Mitochondrial DNA changes in pedunculopontine cholinergic neurons in Parkinson's

Running Head: *PD affects mtDNA in PPN cholinergic neurons*

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Character Count (Title): 80, including spaces

Character Count (Running Head): 43, including spaces

Word Count (Abstract): 100

Word Count (Body): 1,651

Figure and Table Count: 3 Main Figures (1 Color; 2 Black and white)

ABSTRACT

In Parkinson's disease (PD), mitochondrial dysfunction associates with nigral dopaminergic neuronal loss. Cholinergic neuronal loss co-occurs, particularly within a brainstem structure, the pedunculopontine nucleus (PPN). We isolated single cholinergic neurons from post-mortem PPNs of aged controls and PD patients. Mitochondrial DNA (mtDNA) copy number and mtDNA deletions were increased significantly in PD patients compared to controls. Furthermore, compared to controls the PD patients had significantly more PPN cholinergic neurons containing mtDNA deletion levels exceeding 60%, a level associated with deleterious effects on oxidative phosphorylation. The current results differ from studies reporting mtDNA depletion in nigral dopaminergic neurons of PD patients.

1. Introduction

Parkinson's disease (PD) patients typically present with tremor, bradykinesia and rigidity, the onset and progression of which associate with selective loss of nigro-striatal dopaminergic neurons.[1] Interest has grown as to the role of non-dopaminergic neurotransmission in PD. Cholinergic degeneration, affecting the basal forebrain, the nucleus basalis of Meynert and a rostral brainstem structure called the pedunculopontine nucleus (PPN), associates with the onset and development of 'axial' signs and cognitive impairment seen in PD patients.[2-4] Surviving (but susceptible) cholinergic neurons in these nuclei contain aggregated α-synuclein fibrils, forming Lewy bodies and Lewy neurites, a neuropathological hallmark of PD.[3]

The links between mitochondria and PD patho-etiology are well studied.[5] This commenced in the 1980s when the potent respiratory chain inhibitor 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), serving as a prodrug to the neurotoxin MPP+, was shown to cause parkinsonism in illicit drug users,[6] through to identification of complex-I-mediated reactive oxygen species forming within PD brains[7] and continuing with inherited and somatic mitochondrial DNA (mtDNA) variants that were shown to affect PD risk.[8-10] Additionally, changes in mitochondrial DNA copy number (mtCN) was shown to associate with PD pathology, with vulnerable dopaminergic neurons showing depleted levels of wild-type mtCN.[11,12]

Recently, we showed that several mitochondrial respiratory chain proteins are significantly up-regulated in gamma-aminobutyric acid(GABA)ergic and glycinergic PPN neurons from PD post-mortem brains.[3] Conversely, in the same patients, there was a marked reduction of these mitochondrial proteins in the cholinergic neurons of the PPN,

suggesting mitochondrial injury. Moreover, we observed a significant reduction in mitochondrial mass across all three neuronal types, with the most pronounced loss seen in PPN cholinergic neurons.[3] Given the links between PPN cholinergic neuronal deterioration, mitochondrial function and the development of PD, we investigated the role of mtDNA maintenance and stability in cholinergic neurons from the PPN of PD patients and controls.

2. Materials and Methods

Subjects: Post-mortem human brains (n=6 controls; n=6 PD; male:female ratio: 5:1) were obtained from the Newcastle Brain Tissue Resource in Newcastle upon Tyne, UK, which is covered by Newcastle University's Human Tissue Authority license. Ethical approval was granted for this study in 2016 by the Newcastle and North Tyneside Local Research Ethics Committee. All tissue donors gave written informed consent during life. All cases were assessed locally by a Neuropathologist and met the UK-PD Society's brain bank criteria for diagnosing PD. None of the control cases met such criteria and also did not meet operational criteria for diagnosing Alzheimer's disease, with control brains that showed only minimal, age-related tau and β-amyloid pathology. The mean (± standard error of the mean (S.E.M.)) for post-mortem interval (PMI) was 21±4 hours (hrs) for PD cases and 20±3 hrs for controls. The mean (± S.E.M.) age of death was 76±3 years for PD cases and 84±7 years for controls. Between PD and control cases there was no statistically significant differences in PMI or age of death (p=5.2×10^{-1} and p=2.3×10^{-1}, respectively; independent Student's t-test). The mean disease duration for the PD cohort was 8±2 years.

