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Article type : Regular Paper

Mitochondrial complex IV mutation increases ROS production and reduces lifespan in aged mice

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Brief title: **Complex IV mutation, ROS and lifespan**

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/apha.13214

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ABSTRACT

Aim: Mitochondrial DNA (mtDNA) mutations can negatively influence lifespan and organ function. More than 250 pathogenic mtDNA mutations are known, often involving neurological symptoms. Major neurodegenerative diseases share key etiopathogenetic components i.e. mtDNA mutations, mitochondrial dysfunction and oxidative stress.

Methods: Here, we characterized a conplastic mouse strain (C57BL/6J-mtNOD) carrying an electron transport chain complex IV mutation that leads to an altered cytochrome c oxidase subunit III. Since this mouse also harbors adenine insertions in the mitochondrial tRNA for arginine, we chose the C57BL/6J-mtMRL as control strain which also carries a heteroplasmic stretch of adenine repetitions in this tRNA isoform.

Results: Using MitoSOX fluorescence, we observed an elevated mitochondrial superoxide production and a reduced gene expression of superoxide dismutase 2 in the 24-month-old mtNOD mouse as compared to control. Together with the decreased expression of the fission-relevant gene *Fis1*, these data confirmed that the ageing mtNOD mouse had a mitochondrial dysfunctional phenotype. On the functional level, we could not detect significant differences in synaptic long-term potentiation, but found a markedly poor physical constitution to perform the Morris water maze task at the age of 24 months. Moreover, the median lifespan of mtNOD mice was significantly shorter than of control animals.

Conclusion: Our findings demonstrate that a complex IV mutation leads to mitochondrial dysfunction that translates into survival.

Keywords: complex IV mutation, FIS1, lifespan, mitochondrial DNA, ROS, SOD2

INTRODUCTION

Mitochondria represent more than just the major energy production site of our cells. They play a crucial role in cell homeostasis[1], apoptosis[2], cellular stress responses[3], nuclear gene expression[4], immune response[5] and even synaptic transmission[6]. Their genome (mitochondrial DNA; mtDNA) encodes for critical mitochondrial components, i.e. genes coding for oxidative phosphorylation protein subunits, tRNAs and rRNAs[7]. Mutations in mtDNA reportedly cause mitochondrial dysfunction and disease phenotypes. Already decades ago, first reports showed that point mutations / deletions in mtDNA can provoke severe diseases, e.g. Leber's hereditary optic neuritis and mitochondrial myopathy, in humans[8,9]. Since then, heritable pathologies have no longer been exclusively attributed to the nuclear genome. A number of mtDNA variants / mutations have also been associated, for example, with susceptibility to common diseases like type 2 diabetes[10]. As the brain, being to a large extent post-mitotic, is particularly vulnerable to mtDNA mutations[11] and since mitochondrial dysfunction may be due to a higher rate of clonal expansion of mutated mtDNA[12], it is not surprising that mtDNA dysfunction can be identified in some of the most prevalent neurological disorders among adults[13, 14]. The pathogenesis of Parkinson's disease (PD), for instance, is associated with mtDNA deletions[15] and mitochondrial dysfunction[16]. PD, Alzheimer's disease[17] and amyotrophic lateral sclerosis[18] are major neurodegenerative diseases which share key components of their etiopathogenesis with regard of either mtDNA mutation, mitochondrial dysfunction or oxidative stress[19]. Furthermore, a dysmorphic mitochondria have been described as a biomarkers of neurological disease in general[20], and it is thought altered mitochondrial morphology to be linked to detrimental mitochondrial dynamics - i.e. fission and fusion[21, 22].

The diversity and high frequency of mtDNA mutations highlights the necessity of investigating the genetics of mitochondrial dysfunction in depth[23]. The progression of mitochondrial disease is known to vary, depending on the level of heteroplasmy, causing more severe characteristics under outbalance of mutant mtDNA[24, 25]. Possibly, there are also other mechanisms influencing the gap between genotype and phenotype, among which heteroplasmic transcriptional reprogramming was proposed[26].

Remarkably, most of the mtDNA point mutations described apply to mitochondrial tRNA mutations[27]. Point mutations in mitochondrial tRNA genes have been shown to modulate mitochondrial function, and have been linked to several syndromes and grave pathological conditions (e.g. myopathy, encephalomyopathy, ataxia and multi-organ failure)[28]. Specifically, a point mutation in the gene coding for mt-tRNA(Asp) is associated with multisystemic mitochondrial disease[29]. Further, mutations in mt-tRNA(Arg) were reported to result in mitochondrial encephalomyopathy[30, 31]. Lastly, a mt-tRNA(Lys) mutation was related to mitochondrial dysfunction associated with myopathy and exercise intolerance[32].

However, these insights from human diseases emerge from case studies, tracing human phenotypes to mutations of mtDNA (top-down). There are only few studies, however, taking the inverse approach, i.e. to follow up particular mutations to establish their impact on pathogenicity (bottom-up), although

initial whole exome sequencing studies in patients have been undertaken which identified around 1% of mtDNA mutations in neurological patients[33]. Most bottom-up animal studies on mtDNA mutations, in turn, use the mtDNA mutator mouse model, expressing a proofreading deficient version of the nucleus-encoded catalytic subunit of mtDNA polymerase-γ (PolgA). This model accumulates increasing levels of somatic point mutations in mtDNA during its lifetime[34], somewhere in the mitochondrial genomes. In the current study, we take a different approach by characterizing conplastic mouse models, carrying stable point mutations in mitochondrial genes. Importantly, these models carry defined, non-accumulating mutations during their whole life, which is essential, given that the rate of ageing and related phenotypes may be set early in life[35].

Since mtDNA mutations affect oxidative phosphorylation[36-40], we wanted to characterize the conplastic mtNOD mouse with respect to reactive oxygen species (ROS) production and mitochondrial dynamic network formation known as fusion-fission processes. Then, we asked for functional consequences of mitochondrial dysfunction in this model by assessing learning and memory as well as survival.

RESULTS

Aged mtNOD mouse brains harbor elevated superoxide levels

In the present study, we addressed the question whether alterations of mitochondrial DNA (mtDNA), especially when related to the electron transport chain (ETC), could be associated with changes of reactive oxygen species (ROS) production. Here, we characterized the mtNOD mutated mouse carrying a conplastic complex IV mutation leading to an amino acid exchange of the cytochrome c oxidase. To directly quantify mitochondrial superoxide levels in the brain, we performed fluorescence microscopy of MitoSOX-stained sections.

