-synuclein pathology. However, there are significantly higher levels of the respiratory chain subunits in neurons containing α-synuclein pathology.

Conclusions: The finding of increased levels of respiratory chain complex subunits within neurons containing α-synuclein does not support a direct association between mitochondrial respiratory chain dysfunction and the formation of α-synuclein pathology.

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The Pathogenesis of Idiopathic Parkinson Disease (IPD) and dementia with Lewy bodies (DLB) remains unclear. Mitochondrial involvement has been identified as important by several lines of evidence. Defects of the mitochondrial respiratory chain, in particular in the activity of complex I, have been described in homogenized substantia nigra (SN) from patients with IPD.1,2 High levels of deleted mitochondrial DNA have been reported in the SN of elderly control subjects and patients with Lewy body disease (LBD).3,4 Also, genes encoding proteins that affect mitochondrial function have been identified in families with autosomal recessive juvenile parkinsonism5 including PARK2 (parkin) (OMIM 602544), PARK6 (pink1) (OMIM 608309), and PARK7 (DJ-1) (OMIM 602533). These genes have varied roles within mitochondria including the targeting of mitochondria to autophagy,5,7 protection against oxidative stress,7,8 and reactive oxygen species sensing/scavenging.9,10

While mitochondrial dysfunction has a key role to play in the pathogenesis of IPD and DLB, its relationship with the other key pathogenic factor—the accumulation of α-synuclein—is less understood. The accumulation of α-synuclein as filamentous inclusions in neurons (Lewy bodies [LB]) and in dystrophic neurites is the pathological hallmark of both IPD and DLB. In autosomal recessive parkinsonism, homozygous mutations generally result in nigral degeneration not associated with LB pathology,9 although LB pathology has been reported in a compound heterozygous individual carrying pink1 mutations11 and in individuals carrying heterozygous mutations in parkin and pink1 genes.12-14

Recent studies suggest that α-synuclein and mitochondria may have closer interactions than once thought, and their interaction in vitro coincides with mitochondrial dysfunction.15,16 However, much of this data comes from studies examining this relationship in homogenized SN tissue17 or overexpressing cell culture systems17-19 rather than in single human neu-
Formalin-fixed, paraffin-embedded upper midbrain tissue from 5 patients with IPD (mean age, 81.2 years; age range, 75-87 years; mean postmortem delay, 52.3 hours; mean tissue fixation, 2.8 months), 8 patients with DLB (mean age, 75 years; age range, 70-81 years; mean postmortem delay, 39 hours; mean tissue fixation, 1.9 months), and 8 neurologically and pathologically normal control subjects (mean age, 74.5 years; age range, 58-87 years; mean postmortem delay, 30.6 hours; mean tissue fixation, 1.6 months) were included. Control cases showed minimal Lewy body pathology—however, 1 case showed occasional LBs, Lewy neurites, and fine granular inclusions—and no cell loss (0 of 4 [0%-25% cell loss]). Patients with DLB and IPD fulfilled the clinical and neuropathologic criteria for these diseases (Table).20 The use of all human tissue had been consented to by the appropriate local research ethics committee and conformed to the UK Medical Research Council guidelines on the use of tissue in medical research.

**METHODS**

**TISSUE**

**IMMUNOHISTOCHEMISTRY**

Single immunohistochemistry (IHC) for α-synuclein (Figure 1A-D) and mitochondrial markers (Figure 1E and F) has been performed previously.21 A dual IHC assay was designed combining staining for α-synuclein with the detection of a mitochondrial protein (Figure 1G and Figure 2A, and G). We used antibodies to specific subunits of respiratory chain complexes I, II, and IV, and detection of an outer membrane mitochondrial protein, porin, as a marker of mitochondrial density.

Immunocytochemistry was performed according to Mahad et al22 with minor modifications. Antigen retrieval was performed in concentrated formic acid followed by high temperature retrieval in 1 mM ethylenediaminetetraacetic acid (pH 8.0) before blocking with 1% normal goat serum. The α-synuclein primary antibody (1:30) was then applied for 90 minutes (clone KM51; Novocastra; Leica Biosystems). Biotinylated goat antimouse antibodies (Vector Laboratories) were applied, followed by Vectastain Elite avidin-biotin complex (Vector Laboratories) and Vectastain Novared substrate (Vector Laboratories). Following this step, sections were stored in TBS overnight at 4°C. Immunocytochemistry for mitochondrial proteins (eTable, http://www.archneurol.com) was then performed with detection of primary antibodies using the polymer detection system (MenaPath kit; Menarini Diagnostics).

