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ORIGINAL COMMUNICATION

Dopaminergic midbrain neurons are the prime target for mitochondrial DNA deletions

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Abstract Mitochondrial dysfunction is a consistent finding in neurodegenerative disorders like Alzheimer's (AD) or Parkinson's disease (PD) but also in normal human brain aging. In addition to respiratory chain defects, damage to mitochondrial DNA (mtDNA) has been repeatedly reported in brains from AD and PD patients. Most studies though failed to detect biologically significant point mutation or deletion levels in brain homogenate. By employing quantitative single cell techniques, we were recently able to show significantly high levels of mtDNA deletions in dopaminergic substantia nigra (SN) neurons from PD patients and age-matched controls. In the present study we used the same approach to quantify the levels of mtDNA deletions in single cells from three different brain regions (putamen, frontal cortex, SN) of patients with AD (n = 9) as compared to age-matched controls (n = 8). There were no significant differences between patients and controls in either region but in both groups the deletion load was markedly higher in dopaminergic SN neurons than in putamen or frontal cortex ($p < 0.01$; ANOVA). This data shows that there is a specific susceptibility of dopaminergic SN neurons to accumulate substantial amounts of mtDNA deletions, regardless of the underlying clinical phenotype.

Key words Alzheimer's disease · neurodegeneration · mitochondrial DNA · deletions · Parkinson's disease · oxidative stress

Introduction

There is strong evidence for an important contribution of mitochondrial dysfunction in the pathogenesis of neurodegenerative disease and aging (for current reviews, see [26, 27]. In the case of Parkinson's disease (PD), much of this evidence centres on complex I of the mitochondrial respiratory chain. A decreased activity of this oxidoreductase has been reported in SN from PD patients and the administration of complex I inhibitors such as rotenone or MPTP led to clinical and neuropathological features strongly resembling PD [4, 16, 33]. The unravelling of genes leading to hereditary PD syndromes indistinguishable from classical PD have provided further support for the important role of mitochondria in this disease since many of the associated gene products have either direct or indirect links to mitochondrial function [14].

There is also extensive literature suggesting a role for

mitochondrial dysfunction in the pathogenesis of Alzheimer's disease (AD) (for current reviews, see [2, 21, 27, 32]). Many of the proteins with presumed importance in AD pathogenesis such as amyloid precursor protein and amyloid-β have a direct impact on mitochondria, causing mitochondrial dysfunction and impaired energy metabolism [1, 28]. Cytochrome c oxidase (COX), complex IV of the respiratory chain, seems to be particularly involved [29] and indeed, the number of COX-deficient neurons was shown to be increased in brains from AD patients [12, 13]. The pathophysiological role of COX deficiency in AD is unclear though. Recent data from a mouse model expressing mutant amyloid precursor protein and mutant presenilin 1 in a neuron-specific COX-deficient background provide evidence that COX deficiency is not a primary event in AD pathology [17].

Increased production of reactive oxygen species (ROS) are consistent findings in PD and AD research [27]. Since oxidative phosphorylation in mitochondria is the main source of intracellular ROS production and since mitochondrial DNA (mtDNA) is in close proximity to the respiratory chain, it has been proposed that mtDNA damage may be at the core of mitochondrial pathology involved in neurodegenerative disease [11]. Indeed, with quantitative single cell techniques, we were recently able to show high levels of mtDNA deletions in post mortem dopaminergic neurons of PD patients and of age-matched controls [3]. This raised the question whether mtDNA deletions might play a substantial role as a core neurodegenerative process not just in PD but also in other diseases.

In the case of AD, an increased incidence of mtDNA control region mutations has been reported in a post-mortem study [10]. On the RNA level, downregulation of mitochondrially encoded subunits for complex I have been reported both in early and definite stage AD in a post mortem study [30]. Several studies have also reported higher incidence of mtDNA deletions in AD patients' brain homogenates compared to controls [9, 20]. Yet, the reported deletion levels were very low and thus very unlikely to be relevant, considering that mtDNA deletions need to exceed a threshold level of greater than 60 % in order to cause mitochondrial dysfunction [34]. As we have shown in the case of PD, single cell approaches must be employed to obtain an accurate estimate of neuronal mtDNA deletion load.