PPN identification: Serial sections (20 μm) were cut from the left hemisphere of PPN-containing brain blocks, using a cryostat (Bright Instrument Company Ltd., UK). To distinguish the PPN, pairs of sections (first and last in series) for each case were stained with Haematoxylin and Eosin (H&E) (Fig. 1A) and Luxol Fast Blue (LFB) (Fig. 1B), using standard protocols. Paired stained sections were then used to define the PPN's boundaries, noting neighboring neural structures, including the lateral lemniscus (LL), medial lemniscus (ML) and superior cerebellar decussation (SCD).[13]

Single cell isolation: To immunohistochemically stain cholinergic neurons in PPN-containing brain tissue sections, the sections were air-dried for 30 min at room temperature (RT) and then fixed for 20 min in 4% paraformaldehyde (PFA) dissolved in phosphate buffered saline (PBS). The sections were washed with PBS before blocking with 5% normal rabbit serum (S-5000; Vector laboratories, UK) for 30 min, then incubated for 2 hrs at RT with a primary antibody detecting choline acetyltransferase (ChAT, polyclonal goat, 1:150; AB 144P, Millipore, USA). After further washing in PBS, the secondary antibody (peroxidase horse anti-goat, 1:200; PI-9500, Vector laboratories, UK) was applied for 1 hr at RT. Following washes in PBS, 3,3',5,5'-tetramethylbenzidine (TMB) stabilized chromagen (Invitrogen, UK), a substrate of horseradish peroxidase that oxides to form a blue chromagen, was applied to the sections for 10 min at RT and then rinsed well with distilled water. All antibody and serum dilutions were made using Tris Buffered Saline (TBS). Individual PPN cholinergic neurons were isolated using the P.A.L.M. MicroBeam Laser-Capture Microdissection system coupled to an inverted Zeiss microscope (Axiovert 200M, Carl Zeiss, Germany), and individually placed into adhesive cap microfuge

tubes (Carl Zeiss, Germany) containing lysis buffer consisting of 50mM Tris-Hydrochloride with 1% Tween 20 (pH 8.0) and 20mg/ml proteinase K (Thermo Fisher Scientific, UK). Each lysis was immediately centrifuged at 13,000 revolutions/min and subsequently incubated at 55°C for 16 hrs, followed by incubation at 95°C for 10 min. In total, 144 neurons were isolated (n=72 controls; n=72 PD).

mtDNA analysis: Quantification of mtDNA was performed as previously described,[14] via a probe-based[11] multiplex Taqman quantitative polymerase chain reaction (qPCR) to amplify the mitochondrial genes *MTND1* and *MTND4.* mtCN was calculated by absolute quantification of *MTND1*, using the standard curve method, with serial dilutions of PCR-generated templates. PD and control samples (assayed in triplicate) were randomly assigned to each run to limit run-specific stratification.

Statistical analysis: Data were analyzed using SPSS (version 22, SPSS Inc., USA) with data-appropriate tests (detailed in text). Statistical significance was set at $p<5\times10^{-2}$. The choice of which analyses to apply was based on the data type. A correlative analysis was used when two continuous variables were present, for example mtCN and PMI. A one-way ANOVA was used where there was greater than two categories whose means were to be compared, i.e. mtCN versus Braak stage (0-4). Data are expressed as the mean ± S.E.M. The raw mtCN data is available on request

3. Results

Comparison of mtDNA levels from single PPN cholinergic neurons showed a significant increase in mtCN in PD cases compared to controls ($p=2.9\times10^{-2}$, two-way

ANOVA, PD mean = 10,706±1,441 versus control mean = 7,017±825; Fig. 2A). Importantly, no correlation was found between PMI and mtCN in either PD cases or controls (p=5.1×10^{-1} and p=8.7×10^{-1} respectively, linear regression analysis). Furthermore, no significant association was detected between mtCN and PD duration (p=9.7×10^{-1}, linear regression analysis) nor mtCN and Braak stage (p=3.1×10^{-1}, one-way ANOVA; Fig. 2B).

mtDNA deletion levels were also significantly increased in PD cases compared to controls (p=2.5×10^{-2}, two-way ANOVA, PD mean = 21.60±3.04% versus control mean = 17.15±1.99%; Fig. 2C). Again, no correlation was found between PMI and mtDNA deletions in neither cases nor controls (p=9x10^{-2} and p=3.4x10^{-1} respectively, linear regression analysis). However, mtDNA deletion levels correlated with higher Braak staging in PD patients (p=3×10^{-3}, one-way-ANOVA; Fig. 2D). Stratification of individual neurons into high- (i.e. >60% at which level they are likely to exhibit a pathological phenotype[8]) and lower mtDNA deletion levels (i.e. <60%, and therefore less likely to exhibit a pathological phenotype), revealed that PD cases harbored a disproportionately higher number of PPN cholinergic neurons with high mtDNA deletion load compared to controls (p<1×10^{-4}, Fisher's exact test; Fig. 3A).