**IMAGING AND DENSITOMETRIC ANALYSIS**

Dual IHC-stained sections were analyzed using bright field microscopy and Nuance multispectral imaging (CRI). We took low magnification (×4) images of the SN, defined its boundaries, and took 15 random images of neuromelanin-containing neurons for each case. By using single-stain controls for each of the chromatogens used in the IHC protocol (Figure 1J-M), individual spectra for each color can be created using the Nuance software. Owing to the nature of the SN neurons, we generated spectra for 4 chromatogens: blue for hematoxylin, red for α-synuclein, purple for the mitochondrial proteins, and yellow/brown for the neuromelanin. These spectra can then be applied to the dual stained sections and the signal from all the component chromatogens can be extracted.23 The Nuance software generates black and white images for all the component chromatogens and a pseudo-colored composite image (Figure 2). The black and white images can then be used for densitometric analysis.

For each pigmented neuron, we measured the intensity of mitochondrial protein staining (per unit area) and assessed the type/amount of α-synuclein pathology. Mitochondrial protein signals were sampled from a region of the neuron not containing neuromelanin, α-synuclein, or the nucleus. Densitometric analysis is an inverse linear scale ranging from 0 (black) to 250 (white). Values were inverted by subtraction from 250 so that more intense staining gave a higher value. All densitometric values were normalized to the mean control value for each protein studied, calculated for cells stained in the same batch. Normalization was performed by expressing each data point from LBD cases stained at the same time as a percentage of the control mean for that particular protein.

To minimize variation in intensity of immunostaining, we performed staining using 1 antibody for all patients and control subjects on the same day, where possible. We also compared porin immunostaining in neurons with no pathology for both control subjects and patients and showed no difference between the mean values obtained for control subjects and patients (eFigure 1). Therefore, it was reasonable to compare all the data from the LBD cases with the control

| Table. Clinical and Neuropathologic Features of All DLB and PD Cases |
|-------------------|------------------|------------------|
| **Case** | **Disease** | **Cortical Lewy Body Score** | **SN Cell Loss** |
| 1 | DLB | 19 | UMB = 2/4 |
| 2 | DLB | 11 | UMB = 2/4 |
| 3 | DLB | 17 | UMB = 2/4 |
| 4 | DLB | 10 | UMB = 2/4 |
| 5 | DLB | 16 | UMB = 2/4 |
| 6 | DLB | 6, limbic | UMB = 2/4 |
| 7 | DLB | 17, neocortex | UMB = 2/4 |
| 8 | DLB | 19 | UMB = 2/4 |
| 9 | PD | 13 | UMB = 2/4 |
| 10 | PD | 1, brainstem | UMB = 1/4 |
| 11 | PD | 11 | UMB = 1/4 |
| 12 | Mixed PD (with cerebral amyloid angiopathy) | 11 | UMB = 1/4 |
| 13 | PD | NA, severe cortical pathology | LMB = 4/4 |
| 14 | PD | LMB = 2-3/4 |

Abbreviations: DLB, dementia with Lewy bodies; LMB, lower midbrain; NA, not applicable; PD, Parkinson disease; SN, substantia nigra; UMB, upper midbrain.

*The cortical Lewy body score for each patient was assessed from the whole cortical area samples available from each case.

*Cell loss scores 0 of 4 (0%-25%), 1 of 4 (25%-50%), 2 of 4 (50%-75%), and 4 of 4 (75%-100%).
Figure 1. Bright field images of single and dual immunohistochemistry (IHC). Different stages of α-synuclein accumulation/aggregation in substantia nigra (SN) (A-D). Punctate α-synuclein granules (A, arrowheads), irregular inclusion/pre-Lewy body (B, arrowhead), single Lewy body (C, arrow), and multiple Lewy bodies (D, arrow).

E. Some SN neurons are deficient for mitochondrial complex I subunits (arrow). In the other neuron, mitochondrial protein staining is very punctate, representing the mitochondria, and it is absent from the nucleus; single IHC for C120 (purple), hematoxylin (blue), and neuromelanin (yellow/brown).