Fig. 1B shows nuclei from neocortical control sections stained with DAPI (blue color in Fig. 1B). In addition, MitoSOX Red-positive fluorescence is occasionally visible in direct vicinity of these nuclei (red puncta in Fig. 1B). To quantify MitoSOX-staining, we calculated the percentage of the mean fluorescence intensity (MFI) of MitoSOX Red by the MFI of DAPI (Fig. 1A). Thus, we obtained 108.4 ± 2.3% MitoSOX Red in 12-month-old controls (n=5) and 108.5 ± 1.7% in 24-month-old controls (n=5, open box plots in Fig. 1A) indicating that superoxide levels in control animals, relative to cell counts, did not change from the age of 12 months to the age of 24 months. In contrast, brain sections from mtNOD mice exhibited a significant rise in superoxide levels (+ 13.5%) during aging (red puncta in Fig. 1C). While the superoxide level at the age of 12 months (105.0 ± 2.1%, n=5; gray box plots in Fig. 1A) was not distinguishable from control values, the superoxide level at the age of 24 months (118.5 ± 2.5%, n=5) was significantly elevated compared to control sections of the same age ($p < 0.05$, Mann-Whitney U-test) as well as to mtNOD sections from 12-month-old mice ($p < 0.01$, Mann-Whitney U-test, Fig. 1A).

Control, but not mtNOD mutated mice show increased glial fibrillary acidic protein (GFAP) level at 24 months

Regarding the impact of the mtNOD mutation in the brain we furthermore explored the effect of mutation on astrocytic proliferation respectively activation using glial fibrillary acidic protein (GFAP) as a marker. As astrocyte activation is characterized by increased expression of GFAP, we quantified the amount of GFAP in comparison to NeuN (neuronal nuclei) in the hippocampal cell layers CA1 (cornu ammonis area 1), CA3 (cornu ammonis area 3) and DG (dentate gyrus). Interestingly, GFAP expression was increased in control tissue more permanently during aging (12 to 24 months) than in mutated mtNOD (2.9 fold increase in control mice, 1.7 fold increase in mtNOD mice)(Fig. 2), perhaps suggesting reduced reactivity of mtNOD astrocytes.

Superoxide levels in the mtNOD brain are associated with reduced SOD2 gene expression

To obtain more detailed information on age-related mitochondrial changes, we analyzed the gene expression of proteins pivotal in ROS defense as well as in mitochondrial dynamic network formation commonly referred to as fusion and fission (Table 2). Catalase, the cytosolic enzyme involved in hydrogen peroxide degradation showed profoundly increased levels in 24-month-old animals compared to 12-month-old tissue, but without significant differences between the two mouse strains ($p < 0.01$ versus 12-month-old animals for both mouse strains, Mann-Whitney U-test; Fig. 3A). We further analyzed the gene expression of the cytosolic proteins superoxide dismutase 1 (*Sod1*) and glutathion peroxidase (*Gpx*), but did not observe any significant changes (Fig. 3A).

With respect to gene expression of mitochondrial proteins, we found a significant reduction of superoxide dismutase 2 (*Sod2*) transcripts in aged mtNOD mice, while this gene tended to be higher in aged control mice ($p < 0.01$ versus control, Mann-Whitney U-test; Fig. 3B). These data indicate that the elevated ROS levels found in aged mtNOD mouse brains might have partly been mediated by lower *Sod2* levels within the mitochondrial compartment.

Mitochondrial dynamic network formation may be altered in the mtNOD brain

Mitochondrial function may not only be assessed by determining ROS production, but also attributable to the dynamic network formation commonly referred to as fusion and fission. Therefore, we also analyzed the transcription of a number of genes involved in these processes. As shown in Fig. 4A, all three genes involved in mitochondrial fusion (Dynamin-related GTPase Optic atrophy 1, *Opa1*; mitofusin 1 and 2, *Mfn1* and *Mfn2*, respectively) were less expressed in mtNOD mice, but without reaching statistical significance. Importantly, regarding genes related to fission, we found a significantly reduced expression of fission 1 (*Fis1*, $p < 0.05$ versus control, Mann-Whitney U-test; Fig. 4B). These data suggest that genes involved in mitochondrial dynamic network formation may be

partly altered in mtNOD mice, and that this altered expression might contribute to the mitochondrial dysfunction in mtNOD tissue. In addition, we tested further mitochondrial genes involved in mitochondrial transcription and post-translational modifications (data not shown). Except *Tfam*, which was also less expressed in mutated mice, we did not obtain altered expression levels in brain tissue from mtNOD (Table 2).

As increased ROS levels might have an impact on mitochondrial stability and network formation, we next analyzed mitochondrial morphology in 24-month-old mice with MitoTracker Deep Red. Abnormal mitochondrial network homogeneity can be detected by accumulation points of MitoTracker Deep Red fluorescence, which mark abnormal lumping of mitochondria[39]. We quantified pixels over threshold from MitoTracker fluorescence pictures. As expected, we found some higher values in mtNOD brain tissue ($17.7 \pm 1.1 \times 10^5$ pixels, n=5) as compared to controls ($16.0 \pm 1.0 \times 10^5$ pixels, n=5, Fig. 4C), but the difference was not significant.

Learning and memory is not impaired in mtNOD mice

Since mtNOD brain tissue shows a mitochondrial dysfunctional phenotype, we next asked whether mice with this mutation might show altered learning and memory. To this end, we first tested synaptic long-term potentiation (LTP) which is thought to reflect learning mechanisms on the cellular network level. Based on our data on ROS production assessed by MitoSOX staining of superoxide in the tissue, we expected that LTP should be normal until the age of 12 months. Indeed, theta-burst stimulation of CA1 pyramidal cells via Schaffer-collateral activation induced robust LTP in both control (146 ± 7%, n=5) and mtNOD tissue (148 ± 18%, n=7; Fig. 5B). Surprisingly, LTP values did not change at the age of 24 months (Fig. 5A and B), indicating that LTP is unaffected by ROS production or – more likely – that other mechanisms fully compensate for the ROS-mediated effects and thus rescue LTP at this synapse. Interestingly, regarding I/O curves (Fig. 5C and D) these were significantly increased both in 12 and 24 months mtNOD preparations compared to controls, indicating a functional increase in excitability in the hippocampus, without however, effecting plasticity. In line with this, also the paired-pulse ratio (PPR; i.e. amplitudes of fEPSP2 / fEPSP1) were smaller in mtNOD tissue; i.e. 1.42 ± 0.05 (n=5 animals; 24 months) and 1.64 ± 0.35 (n=3; 12 months) in controls and 1.11 ± 0.02 (n=9; 24 months) and 1.19 ± 0.04 (n=7; 12 months) in mtNOD. This difference was significant in the aged group ($p<0.05$ ANOVA on Ranks with Dunn's Post-Test). Both increased I/O cureves and apparent increase in release probability as evidenced by PPR changes may indicate that some compensatory mechanism is occurring to rescue or maintain function by raising excitability.