Thus, unlike recent studies performed on substantia nigra pars compacta (SNpc) dopaminergic neurons taken from post-mortem PD brains, and which reported mtCN depletion,[6,9,10,12] the current findings relating to single PPN cholinergic neurons showed significantly elevated mtCN and mtDNA deletion levels in PD patients compared to controls. We investigated this relationship further by using linear regression analysis. A significant negative correlation was observed between mtCN and mtDNA deletion levels in

neurons taken from controls ($r=-2.5x10^{-1}$, $p=4.9x10^{-2}$), this finding resulting from very few neurons having higher levels of mtDNA deletions. When all data points were included, no significant association was detected between deletion levels and mtCN ($r=5.4x10^{-2}$, $p=6.7x10^{-1}$; Fig. 3A).

An elevation in mtCN in response to the accumulation of mtDNA deletions might be considered evidence for cells' ability to proliferate mtDNA in order to maintain a critical number of wild-type mtDNA molecules to support a variety of physiological processes. This regression analysis was repeated, by excluding neurons containing lower deletion levels (30%), as at these lower levels the neuroprotective processes might not have been triggered yet, thereby inhibiting the ability to detect a correlation between the two variables. The exclusion of neurons with lower mtDNA deletion levels did not reveal a significant relationship between deletion levels and mtCN ($r=0.23$, $p=4x10^{-1}$; Fig. 3B). Taken together, our study reveals an elevation of mtCN and mtDNA deletion levels in PD patients compared to controls; however, a correlation was not detected between mtCN and mtDNA deletion levels in the single cholinergic neurons of the PD patients.

4. Discussion

This is the only study to date that characterizes mtCN and mtDNA deletion levels in cholinergic neurons of the PPN, a neuronal population that is highly vulnerable to cell death in PD patients. The aim was to better understand the role that mtDNA changes play in the loss of PPN cholinergic neurons in PD, which has been shown to be pivotal in the onset and progression of motor and non-motor PD symptoms.

Our investigations revealed that in remaining PPN cholinergic neurons of PD pa-

tients mtCN is increased compared to controls. This is in contrast to studies which reported decreased mtCN levels within remaining SNpc dopaminergic neurons in PD postmortem brains,[8,11,12,15] suggesting for neuronal-type and brain region-specific responses to accumulation of mtDNA mutations in PD patients. However, similar to previous studies performed on SNpc dopaminergic neurons,[8,11,12,15] our data indicate that mtDNA deletions are increased in PD patients compared to controls. Importantly, we show that in PD patients there is a substantial increase in the number of PPN cholinergic neurons containing >60% mtDNA deletion levels, making it highly likely that these neurons should manifest a respiratory chain deficiency.[8]

Our data shows that cholinergic neurons in the PPN, in contrast to reports of depletion in the SNpc of PD cases,[8,11,12,15] appear to increase their mtCN in response to rising deletion levels. This raises the hypothesis that the PPN has a compensatory mechanism designed to maintain a pool of wild-type mtDNA molecules that is not present in the SNpc, or alternatively that the dopaminergic neurons of the SNpc are more vulnerable to rising mtDNA deletion levels than the cholinergic neurons of the PPN. The former appears to be supported by our data, as we detected a seemingly overall elevation in mtCN in the PD patients who also had higher deletion levels (Figs. 2A & B). This may reflect an inability by PPN cholinergic neurons to maintain the required level of wild-type mtDNA past the threshold deletion level, or might result from the smaller number of neurons with high levels of mtDNA deletions which we observed here. Nevertheless, this exploratory observation should be explored further in future studies.

Although not a conclusive indicator of PD, Braak staging indicates disease advancement.[16] In this study, we identified a significant difference between high mtDNA

deletion levels and advanced Braak staging, supporting the hypothesis that mtDNA deletions within PPN cholinergic neurons contribute to PD progression. This is similar to SNpc dopaminergic neurons in PD patients, where Lewy body abundance, associated with advanced Braak staging, coincided with increased mtCN, which was hypothesized as a possible compensatory response against deficient levels of adenosine triphosphate (ATP).[17] In the current study, we found no significant difference in PPN cholinergic neuronal mtCN values between the different Braak stages; however, this is an area that merits additional investigation in future studies.

In conclusion, we show increased mtCN and mtDNA deletion levels within remaining PPN cholinergic neurons in PD patients compared to controls. We further found significantly more cholinergic neurons harboring mtDNA deletion levels that associate with a mitochondrial dysfunction in PD, compared to controls. These findings support the view that mtDNA deletions are frequent in PPN cholinergic neurons and that these might play a role in the death of these neurons in PD patients. Critically, the data suggests that different brain regions and neurochemical cell types vary in their responses to accumulated mtDNA deletions in PD patients, since the data contrasts with prior observations relating to SNpc dopaminergic neurons.