F. All nigral neurons show equal levels of porin; single IHC for porin (control section, purple), hematoxylin (blue), and neuromelanin (yellow/brown).

G. SN neurons showing different stages of α-synuclein accumulation/aggregation and variable degree of deficiency for mitochondrial COXI subunit; granular α-synuclein pathology (red arrow), larger and denser pathology (black arrow), and deficiency for COXI in the neuron lacking any α-synuclein pathology (arrowhead).

H. SN neuron staining using dual IHC for α-synuclein, with the omission of the primary antibody for the mitochondrial proteins. I. SN neuron staining using dual IHC for mitochondrial protein (porin), with the omission of the primary antibody for α-synuclein. Single IHC control samples for each of the chromogens used in this study: hematoxylin (J, blue), α-synuclein (K, red), mitochondrial protein (porin) (L, purple), and neuromelanin (M, yellow/brown). Scale bars represent 20 µm.
cases and to assume that any changes seen were not due to technical variation.

**STATISTICS**

The data sets were not normally distributed and we used a Mann-Whitney U test to compare mitochondrial protein staining in neurons with and without pathology. To ascertain whether the staining intensities for mitochondrial proteins changed between different pathology types, we performed a Bartlett test for equal variances and the Kruskal-Wallis test. To investigate the number of cells that were deficient for certain mitochondrial respiratory chain proteins in cases and control subjects using densometric analysis and without assigning arbitrary ranges, we defined deficient cells as those with a staining intensity falling below the 10th percentile value for the control data for each protein. To determine whether there was a difference between cells with and without pathology, we performed a Fisher exact test.

**RESULTS**

**METHOD VALIDATION**

Absolute quantification of protein expression through the use of densitometric analysis is difficult, but it is the only approach that allows us to look at expression in neurons within the same section and compare with...
control cases. Several publications have shown that antibodies to several respiratory chain complexes (including those used in this article) can detect changes that correlate with a biochemical defect.22,24,25 In addition, we used the same method to explore protein expression in patients with neurodegeneration secondary to primary defects of the mitochondrial genome and we were able to show clear defects of protein expression in neurons that correlate with the biochemical defect seen (eFigure 2).

**MITOCHONDRIAL PROTEIN LEVELS IN SN NEURONS OF NORMAL CONTROL SUBJECTS**

Using brain sections from normal control subjects, we measured the normal expression of different mitochondrial proteins in neurons from Lewy body disease cases. The data have been normalized to the mean staining intensity for the control data and each data point represents 1 neuron.

**Figure 3.** Mitochondrial protein staining is increased in substantia nigra (SN) neurons with α-synuclein pathology. There is a significant increase in the levels of several mitochondrial respiratory chain proteins in neurons containing pathology, although the density of mitochondria (porin) and CII70 staining is uniform. A, Staining intensities per unit area for porin (neurons without pathology [NWOP]; 25th percentile, 104.8; median, 120.72; 75th percentile, 166) (neurons with pathology [NWP]; 25th percentile, 98.9; median, 121.04; 75th percentile, 169.4; \( P = .36 \)) and CII70 in neurons with and without α-synuclein pathology (NWOP: 25th percentile, 93.8; median, 121.45; 75th percentile, 179) (NWP: 25th percentile, 105.1; median, 126.96; 75th percentile, 184.7; \( P = .15 \)). B, Staining intensities for complex I subunits in individual SN neurons with and without α-synuclein pathology (CI19 NWOP; 25th percentile, 74.8; median, 102.58; 75th percentile, 127.7) (NWP; 25th percentile, 103.1; median, 127.63; 75th percentile, 183.3) (CI20 NWOP; 25th percentile, 32.3; median, 72.03; 75th percentile, 120.9) (NWP; 25th percentile, 87.1; median, 115.23; 75th percentile, 136). C, Staining intensities for complex IV subunits in individual SN (COXI NWOP; 25th percentile, 90.5; median, 113.73; 75th percentile, 133.1) (NWP; 25th percentile, 123.2; median, 139.88; 75th percentile, 157.3) (COXIV NWOP; 25th percentile, 77.2; median, 93.89; 75th percentile, 109.2) (NWP; 25th percentile, 91; median, 103.58; 75th percentile, 127.3). *\( P < .001 \). The median of each data set is shown (bar). The data have been normalized to the mean staining intensity for the control data and each data point represents 1 neuron.