Although mitochondrial disorders correlate with cognitive dysfunction[41], LTP at a single synapse may not be a strong biomarker of a cognitive phenotype within the living animal, due to compensatory regulation. To explore cognitive function further, we tested the spatial memory of the mtDNA conplastic mouse strain in the Morris Water Maze (MWM) at 12 and 24 months of age (Fig. 6). We

recorded the swimming distance from the insertion point to the platform on seven consecutive days in order to quantify memory acquisition. Consistent with the MitoSOX and the LTP data, 12-month-old mice did not show differences in MWM learning performance (Fig. 6A). Even though 12-month-old mutant mice had a significantly lower body weight than control mice (Fig. 7B), there was no difference in swimming speed (Fig. 6B) between the two strains. Also the following probe trial without the platform did not disclose differences in spatial memory of the 12-months-old groups. Both strains showed equal spatial memory retention (i.e. spent more time in the target quadrant) (Fig. 6C). In marked contrast, 24-month-old mtNOD mutated mice were regularly unable to complete the MWM task (i.e. stopped active swimming and were in danger of drowning), suggesting poor physical constitution in this strain and precluding cognitive performance comparison among the old age groups. Only one out of ten mice was able to complete all trials of the paradigm, while this rate was inversed and hence significantly higher in controls (8 out of 10, $p < 0.01$, Fisher's exact test, Fig. 6D).

The lifespan of mtNOD mice is significantly shorter

As mitochondrial dysfunctions are heavily implicated in the ageing process[42], we were interested to explore the association of the ETC complex IV mutation with lifespan. A total of 39 mice (19 males, 20 females) carrying the mtNOD mutation (Table 1) were compared to 43 control mice (21 males, 22 females), and the survival curves for both strains are presented in Fig. 7. The median lifespan of mtNOD mutated mice of 803 days (95% C.I. of 759 to 847 days) was significantly shorter than the median survival in control animals (median 855 days, 95% C.I. of 790 to 920 days, $p < 0.001$, Log rank test, Fig. 7 and Table 3). These data indicate that mtNOD mutation leading to altered cytochrome c function impairs survival.

DISCUSSION

Large-scale mtDNA defects are widely known to have a negative impact on lifespan and tissue integrity. The present study asks the question whether also relatively isolated and specific point mutations in the mitochondrial genome can have observable impact on global life parameters such as lifespan and cognitive function. One can readily expect that mutations in genes of the mitochondrial respiratory chain responsible for oxidative phosphorylation can cause mitochondrial dysfunction[43]. In addition, mitochondrial tRNA alterations also seem to mediate mitochondrial dysfunction, keeping in mind that genes coding for tRNAs represent only 10% of the total mtDNA sequence, but are responsible for half of mtDNA mutational diseases[44]. Here, we examined the effect of single nucleotide polymorphism (SNP) in the complex IV gene in the mtNOD mouse and controlled for the adenine repetitions in the tRNA(Arg) found in this mouse by using the mtMRL strain.

The phenotype of the mtNOD mouse

One in vitro parameter, for which mtNOD mice differed in our study, was brain superoxide which showed a strong increase in 24-month-old mice. The other in vitro parameter, for which mtNOD mice differed, was the reduced *Sod2* expression. SOD2 is considered to be a mitochondrial superoxide scavenger, but also, and perhaps even more importantly, its major function is related to redox signalling mechanisms[45]. Therefore, the reduced *Sod2* expression in mtNOD tissue is expected to aggravate the superoxide-related challenge in these mice. Moreover, why did we find higher GFAP levels in the aged mice of the control strain and not in mtNOD? While this is a reactive sign, astrocyte activation seems to be important to maintain or retain brain integrity in the acute phase of traumata and oxidative stress[46]. Thus Daverey and Agrawal[47] showed astrocyte activation after mild oxidative stress and concluded the upregulation of GFAP to be an initial response mechanism of astrocytes to protect themselves under stress conditions. So the increased GFAP level might be a first sign, and the enhanced *Sod2* expression a first defence mechanism, for a challenge to hippocampal function in these mice. However this was not the case in mtNOD mice, perhaps suggesting reduced reactivity of mtNOD astrocytes or a somehow dysregulated redox signalling.

But how can superoxide elevations contribute to the observed phenotype of impaired ability to perform an exercise such as the water maze task? As some of the neocortical functions (mainly sensory perception, generation of motor commands and spatial reasoning)[48] are essential for the water maze task, changes in superoxide content might destabilize the underlying cellular mechanisms, as balanced superoxide levels are known to be important for cellular function[49, 50]. In addition to this, one cannot exclude that peripheral effects on other organ systems harboring the same mutation contributed to reduced exercise ability.

In the present study, we also obtained evidence that the fusion-fission processes are altered in mtNOD mice. *Fis1* was significantly down-regulated in aged mtNOD mice, and other genes such as *Opa1*, *Mfn1* and *Mfn2* showed lower expression levels in these animals. Although the differences for each of the fusion-associated genes were not statistically significant, most probably due to the limited sample size, it is worth to note that there was no gene expressed at higher levels in the mutant mtNOD tissue. In particular, reduced *Mfn2* expression correlated with changes in mitochondrial network formation, since aged mtNOD mice tended to have enhanced network inhomogeneity in hippocampal and neocortical cells. This finding is in line with recent evidence that *Mfn2* not only mediates mitochondrial outer membrane fusion, but also has additional functions potentially required to sustain cellular energy demand[51]. Thus, a set of *Mfn2* knockout mice, developed by Chen *et al.*[52], are either lethal early postnatally (complete KO), or show severe tissue-specific changes with only partial loss of *Mfn2*. Importantly, the loss of *Mfn2* has been linked to the loss of complex IV activity[52] which indicates a link between the ETC and *Mfn2*. Together, we conclude that there is at least a partial dysfunction in the mitochondrial dynamic network formation in mtNOD mice.