In both AD and PD there is strong evidence for a pathophysiological involvement of mitochondrial dysfunction and oxidative stress. We used laser capture microdissection (LCM) of single neurons in three different brain regions in AD patients and in controls to test the hypothesis that mtDNA damage is also involved in AD pathogenesis, following the anatomical distribution of neuropathological changes.

Materials and methods

Frozen brain specimens of the putamen (Put), frontal cortex (FC), and substantia nigra (SN) were obtained from the German brain bank (Brain-Net®) of patients with clinical and neuropathological diagnosis of AD (n = 9) and of age-matched normal controls (n = 8), the latter without history or histological evidence of neurodegeneration (patient data, see Tables 1 and 2). These individuals were completely different from the previously studied English patients (Newcastle upon Tyne Brain Tissue Resource). The study protocol was approved by the appropriate ethics committee and the study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

For AD, only patients with predefined isocortical Braak stage V or VI were chosen [7]. None of these cases showed Lewy body pathology. From each brain area, a series of 10 sections (20 μm thickness) was cut with a cryostat on membrane slides, stained with classical Nissl staining procedure using cresyl violet (Merck, Darmstadt, Germany), and frozen at –20 °C until further processing. From each brain area, 30 neurons were dissected by a LCM system (AS LMD, Leica, Germany). Large pyramidal neurons were identified by standard neuropathological criteria using Nissl staining. For the SN, melanin-containing neurons were chosen from the pars compacta. DNA was extracted with the DNA Micro Kit (Qiagen, Germany) according to the manufacturer's protocol. Quantification of mtDNA deletion levels

Table 1 Individual patient characteristics

No.	Group	Age/sex	Post mortem interval [h]	Braak stage
1	Control	68/M	51	None
2	Control	74/M	40	None
3	Control	78/F	42	None
4	Control	69/M	18	None
5	Control	67/M	14	None
6	Control	63/M	17	None
7	Control	69/F	38	None
8	Control	83/F	39	None
9	AD	73/F	36	V/VI
10	AD	75/F	47	V/VI
11	AD	67/M	47	V/VI
12	AD	80/F	40	V/VI
13	AD	86/M	42	V/VI
14	AD	78/M	36	V/VI
15	AD	63/F	28	V/VI
16	AD	89/M	20	V/VI
17	AD	75/M	48	V/VI

Table 2 Summary of patient characteristics

	Alzheimer disease	Controls
Number of patients	9	8
Patient age* (years ± SD)	76.2 ± 8.2	71.4 ± 6.5
Post mortem delay* (hours ± SD)	38 ± 9	32 ± 14

SD standard deviation, * There was no statistical difference in mean age or mean post mortem delay time between groups (T-Test, $p > 0.1$)

was based on a realtime PCR method described earlier [22]. In short, quantities of the mitochondrial ND1 and ND4 genes of a single cell are compared to one another by means of relative gene quantification according to the delta-delta-CT method (ΔΔCT). As the vast majority of mtDNA deletions comprises the ND4 region and spares the ND1 gene, ND1 can be used as an internal control, whereas a drop in ND4 quantity serves as a measure for the abundance of deletions. Differing from the original method, in order to minimize pipetting errors and to optimize retest reliability, we have further optimized the realtime assay to be run as a duplex realtime PCR experiment with quantification of ND1 and ND4 genes within the same reaction well. With this protocol, there is high correlation with deletion quantification by Southern blot and by the original method [25]. Primers (MWG Biotech, Germany) and sequence specific probes (Applied Biosystems, UK) were: for ND1 (forward primer nt 3485–3504, reverse primer nt 3553–3532, VIC-dye-labelled probe nt 3506–3529) and for ND4 (forward primer nt 12087–12109, reverse primer nt 12170–12140, FAM-labelled probe nt 12111–12138). Final concentrations of primers and probes were 900 nM and 250 nM, respectively. The Taqman Universal PCR Mastermix (Applied Biosystems) was used for the assay in a 25 µl reaction mix per sample. Cycler conditions on an ABI prism 7300 analyser (Applied Biosystems, UK) were 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C followed by 1 min at 60 °C. Of each sample, triplicate runs were performed and the resulting mean values were used for statistical analysis.