**Figure 4.** Mitochondrial complex I 19-kDa subunit staining is increased in neurons containing α-synuclein pathology in all cases. The mean mitochondrial CI19 staining increases in neurons containing α-synuclein pathology compared with those without pathology for 11 of 12 cases. For LB6D, there is a very small decrease in staining intensity in cells with pathology compared with those without pathology, but this may be explained by the small sample size when considering individual cases. LB indicates Lewy body disease.
teins in 5298 single pigmented SN neurons (eFigure 3). There was minimal evidence of LBs and α-synuclein aggregates in these subjects (6.5% of cells contained pathology), which was much lower than in IPD and DLB cases (29% of cells contained pathology [1433 of 4947 neurons]). There was wide variation of staining intensities in normal control individuals for all 6 mitochondrial proteins studied in this investigation.

**MITOCHONDRIAL PROTEIN LEVELS IN SN NEURONS WITH AND WITHOUT PATHOLOGIC α-SYNUCLEIN AGGREGATES**

The data obtained from both IPD and DLB cases showed the same results and because IPD and DLB are postulated to be on a clinical spectrum, we pooled all the data from these cases to ensure robust statistical significance. From this point forward, LBD refers to any data from both IPD and DLB cases. We analyzed a total of 4947 single SN neurons from LBD cases.

Porin IHC showed that the median and range of the relative mitochondrial density were equal in neurons with and without α-synuclein pathology in LBD cases (P = .36) (Figure 3A). Therefore, any differences in intensity of respiratory chain subunit staining between neurons with and without pathology were unlikely due to changes in overall mitochondrial mass. Staining for complex II 70 kDa showed a slight increase in cells with pathology (P = .05), but this was less significant than for other proteins studied. This is relevant because complex II is the only complex of the electron transport chain that contains no subunits encoded by mitochondrial DNA and in fact, forms the basis of our cytochrome C oxidase (COX)/succinate dehydrogenase activity assay.

For all other mitochondrial respiratory chain proteins studied (CI19, CI20, COXI, and COXIV), the median intensity of staining was significantly greater in those cells containing α-synuclein pathology than in those without (P < .001) (Figure 3). Results for both complex I subunits show that a large number of the cells without pathology were also seen for complex IV. The relative mitochondrial density were equal in neurons with and without pathology. This further validates our observation because the staining intensities of neurons of a single case are the same as when we consider the whole group.

![Figure 3](attachment:figure3.png)

**Figure 3.** A) Staining intensity for CI19 subunit and porin in neurons with and without pathology. The colors represent different relative staining intensities. Red indicates no staining, yellow indicates minimal staining, and green indicates strong staining. B) Staining intensity for Complex IV subunit I in neurons with and without pathology. The colors represent different relative staining intensities. Red indicates no staining, yellow indicates minimal staining, and green indicates strong staining.

To examine the relationship between the expression of mitochondrial proteins and the extent of α-synuclein pathology, we graded the density of α-synuclein staining as few granules (+/−), granules or a small pre-Lewy body (+), larger pre-Lewy body (+++), and very dense pre-Lewy or large Lewy body (++++). The only statistically significant differences between data sets occurred when considering the staining for CI19 (Figure 5A) and porin (between + vs +++ and + + vs ++++ [P < .05]). For all other mitochondrial proteins, there was no statistical difference (P > .05) (Figure 5B; eg, COXI). From these results, it appears that in individual SN neurons, increased levels of expression of the majority of mitochondrial proteins is not related to the progression of α-synuclein aggregates from granules to Lewy bodies and is certainly not as dramatic as observed between cells with and without pathology.

![Figure 4](attachment:figure4.png)

**Figure 4.** A) Complex I 19-kDa subunit staining in neurons containing α-synuclein pathology compared with those devoid of pathology. This further validates our observation because the staining intensities of neurons of a single case are the same as when we consider the whole group. B) Complex IV subunit I staining in neurons containing α-synuclein pathology compared with those devoid of pathology. This further validates our observation because the staining intensities of neurons of a single case are the same as when we consider the whole group.
Since previous data suggest a role for complex I deficiency in PD, we determined the number of neurons that were deficient for complex I subunits (Figure 6). In normal aged SN, 9.94% of neurons (36 of 362) are deficient for CI20, while 9.85% are deficient for CI19. In LBD, we determined that 18.0% of neurons contain-
ing no pathology (66 of 366) are deficient for CI19, while only 0.9% of neurons containing α-synuclein pathology (1 of 107) are deficient (P < .001). For CI20, 10.4% of all LBD cells containing no pathology (31 of 298) are deficient compared with only 1.5% of α-synuclein–containing neurons (2 of 126) (P < .001). Thus, very few neurons containing α-synuclein pathology are deficient for complex I subunits and that deficiency occurs predominantly in neurons that do not contain pathology.

