

Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons

Yevgenya Kraytsberg¹, Elena Kudryavtseva¹, Ann C McKee^{2,3}, Changiz Geula¹, Neil W Kowall^{2,3} & Konstantin Khrapko¹

Using a novel single-molecule PCR approach to quantify the total burden of mitochondrial DNA (mtDNA) molecules with deletions, we show that a high proportion of individual pigmented neurons in the aged human substantia nigra contain very high levels of mtDNA deletions. Molecules with deletions are largely clonal within each neuron; that is, they originate from a single deleted mtDNA molecule that has expanded clonally. The fraction of mtDNA deletions is significantly higher in cytochrome *c* oxidase (COX)-deficient neurons than in COX-positive neurons, suggesting that mtDNA deletions may be directly responsible for impaired cellular respiration.

Somatic mutations in mtDNA have been hypothesized to be responsible for some aspects of the aging process^{1,2}. Indeed, mtDNA mutations are capable of producing aging-like phenotypes in mitochondrial diseases ranging from cardiomyopathy to neurodegeneration: for example, in mitochondrial diseases. Recently, multiple aging phenotypes have been demonstrated in transgenic mice with an increased rate of somatic mtDNA mutations (reviewed in ref. 3). However, a major challenge to the mtDNA mutational theory of aging has been the presumably low abundance of mtDNA mutations in normally aging tissues^{3,4}, such that their physiological significance may be questionable. It was shown over a decade ago that a particular

type of mtDNA mutations, mtDNA deletions, are distributed in a highly nonuniform manner among different tissues and within the same tissue, particularly among different areas of the brain^{5,6}. Some brain areas have been reported to sustain a few orders of magnitude more deletions than others. It is tempting to hypothesize that mtDNA mutations, although rare on average, may reach sufficiently high concentrations in specific cell types to significantly impair cellular processes in an age-dependent manner. Measurements of mtDNA deletions are technically difficult, however, and reliable quantitative studies have focused on a single 'common' type of deletion^{5,6}. Thus, the absolute burden of all possible mtDNA deletions, the most important parameter with respect to physiological relevance of these mutations, remained unknown.

The substantia nigra, the primary site of neurodegeneration in Parkinson disease, sustains particularly high levels of 'common' mtDNA deletions compared with other brain areas⁶. Notably, a large proportion of pigmented neurons in substantia nigra have been shown by immunohistochemistry to lose COX, an mtDNA-encoded enzyme, with increasing age⁷. No direct relationship between these defects and mtDNA mutations has yet been reported, although our previous work⁸ suggested that individual pigmented neurons may contain very high quantities of all mtDNA deletions combined. To more fully explore this relationship, we developed a new approach to quantify the total cellular burden of mtDNA deletions using single-molecule PCR (smPCR), as described in the **Supplementary Note** online. We then used this quantitative smPCR method to directly measure mtDNA deletions in individual neurons of substantia nigra of various ages and compare the mutational loads of COX-positive and COX-deficient neurons. We and others have previously used this smPCR approach to perform mutational analysis in other systems (reviewed in ref. 9).

We collected 80 individual cells from human substantia nigra specimens, aged 33–102 years, and determined the percentage of deleted mtDNA molecules in each cell in order to assess the

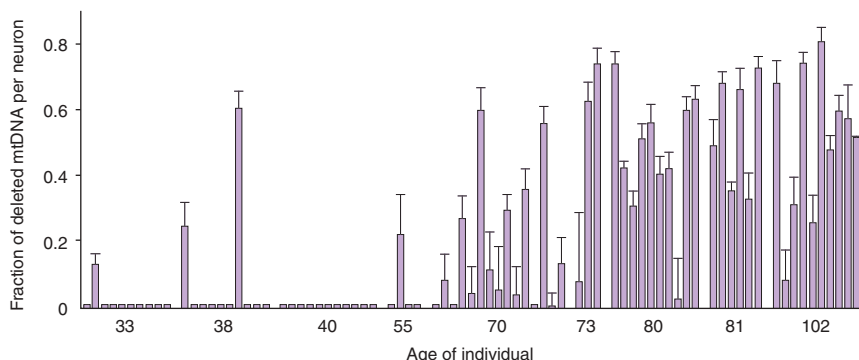


Figure 1 Fraction of deleted mtDNA in individual pigmented neurons of substantia nigra from subjects of different ages. Each bar represents the mutant fraction in a single cell as determined by single-molecule PCR or extended PCR. Bars representing neurons from the same individual of a certain age are grouped, and the age is indicated under the group. Error bars represent standard error with respect to repeated measurements of the same cell ($n \geq 3$). Zero-height bars represent cells that were determined to be deletion-free by extended PCR. See **Supplementary Note** for methodological details.