With respect to learning and synaptic plasticity, we had expected to see a correlation between spatial learning and in vitro parameters of hippocampal cell alterations, e.g. superoxide levels, with synaptic plasticity (measured as LTP) as the link in between. Puzzlingly, LTP was not distinguishable between mtNOD mice and controls at both ages tested. How can the lack of ROS effect on LTP be explained? The hippocampal formation has been identified for many years as a key structure in consolidation of spatial memory[53]. LTP, initially established in vitro by Andersen and Lømo[54], was suggested to be one main cellular mechanism of learning[55] because this postsynaptic plasticity form shows stable enhancement of synaptic transmission after many hours[55, 56]. ROS was shown to play an important role in the expression, but also the suppression of LTP[49]. Our results, a lack of differences in LTP together with reduced lifespan and exercise ability, may be in agreement with the so called 'Red Queen Theory' which was initially put forward by Agnati *et al.*[57] in 1992: Neuronal plasticity is not only regarded as the basis for learning and memory, but also important for the compensatory responses to degenerating processes during ageing. If there is a maximal capability of neuronal plastic responses, this capacity will have to be allocated both to store new information and to compensate for degenerating processes to face continuously changing demands. During ageing this capability declines until 'pathological ageing' will be the final outcome of a reduction of plasticity or to an enhancement of the degenerative processes. Thus, a lack of differences in LTP may support a compensatory mechanism preserving for learning and memory, while another fails to protect against degenerative processes leading to reduced exercise ability and finally to reduced lifespan. Fittingly, an increasing number of studies show that indeed the link between learning behavior and LTP is far from being direct. Thus, a review by Lynch[58] lists both, studies confirming correlations between cognitive ability and LTP, and others dismissing them. An example for such dissociation between learning performance and LTP was described by Huang *et al.* [59] for a mouse model of ageing, where in fact reduced learning performance was associated with increased LTP. We suggest that ROS might exert concurring effects on synaptic plasticity, thereby blurring the relationship between in vitro LTP and in vivo learning behavior.

Relevance of complex IV mutation in the mt-tRNA(Arg) background

As our study shows, the mtNOD mouse shows clearly reduced lifespan and exercise ability in the course of ageing. However, this mouse harbors two genetic alterations, on the one hand the complex IV mutation and on the other hand repetitive adenines in the mt-tRNA(Arg) gene. Since mt-tRNA mutations are reported to be related to mitochondrial dysfunction associated exercise intolerance[28, 32], we chose the mtMRL strain also having repetitive adenines in the mt-tRNA(Arg) to compensate for this tRNA mutation. Thus, the differences we obtained can be attributed to the complex IV mutation which seems to be pathophysiologically relevant.

What is known about complex IV mutation and lifespan? As there is no existing mouse model carrying only the SNP at nt9348 leading to the alteration of cytochrome c oxidase subunit III, a real control to

this strain is lacking. Nevertheless, from other mouse models harboring a respiratory chain mutation, i.e. a complex IV deficiency, we know a wide range of different phenotypes[43]. Radford *et al.*[60] developed a mouse model in which the nuclear encoded subunit *CoxVIaH* was knocked out: *CoxVIaH*$^{-/-}$ mice displayed a reduction in complex IV activity to about 23 % of the controls in cardiac tissue. Interestingly, these mice were viable and had normal lifespan. SURF1, in turn, is a protein known to participate in the early assembly steps of complex IV, and mutations in *Surf1* cause severe neurological diseases in humans such as Leigh Syndrome, a disorder usually resulting in early lethality. *Surf1* knockout mice, generated by Dell'agnello *et al.* [61], showed mild complex IV deficiency (30-40 % of control values) in brain, heart, liver and skeletal muscle. Surprisingly, these mice were phenotypically normal with even enhanced memory performance and a prolonged lifespan (5 months longer than control). *Sco2* encodes a chaperone that is required for assembly and function of complex IV. Whereas most *Sco2* deficient humans die in infancy, Yang *et al.*[62] created two mouse models (*Sco2KI/KI* and *Sco2KO/KI*) that showed complex IV deficiency in heart, muscle, brain and liver and also an accumulation of complex IV assembly intermediates in brain and liver, but no reduction of lifespan. One important exception of a complex IV deficiency with reduced lifespan is the *Cox10* knockout mouse. The product of the *Cox10* gene catalyzes the first step of the biosynthesis of heme A, an essential prosthetic group for the function of complex IV. Diaz *et al.*[63] developed a set of tissue-specific *Cox10* knockout mice, e.g. the *Cox10-Mlc-1f* KO (a myopathy model). These mice have a severe complex IV deficiency in skeletal muscle (13 % of residual activity) progressing with age and a very early death at about 6 months. As another exception, *Cox10-CaMKIIa* knockout mice serve as an encephalomyopathy model, because these animals develop a progressive complex IV deficiency which is restricted to forebrain structures[64]. They show behavioral abnormalities and severe cortical atrophy which results in premature death between 10 to 12 months of age. Thus, while most humans with complex IV mutations show reduced lifespan, only some mouse models share this phenotype. In this regard, it is important to note that our mtNOD mouse with a complex IV mutation has enhanced ROS levels and reduced lifespan.

Taken together, we conclude that it is the complex IV mutation which entails a shorter-lived phenotype with poor physical performance at old age. Future studies could address the question whether the detrimental effect of this mutation on lifespan and exercise ability is due to an early onset, corresponding to early life effects as discussed by Ross *et al.* [35], or to a breakdown just in time with the occurrence of the phenotype at the age of 24 months, such as an accumulation over lifetime that destroys a system balance when a certain threshold is exceeded.

MATERIAL AND METHODS

Animal housing and care

All experiments were performed according to the guidelines of the local animal use and care committee, which also approved this study (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, permit number: 7221.3-1.1-059/12; Animal Care and Use Committee Lübeck, permit number: V242-7224. 122-5, Kiel, Germany). Animals were housed in groups up to 4 animals per cage. Cages were equipped with nesting material and red polycarbonate houses (i.e. environmentally enriched conditions). Animals were kept under stable surroundings (room temperature 23 ± 2 °C, relative humidity 40 ± 5 %, day-night rhythm with illumination 6 a.m. – 6 p.m.). Water and food were available ad libitum. For experiments, animals were taken from the housing unit in randomized fashion to reduce systematic bias.