COMMENT

Our data shows that within individual nigral neurons, the presence of pathologic α-synuclein aggregates is associated with significantly higher expression of respiratory chain subunits. α-Synuclein pathology and deficiencies of mitochondrial respiratory chain subunits, which are 2 factors thought to play important roles in the pathogenesis of synucleinopathies, appear to affect different populations of nigral neurons.

Although we only studied protein expression levels in fixed tissues, the expression of a number of the selected proteins reflects the activity of respiratory chain complexes within mitochondria.22 We had expected that mitochondrial dysfunction would lead directly to α-synuclein pathology. A deficiency of mitochondrial respiratory chain subunits could lead to adenosine triphosphate depletion, which would impair a number of cellular processes including protein degradation through both chaperone-mediated autophagy and the ubiquitin proteasome system.30 Defects of mitochondrial respiratory chain subunits can cause increased free radical production in cell culture31–33 and potentially lead to damage of intracellular proteins and increased load on the proteasome.

There are a number of potential explanations for why cells containing α-synuclein pathology appear to have increased expression of mitochondrial respiratory chain proteins, with no increase in overall mitochondrial density; we discuss 3 of them. First, it is feasible that mitochondrial dysfunction and α-synuclein accumulation provide a toxic double hit for neurons. Those cells showing both severe mitochondrial dysfunction and α-synuclein accumulation are lost thus, explaining why it is extremely rare to find cells with both low levels of mitochondrial protein expression and α-synuclein pathology. However, data from this study examining the relationship between different stages of α-synuclein accumulation and mitochondrial dysfunction would not support this.

Second, there is a possibility that normal mitochondrial function is required for the accumulation of α-synuclein into Lewy bodies. Aggregating protein needs to be transported along axons/neurites to the cell body, where it is proposed that it accumulates into aggresomes, which eventually form Lewy bodies. Recruitment of mitochondria and ubiquitin proteasome system proteins then occurs, facilitating the clearance and degradation of aggregating protein.33 The transport and clearance of α-synuclein will require mitochondrial adenosine triphosphate and thus, it can only occur in cells with normal mitochondrial function. A situation may then arise whereby α-synuclein clearance cannot cope with the increased levels of aggregating protein and the Lewy body forms. In this context, the observation of little or no α-synuclein pathology in the respiratory chain deficient SN neurons would seem entirely fitting.

A final consideration is the type of α-synuclein being investigated. There still remains some debate as to which of the α-synuclein forms is potentially toxic. α-Synuclein can exist in many forms, some of which have the ability to interact with lipid membranes and cause the formation of pores within them. We have only examined insoluble forms of α-synuclein, and it is possible that it is these forms that are less harmful. In neurons, damaged α-synuclein forms soluble oligomers and proteofibrils, which have been shown to damage membranes and have other detrimental effects. Neurons that show mitochondrial dysfunction may be unable to aggregate these toxic forms of α-synuclein. Again, it could potentially be the same double-hit hypothesis, as previously mentioned, of increased toxic α-synuclein and mitochondrial dysfunction leading to cell death. Neurons with normal mitochondrial function may be better equipped to respond to the toxic α-synuclein species and bring them together into a LB, thus protecting the cell from the damaging effects of soluble forms of this protein. α-Synuclein is believed to have detrimental influences on mitochondrial function but again, the form of α-synuclein responsible remains unclear.

While many previous studies have found an association between mitochondrial dysfunction and the accumulation of α-synuclein in homogenized tissue, our data shows that at the single cell level, these 2 pathologies seem not to occur in the same cells. The data presented in this article shows that while mitochondrial dysfunction and α-synuclein pathology are important in LBD, mitochondrial dysfunction appears not to be the catalyst for α-synuclein accumulation at the single cell level.

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Online-Only Material: The eFigures and eTable are available at http://www.archneurol.com.

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