Acknowledgements

ISP receives grant support from the Rosetrees Trust and the British Pharmacological Society. GH is a Parkinson's UK Senior Research Fellow (F-1202).

Abbreviations used: ATP, adenosine triphosphate; ChAT, choline acetyltransferase; CI, confidence interval; GABA, gamma-aminobutyric acid; H&E, haematoxylin and eosin; hrs, hours; LFB, luxol fast blue; LL, lateral lemniscus; mtCN, mitochondrial DNA copy number;

mtDNA, mitochondrial DNA; ML, medial lemniscus; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PFA, paraformaldehyde; PBS, phosphate buffered saline; PD, Parkinson's disease; PPN, pedunculopontine nucleus; PMI, post-mortem interval; qPCR, quantitative polymerase chain reaction; RT, room temperature; S.E.M., standard error of the mean; SNpc, substantia nigra pars compacta; SCD, superior cerebellar decussation; TBS, Tris Buffered Saline; TMB, 3,3',5,5'-tetramethylbenzidine

Author Contributions: I.-S.P., J.L.E. and G.H. conceived and designed the project. A.G.B., A.P., J.L.E., L.G., C.M.M., G.H., and I.-S.P. collected, analyzed and interpreted the data. A.G.B., A.P., J.L.E., L.G., C.M.M., G.H., and I.-S.P. wrote the paper.

Potential Conflicts of Interest: Nothing to report.

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Figure and table legends:

Figure 1: A low (2.5× air-based) objective lens was used to capture tiled images, **(A)** representing the most rostral and **(B)** immediately adjacent section, along the rostro-caudal axis that cryostat sections were collected. The PPN and surrounding neural structures were visualized with **(A)** H&E and **(B)** LFB staining. Mapped outlines indicate the anatomical location of the PPN in relation to major surrounding structures, including the LL, ML and SCD. The insets show magnified (20× air-based objective) images of the **(Ai)** H&E and **(Bi)** LFB stained sections, illustrating the Ch5 cholinergic neurons of the PPN.[18] Scale bars: **(A, B)** 500 μm and **(Ai, Bi)** 75 μm. **(C)** Isolation of individual cholinergic neurons from the PPN, using laser-assisted microdissection. **(Ci)** Prior to dissection, individual cholinergic neurons were viewed with a Brightfield inverted microscope (Carl Zeiss) at high (40×) magnification. Neurons were collected based on ChAT immunoreactivity and typical morphology. Neurons were manually circumscribed by using the "draw shape" tool of the software interface. **(Cii)** Laser energy pulses were then applied to separate out an outlined neuron from the surrounding tissue. **(D)** Successful cell capture was confirmed by microscopically viewing the tube's cap, shown at **(Di)** 5× and **(Dii)** 20× magnification. Scale bars: **(Ci, Cii)** 75 μm, **(Di)** 50 μm and **(Dii)** 75 μm.

Figure 2: (A) A data scatter plot shows a significant increase in mtCN in PPN cholinergic neurons of PD compared to controls (p=2.9×10^{-2}). There was no significant difference in these values between individual cases, although more stratification was observed between the PD cases (p= 5.2×10^{-1}). **(B)** A box plot shows no significant trend between increased mtCN and later Braak stage for the PD cohort (p=3.1×10^{-1}). The error bars represent the 95% confidence interval (CI) of the mtCN values for each case.

Dots above the error bars represent values greater than the upper 95% CI. **(C)** A data scatter plot shows a significant increase in %mtDNA deletions in PD patients compared to controls ($p=2.5\times10^{-2}$), with no significant difference in such values between individual cases ($p=7.8\times10^{-2}$) in either the PD or control cohorts. Significantly more PPN cholinergic neurons harbored mtDNA deletions >60%, compared to controls ($p<1\times10^{-4}$). **(D)** A box plot of % mtDNA deletion against Braak stage shows a positive, statistically significant relationship between mtDNA deletion levels and advanced Braak stage ($p=3\times10^{-3}$). The error bars represent the 95% CI of the mean percentage deletion value for each case. Dots above the error bars represent values greater than the upper 95% CI.

Figure 3: The figure depicts the relationship between mtDNA deletion levels and mtCN for **(A)** all PPN cholinergic neurons taken from PD patients and controls, as well as **(B)** in PPN cholinergic neurons where mtDNA deletion levels exceeded 30%.