Conplastic mouse strains

Conplastic mouse strains were generated as described previously[65]. The C57BL/6J-mt$^{NOD/LtJ}$ (referred to as mtNOD) carries an electron transport chain (ETC) complex IV mutation at mt.9348 (9348 G>A) in mt-Co3 that leads to an alteration of cytochrome c oxidase subunit III (Val-Ile exchange) (Table 1). Additionally, there is an adenine insertion at mt.9821 in the dihydrouracil (DHU) loop of mitochondrial tRNA-Arg[66]. To analyze the effect of the complex IV mutation, a control strain was needed that also carries the tRNA mutation. Therefore, the C57BL/6J-mt$^{MRL/MpJ}$ (referred to as control) strain was chosen which carries a heteroplasmic stretch of adenine repetitions at mt.9821. As described by[67] the poly(A) tract in the DHU arm of the tRNA(Arg) varies, where the prevailing variants are 10, 11 and 12 A. Checking for age-related changes by sequencing the mitochondrial genome revealed an additional heteroplasmic T to C mutation in mt.3900. This effects mitochondrial tRNA(Met) and was predicted to increase the length of the tRNA stem and decrease the size of the single stranded TΨC loop but not to change tRNA stability[67]. Heteroplasmy at this site ranged from 41 % to 87 %, with an average of 59 %, and did not change with age. As Chomyn *et al.*[68] described a 90 % threshold level for tRNA mutations to cause respiratory chain dysfunction, it seems rather unlikely that this mutation contributes much to the phenotype of control strain.

Preparation of brain slices for electrophysiology and staining

Mice were deeply anesthetized via diethyl ether inhalation (Mallinckrodt Baker, Deventer, Netherlands) and thereupon decapitated. The brain was quickly removed and transferred into chilled and oxygenated (carbogen 95% O_2 / 5% CO_2) dissection solution (87 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 10 mM D-glucose, 75 mM sucrose adjusted

to pH 7.4 and 326-328 mosmol/l H_2O). The brain was divided into hemispheres and glued to a vibratome (Integraslice 7550 MM, Campden Instruments Ltd., UK). Slices were prepared in chilled and oxygenated artificial cerebrospinal fluid (aCSF) - plane: transversal for hippocampal formation (400 µm) and coronal for neocortex (500 µm) respectively. ACSF was comprised of 124 mM NaCl, 26 mM $NaHCO_3$, 3 mM KCl, 1.25 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 10 mM D-glucose adjusted to pH 7.4 and 304-312 mosmol/l H_2O. After preparation, slices were transferred into a submerged-type ACSF-storage chamber for maintenance and kept for 1.5 hours, before starting recordings and staining procedures.

Quantification of mitochondrial superoxide

Quantification of mitochondrial superoxide was done as described before[69]. Acute brain slices (400 µm thick transversal slices for hippocampal formation and 500 µm thick coronal slices for neocortex respectively) were incubated in 1 µM MitoSOX Red (Life Technologies, Darmstadt, Germany) in oxygenated aCSF for 15 minutes at room temperature and protected from light. After washing for 5 minutes in aCSF, slices were directly fixed in 3.7% paraformadehyde, cryo-protected with 30% sucrose in 1 x phosphate buffered saline (PBS) overnight and frozen. Probes were cut into 10 µm slices, counterstained and mounted with ProLong Gold Antifade Reagent containing DAPI (Life Technologies). Quantifying analysis of ROS levels relative to nucleic area stained with DAPI were performed by confocal laser scanning microscopy (Fluoview FV10i, Olympus) with constant laser and sensitivity positions. Ten slices of each animal with six ROIs each (two ROIs from three different regions) were analyzed. In hippocampal formation ROIs were taken in cell layers CA1 (Cornu Ammonis area 1), CA3 (Cornu Ammonis area 3) and DG (dentate gyrus). In neocortical slices, cell layers I / II, III / IV and V/VI were analyzed. To increase signal to noise ratio and statistical power, values for cortex and hippocampus were pooled.

GFAP to NeuN-ratio

Hippocampal slices were fixed in 3.7% paraformaldehyde, cryo-protected with 30% sucrose in 1 x PBS at 4 °C overnight and frozen. Probes were cut into 20 µm slices, blocked with 10% bovine serum albumin, 0.05% Triton X-100 for 1 h at room temperature and incubated with rabbit polyclonal anti-NeuN antibody (1:400; 1 h at 37 °C; Abcam (Cambridge, UK)). Sections were washed three times in PBS for 15 min and exposed to mouse monoclonal anti-GFAP antibody (1:400; 1 h at 37 °C; Abcam). Washing was followed by application of secondary fluorescent-labeled antibodies Cy3 goat anti-rabbit IgG and Cy5 goat anti-mouse IgG (1:2000; 1 h at 37 °C; Thermo Fisher Scientific, Waltham, USA). Quantification was conducted with fluorescence microscopy (Leica DMI 6000B (Wetzlar, Germany)). Values are composed of samples from five animals per strain. Two slices of each animal with three pictures each were analyzed.

Gene expression analysis

Gene expression analysis was done as described before[39]. A sample of 30 mg of brain tissue (whole brain) was homogenized in Lysing Matrix D tubes with a Fast PrepTM homogenizer (MP Biomedicals, Solon, USA). RNA was isolated and purified using an RNeasy® Mini Kit (Qiagen, Hilden, Germany). The Maxima™ First Strand cDNA synthesis kit for RT-qPCR (Thermo Scientific, Darmstadt, Germany) was used to synthesize cDNA. For real-time PCR, cDNA solutions containing TaqMan Universal Master Mix (Applied Biosystems, Darmstadt, Germany) and the corresponding TaqMan® Gene Expression Assay (Applied Biosystems) of primer and gene probe (Table 2) were amplified and detected using a PikoReal Real-Time PCR System (Thermo Scientific). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a housekeeping gene for nuclear-encoded genes. Gene expression values were calculated from the Cq values in the Thermo Scientific PikoReal Software 2.1 (Thermo Scientific).