¹Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215, USA. ²Boston University School of Medicine, Boston, Massachusetts 02118, USA. ³Geriatric Research Education and Clinical Center, Bedford Veterans Affairs Medical Center, Bedford, Massachusetts 01730, USA. Correspondence should be addressed to K.K. (khrapko@hms.harvard.edu).

Received 13 December 2005; accepted 6 March 2006; published online 9 April 2006; doi:10.1038/ng1778

distribution of fractions of deleted mtDNA that accumulate in individual pigmented neurons with age (Fig. 1). The number of mtDNA deletions was significantly different between old and young tissues ($P < 0.0001$ by two-sample, two-tailed, homoscedastic t -test, regardless of cutoff age, 40 to 69 years). Moreover, there was a very high absolute prevalence of mtDNA deletions in neurons from aged substantia nigra (Fig. 1). In addition, in many neurons, the fraction of deletions exceeded 60%, which is believed to be the phenotypic threshold (the fraction above which mtDNA deletions impair respiratory function; reviewed in ref. 10). Younger pigmented neurons occasionally accumulate high fraction of deletions, as in one of the cells from a 38-year-old (Fig. 1), but such events are rare. Several other cell types, including pyramidal neurons of the cerebral cortex, cerebellar Purkinje cells and large neurons of the dentate nucleus of aged individuals usually contained undetectable deletion levels, consistent with the notion that the accumulation of mtDNA deletions is highly cell type-specific^{5,6}. Similar results have been reported independently by another group using a different approach (real-time PCR) to quantify mtDNA deletions¹¹.

We then explored whether the highly abundant mtDNA deletions that we observed have an effect on COX activity. A large representative study⁷ on four areas of substantia nigra from the brains of 36 subjects aged 30–99 years old has shown that up to 30% of pigmented neurons in the older brains are COX deficient, albeit with a large case-to-case variation. Capitalizing on these data, we attempted to determine whether COX deficiency develops in cells with the highest fractions of mtDNA deletions. Because it is particularly important to address this question in the most affected individuals, we selected an 80-year-old brain with high frequency of COX-deficient neurons for a detailed cell-by-cell study. A tissue section from this individual stained for COX activity (Fig. 2a) shows that a local population of pigmented neurons contains about 30% COX-deficient cells; that is, cells with decreased, but not necessarily absent, COX activity (Supplementary Note online). We collected individual COX-positive and COX-deficient neurons and measured the fraction of deletions in each cell (Fig. 2b). In accord with our hypothesis, COX-positive cells contained a significantly lower fraction of deletions than COX-deficient cells ($P < 0.0001$; two-sample, two-tailed, homoscedastic t -test). Notably, the fractions of deletions in all COX-deficient cells were not only higher than in COX-positive cells but they actually exceeded the 60% threshold and thus might be sufficient to cause the observed COX deficiency, although more data will be necessary to confirm this observation.

The difference in distribution of mtDNA deletions between COX-positive and COX-deficient neurons strongly implies that the mtDNA deletions may be one of the primary causes of the COX defect. However, an alternative possibility is that some other local primary defect (such as increased free radical production or a defect in DNA maintenance) might have caused secondary accumulation of deletions and the development of respiratory deficiency. To evaluate this possibility, we assessed, in individual neurons, the distribution of mtDNA deletions by type in individual neurons. The DNA of individual neurons was subjected to 'extended' PCR capable of amplifying the entire mitochondrial genome¹². Although extended PCR is not quantitative, it helps to determine the specific types of mtDNA deletions present in a cell. A typical example of such an analysis is presented in the Supplementary Note online. In most cases, a neuron either contained no deletions or contained a single species of deletion that showed up repeatedly in several independent PCRs. The presence of multiple identical molecules of deleted mtDNA in a cell implies that mtDNA deletions are clonal; that is, they originate from a single initial mutant molecule that has multiplied. Thus, expansion of preexisting mutant molecules, rather than ongoing *de novo* mutational