To quantify the relative amount of ND1 to ND4 the following equation was used: $R = 2^{-\Delta\Delta Ct}$, where R is the calculated relative copy number and ΔΔCT is the CT_{ND4}-$_{ND1}$ (after subtracting the ΔCt from a control sample with no deleted mtDNA ("standard DNA" extracted from blood from a 35 year old healthy male). Using this undeleted standard DNA, a dilution series over 5 orders of magnitude was established for ND1 and ND4 in triplicate runs in order to show similarity of PCR reaction efficiencies as a prerequisite for relative quantification with the ΔΔCT method (Fig. 1).

All statistical analyses were performed with SPSS 12.0 for Windows. Depending on the number of groups to be compared either a two-sided t-test (for 2 groups, i.e. comparison of both patient groups) or an ANOVA test (for 3 groups, i.e. comparison of the 3 brain areas within one patient group) was used to look for significant differences, followed by a post hoc Scheffe test to further characterize this difference, where applicable (ANOVA).

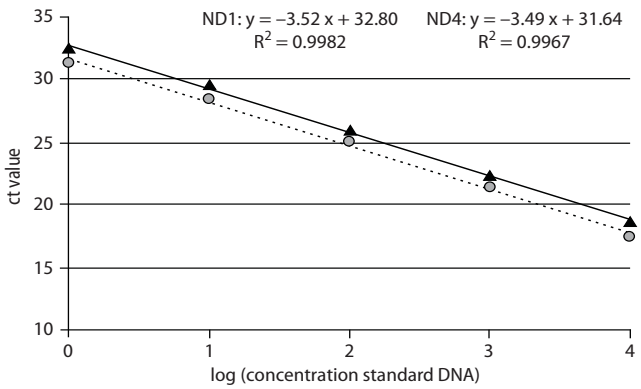

Fig. 1 Realtime PCR of ND1(▲) and ND4 genes (●) from a dilution series of blood DNA over 5 orders of magnitude. Trendlines for ND1 (solid line) and ND4 (dotted line) are parallel, proving similar reaction efficiencies for the two genes, thereby enabling relative gene quantification. The slope in the reaction equations in the upper right hand corner gives reaction efficiency, which is comparable for ND1 (–3.52) and ND4 (–3.49)

Results

A total of 1530 single neurons (30 per brain region, i.e. 90 per patient) were microdissected, pooled for each brain region, and analyzed for mtDNA deletion levels. Deletion levels of the SN were consistently higher than those of the other brain regions in patients with AD (32 % ± 11 % in SN versus 13 % ± 10 % in FC and 14 % ± 9 % in Put, $p < 0.01$) and controls (35 % ± 10 % versus 14 % ± 10 % and 14 % ± 10 %, $p < 0.01$; Fig. 2). There was no difference in mtDNA deletion levels per brain region between groups.

Discussion

As in our previous study in PD patients and controls, we were able to detect high levels of mtDNA deletions in neurons of the SN of AD patients and of age-matched controls, using quantitative single cell techniques. Underlining the importance of such single cell approaches, the reported deletion levels are much higher than those of previous studies which investigated tissue homogenates rather than individual neurons [9, 20]. Unlike our observations in PD, we did not find good correlation between the major affected region (frontal cortex) in patients with AD and high levels of deleted mtDNA. In this study we did not specifically look at COX normal or COX deficient neurons. What we did observe in these patients was that the neurons of the SN are particularly prone to develop high levels of mtDNA deletions.