Quantification of mitochondrial network structure

Acute brain slices (400 µm thick transversal slices for hippocampal formation and 500 µm thick coronal slices for neocortex respectively) were handled as described above. Probes were cut into 10 µm slices, stained with MitoTracker Deep Red (Molecular Probes) in 1:20000 dilution for 30 min at room temperature and protected from light. Slices were counterstained and mounted with ProLong Gold Antifade Reagent containing DAPI (Life Technologies). Quantifying analysis was performed by confocal laser scanning microscopy (Fluoview FV10i, Olympus) with constant laser and sensitivity positions. The inhomogeneity of the mitochondrial network structure was determined with MitoTracker Deep Red fluorescence intensity as described before[39]. The amount of pixels with a higher intensity than a defined threshold value was measured using ImageJ 1.51k quantification software. Values are composed of samples from five animals per strain. Four slices per animal with three pictures each were analyzed. Again, to increase signal to noise ratio and statistical power, values for cortex and hippocampus were pooled.

Spatial learning (Morris Water Maze)

Assessment of spatial learning abilities as a paradigm of cognitive function took place in an open-field variant of the Morris Water Maze (MWM) as described before[69]. Testing occurred in a separate room with stable temperature (air: 22 ± 1 °C, water: 21 ± 1 °C), brightness (110 lux) and minimal noise. For habituation mice were housed in the testing room 2 days prior to MWM experiments. They were selected randomly and all MWM experiments were done blinded to reduce bias.

Mice were checked for their general state of health. Only animals in good general condition (generally healthy and free of open wounds) were used for the Morris Water Maze task. As visual ability is essential for spatial learning in the MWM, mice were additionally checked for sight disorders[70] and if so, excluded. For MWM experiments visible cues (black geometrical symbols; 0.5 x 0.5 m) were attached to each wall. Mice had to reach a hidden platform (ø 7.5 cm) to escape the tank (ø 1.1 m) filled with opaque water. A submerged platform (target), approximately 1 cm below the surface of water was placed in a specific quadrant. Mice were randomly released from eight starting locations. After first day of habituation each mouse was tested six trials per day, seven consecutive days. Each trial had a time limit of 60 s, followed by 30 s remaining upon the platform and resting in a neutral cage for 60 s. Mice were gently guided to platform by hand, in case of failing to reach the target within time limit. Testing started at 09:00 a.m. each day. Tracks were recorded with a camera for subsequent data analysis with the software Etho Vision 3.1 (Noldus, Netherlands). Following MWM a probe trial was done on day 8. The platform was removed and mice were allowed to search for it during 2 trials (30 s each).

Field potential recordings for analysis of synaptic plasticity

Evoked field potential recordings were performed as described before[69] in an interface-type recording chamber (BSC-BU, Harvard Apparatus Inc., U.S.A.). During recording, slices were perfused with pre-warmed (Haake C10, Electron Corporation GmbH, Germany) and oxygenated (carbogen, 95% O_2 / 5% CO_2) ACSF with a continual flow of 2-3 ml/min (Perimax, Spetec GmbH, Germany). The temperature of the fluid in the recording chamber was kept constant at 32 ± 1°C (TC-10, npi electronic GmbH, Germany). Schaffer collaterals were stimulated with a bipolar platinum electrode (PT-2T, Science Products GmbH, Germany). Stimulation was controlled by a Master-8 pulse stimulator (A.M.P.I., Jerusalem, Israel) connected to a stimulus isolator (A365, WPI Inc., USA), applying a paired-pulse protocol with 40 ms inter-pulse interval (IPI) and an inter-stimulus interval (ISI) of 30 s (0.033 Hz). Baseline stimulation strength was determined generating input-output curves until saturation of amplitude of field excitatory postsynaptic potentials (fEPSP); for further stimuli, intensity was reduced to half-maximum intensity (12 months control: 116 ± 34 µA ; mtNOD: 85 ± 29 µA; 24 months control: 123 ± 41 µA; mtNOD: 98 ± 38 µA).

Following 10 minutes of stable baseline recording, theta-burst stimulation (TBS; 3 trains 20 s apart; train of 10 epochs at 5 Hz containing 5 pulses each; duration 150 µs at 100 Hz) was used to evoke long-term-potentiation. Field EPSPs were recorded in stratum radiatum of the CA1 subfield using borosilicate glass pipettes (GB150-8P Science Products GmbH, Hofheim am Taunus, Germany) with a tip resistance of 2-3 MΩ (pulled with PIP5 puller from HEKA Elektronik, Lambrecht, Germany), filled with ACSF containing an Ag/AgCl wire. Evoked fEPSPs were amplified and filtered at 1 kHz (EXT-10-2F, npi electronic GmbH). Recordings were digitized (Micro 1401mkII, CED Ltd., Cambridge, UK) and

analyzed using Signal 2.16 (CED Ltd.). Slope of fEPSPs were measured, displayed and plotted relative to mean of baseline.

Lifespan study design

Fourty-three control mice (21 male, 22 female) and fourty-two mtNOD mice (20 male, 22 female) were used for a longitudinal study to evaluate lifespan. All of them experienced a Morris Water Maze task once in their lifetime. Animals were housed as described above and periodically inspected by certified personnel until natural death. A mouse showing more than one of the following clinical signs was determined moribund[36]: inability to eat or drink; severe lethargy (reluctance to move when gently prodded with forceps); severe balance or gait disturbance; rapid weight loss; an ulcerated or bleeding tumor; enlarged abdomen. Moribund mice were killed and the age was taken as the best available estimate of its natural lifespan. Three mtNOD mice (1 male, 2 female) died before day 500 and were excluded from statistical analysis of lifespan differences because their cause of death was likely not due to aging[71].

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics 22. A significance level of $p < 0.05$ was used to evaluate the null hypothesis. Survival curves are shown as Kaplan-Meier plot. Analysis was done by Log Rank test (Mantel-Cox). For analysis of MWM acquisition phase and I/O curves, a mixed ANOVA was used. The proportion of animals that passed respectively failed in finishing Morris Water Maze task was analyzed by Fisher's exact test. Comparisons of independent groups were performed with Mann-Whitney-U test. Results are shown as Box-whisker plots.