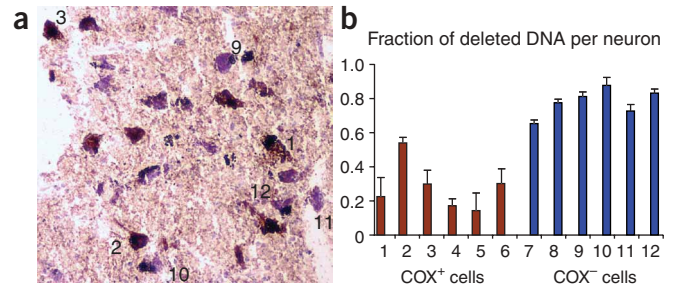


Figure 2 Clonal expansions of mtDNA deletions are associated with COX defects in individual neurons. (a) COX-specific immunostaining of the substantia nigra pars compacta of an 80-year-old (Cresyl violet counterstain). COX-positive cells appear brown, whereas COX-deficient neurons appear violet owing to counterstaining with Cresyl violet. Dense black granules are the neuromelanin aggregates characteristic of pigmented neurons. Cells identified as COX-positive or COX-deficient were individually collected by laser capture microdissection and analyzed for mtDNA deletions. (b) Mutational analysis of individual neurons by single-molecule PCR. Each bar represents the fraction of deletions in a single neuron; brown and blue bars represent COX-positive and COX-deficient neurons, respectively. Numbers under the bars correspond to the numbers assigned to individual collected cells, some of which are visible in a, though not all the collected cells fit within the field of view. Error bars represent standard error ($n \geq 3$).

events (as one would expect in the case of a local DNA maintenance defect or increased free radical production), is primarily responsible for accumulation of mtDNA deletions in individual neurons.

In conclusion, here we have provided evidence for direct involvement of mtDNA deletions in the development of COX defects in the aged human substantia nigra. The relationship between these COX defects and aging awaits further investigation. It is worth noting, however, that the incidence of mild parkinsonian signs (MPS), a movement disorder common in the older population (prevalence of 25% over 65 years of age and 50% over 85 years of age) associated with significant morbidity and excessive mortality¹³, is similar to the percentage of aged individuals with an elevated fraction of COX-deficient neurons in substantia nigra (~40% over 80 years old)⁷. COX defects may affect performance of substantia nigra either by rendering the affected neurons dysfunctional via disrupted energy supply or by prompting neuronal death. Either of these end points is expected to predispose to MPS, as MPS have been shown to correlate with decreased neuron counts in substantia nigra¹⁴.

The influence of mtDNA deletions may not be limited to substantia nigra. The putamen and the caudate nucleus^{5,6} have been reported to contain high fractions of the 'common' mtDNA deletion and thus are likely to contain cell types significantly affected by mtDNA deletions. Our preliminary results indicate that respiratory defects in specific neurons from other brain areas are also caused by clonal expansion of mtDNA deletions. Therefore, we believe that a cell type-specific quantitative study of mtDNA mutations in the brain may uncover additional critical areas where aging is driven by mtDNA mutations. These studies are of practical importance, as procedures aimed at alleviating the effects of mtDNA mutations are currently in development (reviewed in ref. 15).

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

The authors are grateful to A. Griner, A. Kraysberg, and A. Vaysburd for help in experiments; E. Richfield (Rutgers University) for tissue samples and critical

review of the manuscript; W. Kunz for communicating his histochemistry protocols and O. Kocher (Beth Israel Deaconess Medical Center) for granting access to a laser capture microscope. This work was supported in part by US National Institutes of Health grants ES11343 and AG19787 to K.K. and AG13846 (Boston University Alzheimer Disease Center) to N.W.K. and the Department of Veteran Affairs.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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