Regional variation in mitochondrial DNA copy number in mouse brain

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A B S T R A C T
Mitochondria have their own DNA (mitochondrial DNA [mtDNA]). Although mtDNA copy number is dependent on tissues and its decrease is associated with various neuromuscular diseases, detailed distribution of mtDNA copies in the brain remains uncertain. Using real-time quantitative PCR assay, we examined regional variation in mtDNA copy number in 39 brain regions of male mice. A significant regional difference in mtDNA copy number was observed (P<4.8×10−35). High levels of mtDNA copies were found in the ventral tegmental area and substantia nigra, two major nuclei containing dopaminergic neurons. In contrast, cerebellar vermis and lobes had significantly lower copy numbers than other regions. Hippocampal dentate gyrus also had a relatively low mtDNA copy number. This study is the first quantitative analysis of regional variation in mtDNA copy number in mouse brain. Our findings are important for the physiological and pathophysiological studies of mtDNA in the brain.

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1. Introduction

Mitochondria play important roles in energy production, apoptosis, calcium signaling, as well as synaptic transmission, and neuroplasticity in neurons [1]. Mitochondrial dysfunction induces various mitochondrial diseases and may also be linked to neurodegenerative disorders, such as Parkinson’s disease (PD), Alzheimer’s disease, and Huntington’s disease [2–4]. These mitochondrial dysfunctions can result from heteroplasmic mitochondrial DNA (mtDNA) mutations, including point mutations and deletions in mtDNA [4,5]. A mitochondrion contains a number of mtDNA copies, and mtDNA depletion also causes mitochondrial diseases [5–10]. It is possible that differences in mtDNA copy number among brain regions lead to association with region-dependent mitochondrial function and disease susceptibility. However, the distribution of mtDNA copies in the brain has not been studied in detail so far.

In the present study, we determined mtDNA copy number in 39 representative brain regions of adult mice using a micropunch technique and real-time quantitative PCR (qPCR). The results demonstrate that mtDNA is enriched in substantia nigra (SN) and the ventral tegmental area (VTA), where dopaminergic neurons are located.

2. Materials and methods

2.1. Animals

Male C57BL/6j mice were kept in the laboratory under light/dark conditions of 12 h:12 h (lights on at 8:00 a.m.). The laboratory was air-conditioned, and temperature and humidity were maintained at approximately 22–23 °C and 50–60%, respectively. From age 20 to 28 weeks, mice were individually housed in cages (24 cm wide×11 cm deep×14 cm high) equipped with a steel wheel (5 cm wide×14 cm in diameter). All animal experiments were performed in accordance with the protocols approved by the Animal Experiment Committee of RIKEN (Wako, Saitama, Japan). All efforts were made to minimize the number of animals used and their suffering.

2.2. Micropunch

Transcardiac perfusion fixation was performed with 4% paraformaldehyde in phosphate-buffered saline (PBS), and then brains were immersed in PBS.

Slices (0.5 mm thick) of mouse brain were generated using a mouse brain matrix (Neuroscience, Tokyo, Japan), and 39 regions (Table 1, Fig. 1) were punched out bilaterally from the fixed slices under a stereomicroscope with a handmade microdissecting needle (gauge 0.5 mm), which has thinner wall than commercially available micropunchers, to pick up adjacent regions from one slice. The anatomical nomenclature is based on the atlas of Franklin and Paxinos.
(Fig. 1) [11]. After overnight incubation of each micropunched region in lysis buffer (1 punch/25 μl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.5% Triton X-100, and 500 μg/ml protease K [Roche Applied Science, Mannheim, Germany]) at 55 °C, samples were incubated at 65 °C over 6 h to de-crosslink, followed by heat inactivation at 96 °C for 10 min and addition of an equal amount of 10 mM Tris–HCl (pH 8.0).

2.3. Real-time qPCR analysis

Real-time qPCR analysis was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Premix Ex Taq reagent (Takara Bio, Otsu, Japan) according to the manufacturer’s instructions. Each PCR product was separately amplified from 2 μl of lysate of micropunched sample in a 10-μl reaction containing 5 μl of 2 × SYBR Premix and 0.2 μM of each primer.

For estimation of amounts of mtDNA in mice, the control region (D-loop) of mouse mtDNA was amplified using the primer pair D1 (5'-CCC AAG CAT ATA AGC TAG TAC-3') and D2 (5'-ATA TAA GTC ATA TTT TGG GAA CTA C-3'). Using this primer set, the thermal cycling protocol used was 95 °C for 20 s, 55 °C for 20 s, 72 °C for 80 s for 30 cycles after an initial denaturation at 95 °C for 1 min.

These real-time qPCRs were carried out in quadruplicate for all measurements. After confirmation of the single major peak representing the specific amplification with the melting curve, the quantities of each PCR product were calibrated by a linear regression model, using standard curves calculated between Ct values and the log of concentrations of standard pcR2.1-TOPO plasmids (Invitrogen, Carlsbad, CA, USA) containing each PCR fragment. In every run, the high linearity ($R^2$>0.99) of the standard curves was verified by amplifications of each PCR product from dilution series of the plasmids made up to 10 ng by pcR2.1-TOPO plasmid. Ct values of all samples were within the linear range.

The relative number of mtDNA copies per cell was calculated as the normalized ratio of D-loop/apoB gene to a median value of 39 brain regions. In all samples examined, PCR products both of the D-loop in mtDNA and of the apoB gene in nuclear genome were amplified within the linear range of assays.