Acknowledgments

The authors wish to thank Anne-Marie Neumann, Tina Sellmann and Hanka Schmidt for excellent technical assistance.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

RK designed the experiments, overall design of collaborative project ROSAge was carried out by GF, SI and RK. GR, TK and RK wrote the main text of the manuscript. All figures were prepared by GR. Mitochondrial ROS analysis and MitoTracker deep Red analysis were performed by GR. Gene expression analysis was done by CZ. Experiments on animal behavior were done by JM, extracellular LTP measurements were carried out by TT and JM. All authors contributed to interpretation of data; all authors reviewed the manuscript.

Funding

This study was supported by Federal Ministry for Education and Research (BMBF) grant ROSAge 31P6662, and FORUN programme funding no. 889062. GF received funding from the European Union's Horizon 2020 research and innovation programme under Grant agreement No 633589.

REFERENCES

1. Duchen MR: Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death. *J Physiol,* 516 (Pt 1): 1-17, 1999.
2. Desagher S, Martinou JC: Mitochondria as the central control point of apoptosis. *Trends Cell Biol,* 10: 369-377, 2000.
3. Nunnari J, Suomalainen A: Mitochondria: in sickness and in health. *Cell,* 148: 1145-1159, 2012.
4. Zhang F, Broughton RE: Mitochondrial-nuclear interactions: compensatory evolution or variable functional constraint among vertebrate oxidative phosphorylation genes? *Genome Biol Evol,* 5: 1781-1791, 2013.
5. Weinberg SE, Sena LA, Chandel NS: Mitochondria in the regulation of innate and adaptive immunity. *Immunity,* 42: 406-417, 2015.
6. Sun T, Qiao H, Pan PY, Chen Y, Sheng ZH: Motile axonal mitochondria contribute to the variability of presynaptic strength. *Cell Rep,* 4: 413-419, 2013.
7. Lagouge M, Larsson NG: The role of mitochondrial DNA mutations and free radicals in disease and ageing. *J Intern Med,* 273: 529-543, 2013.
8. Holt IJ, Harding AE, Morgan-Hughes JA: Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. *Nature,* 331: 717-719, 1988.

9. Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, Lezza AM, Elsas LJ, Nikoskelainen EK: Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science,* 242: 1427-1430, 1988.

10. Poulton J, Luan J, Macaulay V, Hennings S, Mitchell J, Wareham NJ: Type 2 diabetes is associated with a common mitochondrial variant: evidence from a population-based case-control study. *Hum Mol Genet,* 11: 1581-1583, 2002.

11. Cortopassi GA, Shibata D, Soong NW, Arnheim N: A pattern of accumulation of a somatic deletion of mitochondrial DNA in aging human tissues. *Proc Natl Acad Sci USA,* 89: 7370-7374, 1992.

12. Larsson NG: Somatic mitochondrial DNA mutations in mammalian aging. *Annu Rev Biochem,* 79: 683-706, 2010.

13. Gorman GS, Schaefer AM, Ng Y, Gomez N, Blakely EL, Alston CL, Feeney C, Horvath R, Yu-Wai-Man P, Chinnery PF, Taylor RW, Turnbull DM, McFarland R: Prevalence of nuclear and mitochondrial DNA mutations related to adult mitochondrial disease. *Ann Neurol,* 77: 753-759, 2015.

14. Lin MT, Beal MF: Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature,* 443: 787-795, 2006.

15. Kraytsberg Y, Kudryavtseva E, McKee AC, Geula C, Kowall NW, Khrapko K: Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. *Nat Genet,* 38: 518-520, 2006.

16. Sterky FH, Lee S, Wibom R, Olson L, Larsson NG: Impaired mitochondrial transport and Parkin-independent degeneration of respiratory chain-deficient dopamine neurons in vivo. *Proc Natl Acad Sci USA,* 108: 12937-12942, 2011.

17. Oka S, Leon J, Sakumi K, Ide T, Kang D, LaFerla FM, Nakabeppu Y: Human mitochondrial transcriptional factor A breaks the mitochondria-mediated vicious cycle in Alzheimer's disease. *Sci Rep,* 6: 37889, 2016.

18. Chang Y, Kong Q, Shan X, Tian G, Ilieva H, Cleveland DW, Rothstein JD, Borchelt DR, Wong PC, Lin CL: Messenger RNA oxidation occurs early in disease pathogenesis and promotes motor neuron degeneration in ALS. *PLOS ONE,* 3: e2849, 2008.

19. Federico A, Cardaioli E, Da PP, Formichi P, Gallus GN, Radi E: Mitochondria, oxidative stress and neurodegeneration. *J Neurol Sci,* 322: 254-262, 2012.

20. Burte F, Carelli V, Chinnery PF, Yu-Wai-Man P: Disturbed mitochondrial dynamics and neurodegenerative disorders. *Nat Rev Neurol,* 11: 11-24, 2015.

21. Knott AB, Perkins G, Schwarzenbacher R, Bossy-Wetzel E: Mitochondrial fragmentation in neurodegeneration. *Nat Rev Neurosci,* 9: 505-518, 2008.

22. Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, Walzer G, Stiles L, Haigh SE, Katz S, Las G, Alroy J, Wu M, Py BF, Yuan J, Deeney JT, Corkey BE, Shirihai OS: Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *EMBO J,* 27: 433-446, 2008.

23. Picard M, Wallace DC, Burelle Y: The rise of mitochondria in medicine. *Mitochondrion,* 30: 105-116, 2016.

24. Grady JP, Campbell G, Ratnaike T, Blakely EL, Falkous G, Nesbitt V, Schaefer AM, McNally RJ, Gorman GS, Taylor RW, Turnbull DM, McFarland R: Disease progression in patients with single, large-scale mitochondrial DNA deletions. *Brain,* 137: 323-334, 2014.

25. Parikh S, Goldstein A, Koenig MK, Scaglia F, Enns GM, Saneto R, Anselm I, Cohen BH, Falk MJ, Greene C, Gropman AL, Haas R, Hirano M, Morgan P, Sims K, Tarnopolsky M, Van Hove JL, Wolfe L, DiMauro S: Diagnosis and management of mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. *Genet Med,* 17: 689-701, 2015.

26. Picard M, Zhang J, Hancock S, Derbeneva O, Golhar R, Golik P, O'Hearn S, Levy S, Potluri P, Lvova M, Davila A, Lin CS, Perin JC, Rappaport EF, Hakonarson H, Trounce IA, Procaccio V, Wallace DC: Progressive increase in mtDNA 3243A>G heteroplasmy causes abrupt transcriptional reprogramming. *Proc Natl Acad Sci USA,* 111: E4033-E4042, 2014.