The relationship between mtDNA deletions and the major neuropathological features in AD is likely to be complex. Our findings show that there is not a simple relationship with the site of the most severe AD pathology and deletion load in neurons. However, the finding

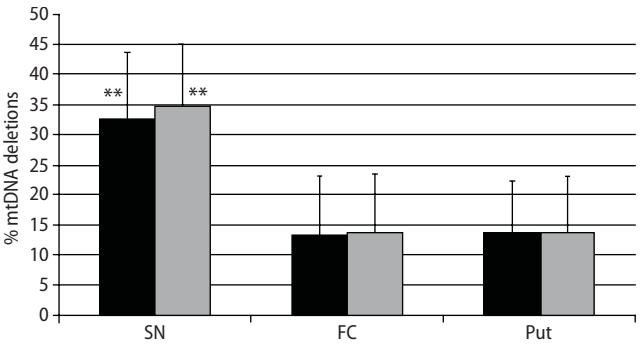

Fig. 2 Levels of mtDNA deletions in 30 microdissected neurons per subject in AD patients (black bars) and in age-matched controls (grey bars) in three different brain areas (SN substantia nigra, FC frontal cortex, Put putamen; error-bars indicate the standard deviation). Note that deletion levels are consistently higher in the SN compared to the other two brain areas in both patient groups (** indicates ANOVA level of significance < 0.01 when comparing deletion levels of the SN with those of the FC and Put)

of increased numbers of COX deficient neurons in AD suggests that in some neurons there are high levels of mtDNA deletions [13]. It is also not known what the fate of these neurons is. In patients with primary mtDNA mutations cell loss is evident in many brain regions suggesting that mtDNA mutations can lead to neuron cell death [5].

Even though we did not find significant group differences, the observed regional pattern of mtDNA damage is striking. Irrespective of patient group, the SN always harboured significantly higher deletion levels than the basal ganglia (Put) or the frontal cortex. The cell type, which we isolated from the SN by LCM was characterized by the appearance of neuromelanin. Even though we did not employ immunostaining for cell characterization, it can be safely assumed that we studied the dopaminergic class of midbrain neurons, which are at the focus of neurodegeneration in PD [23]. Our findings therefore confirm that the dopaminergic neurons of the SN are particularly prone to accumulate mtDNA deletions. Yet, this type of mitochondrial damage is far from being specific for pigmented neurons as shows our detection of substantial amounts of mtDNA deletions in neurons of the other two investigated brain areas.

It is yet unclear what constitutes the high susceptibility of dopaminergic neurons for the accumulation of somatic mtDNA deletions. It is tempting to speculate that the catecholamine metabolism as such is responsible for this increased vulnerability. It is well recognized that dopamine metabolism leads to the production of ROS [8]. Interestingly, in the context of the presumed role of complex I in PD, dopamine and some of its metabolites can act as inhibitors of complex I [18, 24]. It has also been shown that neuromelanin, which is a byproduct of dopamine metabolism, induces oxidative stress in mitochondria through the release of iron [35]. The SN is a preferential candidate for ROS-mediated damage, because it contains not only neuromelanin and iron, but also oxidizable dopamine, polyunsaturated fatty acids as well as a relatively low antioxidant defence system [15]. In addition, gene expression profiling of rat SN dopamine neurons revealed that they had higher RNA levels of genes related to energy metabolism, suggesting that they might utilize more oxygen [19]. This is reflected by the fact that SN neurons are reported to fire 50 % more often than other midbrain dopaminergic neurons [36]. Since the mitochondrial respiratory chain is the main site for ROS production, this higher metabolic activity might point towards an increased risk for oxidative damage to nucleic acids in dopaminergic SN neurons [6, 31].

In summary, we were able to show high mtDNA deletion levels in the SN of patients with AD and controls, significantly exceeding those in other brain areas. This consistent pattern suggests that the dopaminergic midbrain neurons are especially vulnerable for mtDNA damage. Further studies on the single cell level are warranted to further explore this susceptibility.

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