2.4. Statistical analysis

The results of quantitative experiments were analyzed by nonparametric tests after use of the Kolmogorov–Smirnov test for confirmation of a normal distribution. A two-tailed test was used for exploratory analysis. Statistical significance was determined using KyPlot 4.0 (KyensLab, Tokyo, Japan). Relative mtDNA copy numbers in several regions did not show a statistically normal distribution (Kolmogorov–Smirnov test, $P<0.05$). Therefore, we used nonparametric tests for detection of statistical significance among brain regions. To determine detailed significant regional differences in mtDNA copy number, we used the nonparametric multiple comparison Steel–Dwass test ($\alpha=0.05$).

3. Results

The average relative mtDNA copy number in the 39 brain areas was between 0.0847 and 2.54 (Fig. 1). There was a statistically significant regional variation in mtDNA copy number among the 39 brain regions (Kruskal–Wallis test, $P=4.8 \times 10^{-35}$) (Fig. 2). In VTA, the mtDNA copy number (median = 2.62, average ± SD = 2.54 ± 0.80) was significantly higher than in 24 other regions. The relative mtDNA copy number in SN (median = 2.12, average ± SD = 2.23 ± 0.65) was higher than in 19 other regions. Moreover, the intermediate nucleus (IP) showed a significantly higher mtDNA copy number (median = 1.76, average ± SD = 1.86 ± 0.53) than did 14 other brain regions.

In contrast, a significantly lower mtDNA copy number was observed in the anterior cerebellar lobe (Cb-lobe) (median = 0.9695, average ± SD = 0.0847 ± 0.0492) than in most other regions. MtDNA copy number in cerebellar vermis (Cb-vermis) (median = 0.0857, average ± SD = 0.144 ± 0.157) was lower than in 28 other brain regions. In the dentate gyrus (DG), mtDNA copy number (median = 0.321, average ± SD = 0.384 ± 0.196) was significantly lower than in 18 other brain regions.

In addition to these statistical differences, there were significant differences between caudate putamen–medial (CP-m) (median = 0.594, average ± SD = 0.567 ± 0.236) or habenular nucleus (Hb) (median = 0.325, average ± SD = 0.408 ± 0.284) and anterior amygdaloïd complex–lateral (A Amy-l) (median = 1.31, average ± SD = 1.36 ± 0.48) or motor cortex–intermediate layers (Motor-in) (median = 1.31, average ± SD = 1.33 ± 0.40). No significant differences were detected between other brain regions.
4. Discussion

In this study, we first quantitatively demonstrated that there is a large regional variation in mtDNA copy number in the brain. Study results could be affected by regional variation in the neuron/glia ratio, if there is a difference in mtDNA copy number between neuron and glia. An *in situ* mtDNA hybridization study showed stronger signals from gray matter than from white matter in striate cortex of primates [12], and an *in situ* PCR study also showed stronger signals from neurons than from glial cells [13]. However, a study using qPCR analysis showed no statistically significant difference in mtDNA copy number between gray matter and white matter of human postmortem brains [14]. In addition, the mtDNA copy number in corpus callosum, containing mostly glial cells, was comparable to that in other brain regions in this study. The results of these quantitative analysis studies suggest that the 30-fold difference in mtDNA copy number between VTA and cerebellum cannot be solely accounted for by regional variation in neuron/glia ratio.

The highest mtDNA copy numbers were observed in VTA and SN, two major midbrain nuclei containing dopaminergic neurons. This finding might be relevant to the fact that mitochondrial dysfunction and mtDNA mutations are implicated in PD, caused by degeneration of dopaminergic neurons of SN [2,3,15]. PD is also a frequent complication of mitochondrial diseases [4,5]. Degeneration of VTA neurons is reported to be associated with depression [16], which is also a frequent comorbid condition of mitochondrial disease [5]. It remains unclear why VTA and SN contain particularly high copy number of mtDNA. It is plausible that vulnerability of mtDNA to oxidative stress resulting from the metabolism of dopamine in these two nuclei may be relevant to the present finding [17]. Meanwhile, it has recently been shown that high mtDNA copy number induces increased mtDNA deletion formation, which might be caused by increased mtDNA replication [18]. If high mtDNA copy number in SN indicates faster turnover, it might be relevant to the fact that SN is particularly susceptible to defects of the clearance of damaged mitochondria, due to mutations of Parkin and PINK1 [5,19,20].

On the other hand, cerebellar vermis and lobes had particularly low levels of mtDNA. The significantly lower mtDNA copy number in cerebellum is consistent with other reports that cerebellum showed lower level of mtDNA copies than several brain areas of rat or human [21,22]. A number of mitochondrial diseases associated with mtDNA depletion and mutations affect the cerebellum [4,5,23]. Although it is still unclear that both the regions with high mtDNA copy number such as SN and those with low copy number such as cerebellum are relevant to mitochondrial diseases, it would coincide with the fact that both depletion and increased copy number of mtDNA are reported to cause mitochondrial dysfunction [8,18]. It seems reasonable that mutation of
mtDNA in smaller copy number can affect the mitochondrial function in the brain regions having lower copy number of mtDNA.

In the present study, we fixed the tissues with 4% paraformaldehyde, because it is difficult to capture more than one region at equal conditions from a frozen or fresh brain section due to its fragility. This approach also allowed us to identify brain structures clearly; we were able to pick up all brain regions from one animal at once. Moreover, we confirmed that paraformaldehyde fixation did not affect the linearity of quantification of each PCR product ($R^2=0.91$, Supplementary Figure 2) and relative amount of mtDNA per nucleus can be compared among brain regions using fixed samples. By introducing de-crosslinking, we could measure mtDNA copy number. This methodology made it possible to describe the distribution in detail.

Our findings on regional variation in mtDNA copy number in the brain provide important information on regional differences in vulnerability to mitochondrial dysfunction.

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