27. Schon EA, DiMauro S, Hirano M: Human mitochondrial DNA: roles of inherited and somatic mutations. *Nat Rev Genet,* 13: 878-890, 2012.

28. Yarham JW, Elson JL, Blakely EL, McFarland R, Taylor RW: Mitochondrial tRNA mutations and disease. *Wiley Interdiscip Rev RNA,* 1: 304-324, 2010.

29. Lehmann D, Schubert K, Joshi PR, Baty K, Blakely EL, Zierz S, Taylor RW, Deschauer M: A novel m.7539C>T point mutation in the mt-tRNA(Asp) gene associated with multisystemic mitochondrial disease. *Neuromuscul Disord,* 25: 81-84, 2015.

30. Roos S, Darin N, Kollberg G, Andersson GM, Tulinius M, Holme E, Moslemi AR, Oldfors A: A novel mitochondrial tRNA Arg mutation resulting in an anticodon swap in a patient with mitochondrial encephalomyopathy. *Eur J Hum Genet,* 21: 571-573, 2013.

31. Uusimaa J, Finnila S, Remes AM, Rantala H, Vainionpaa L, Hassinen IE, Majamaa K: Molecular epidemiology of childhood mitochondrial encephalomyopathies in a Finnish population: sequence analysis of entire mtDNA of 17 children reveals heteroplasmic mutations in tRNAArg, tRNAGlu, and tRNALeu(UUR) genes. *PEDIATRICS,* 114: 443-450, 2004.

32. Blakely EL, Swalwell H, Petty RK, McFarland R, Turnbull DM, Taylor RW: Sporadic myopathy and exercise intolerance associated with the mitochondrial 8328G>A tRNALys mutation. *J Neurol,* 254: 1283-1285, 2007.

33. Yang Y, Muzny DM, Xia F, Niu Z, Person R, Ding Y, Ward P, Braxton A, Wang M, Buhay C, Veeraraghavan N, Hawes A, Chiang T, Leduc M, Beuten J, Zhang J, He W, Scull J, Willis A, Landsverk M, Craigen WJ, Bekheirnia MR, Stray-Pedersen A, Liu P, Wen S, Alcaraz W, Cui H, Walkiewicz M, Reid J, Bainbridge M, Patel A, Boerwinkle E, Beaudet AL, Lupski JR, Plon SE, Gibbs RA, Eng CM: Molecular findings among patients referred for clinical whole-exome sequencing. *JAMA,* 312: 1870-1879, 2014.

34. Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, Bohlooly Y, Gidlof S, Oldfors A, Wibom R, Tornell J, Jacobs HT, Larsson NG: Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature,* 429: 417-423, 2004.

35. Ross JM, Coppotelli G, Hoffer BJ, Olson L: Maternally transmitted mitochondrial DNA mutations can reduce lifespan. *Sci Rep,* 4: 6569, 2014.

36. Hirose M, Schilf P, Lange F, Mayer J, Reichart G, Maity P, Johren O, Schwaninger M, Scharffetter-Kochanek K, Sina C, Sadik CD, Köhling R, Miroux B, Ibrahim SM: Uncoupling protein 2 protects mice from aging. *Mitochondrion,* 30: 42-50, 2016.

37. Kretzschmar C, Roolf C, Timmer K, Sekora A, Knubel G, Escobar HM, Jaster R, Müller S, Fuellen G, Köhling R, Junghanss C: Uncoupling protein 2 deficiency results in higher neutrophil counts and lower B-cell counts during aging in mice. *Exp Hematol,* 44: 1085-1091, 2016.

38. Kretzschmar C, Roolf C, Timmer K, Sekora A, Knubel G, Murua EH, Fuellen G, Ibrahim SM, Tiedge M, Baltrusch S, Jaster R, Köhling R, Junghanss C: Polymorphisms of the murine mitochondrial ND4, CYTB and COX3 genes impact hematopoiesis during aging. *Oncotarget,* 7: 74460-74472, 2016.

39. Niemann J, Johne C, Schröder S, Koch F, Ibrahim SM, Schultz J, Tiedge M, Baltrusch S: An mtDNA mutation accelerates liver aging by interfering with the ROS response and mitochondrial life cycle. *Free Radic Biol Med,* 102: 174-187, 2017.

40. Roolf C, Kretzschmar C, Timmer K, Sekora A, Knubel G, Murua EH, Fuellen G, Ibrahim SM, Tiedge M, Baltrusch S, Müller S, Köhling R, Junghanss C: Polymorphism in Murine mtATP8

Gene Correlates with Decreased Reactive Oxygen Species in Aging Hematopoietic Cells. *In Vivo,* 30: 751-760, 2016.

41. Finsterer J: Cognitive dysfunction in mitochondrial disorders. *Acta Neurol Scand,* 126: 1-11, 2012.

42. Trifunovic A, Larsson NG: Mitochondrial dysfunction as a cause of ageing. *J Intern Med,* 263: 167-178, 2008.

43. Torraco A, Peralta S, Iommarini L, Diaz F: Mitochondrial Diseases Part I: mouse models of OXPHOS deficiencies caused by defects in respiratory complex subunits or assembly factors. *Mitochondrion,* 21: 76-91, 2015.

44. Moreno-Loshuertos R, Ferrin G, Acin-Perez R, Gallardo ME, Viscomi C, Perez-Martos A, Zeviani M, Fernandez-Silva P, Enriquez JA: Evolution meets disease: penetrance and functional epistasis of mitochondrial tRNA mutations. *PLOS Genet,* 7: e1001379, 2011.

45. Zou X, Ratti BA, O'Brien JG, Lautenschlager SO, Gius DR, Bonini MG, Zhu Y: Manganese superoxide dismutase (SOD2): is there a center in the universe of mitochondrial redox signaling? *J Bioenerg Biomembr,* 49: 325-333, 2017.

46. Pekny M, Pekna M: Astrocyte reactivity and reactive astrogliosis: costs and benefits. *Physiol Rev*, 94: 1077-1098, 2014.

47. Daverey A, Agrawal SK: Curcumin alleviates oxidative stress and mitochondrial dysfunction in astrocytes. *Neuroscience,* 333: 92-103, 2016.

48. Lodato S, Arlotta P: Generating neuronal diversity in the mammalian cerebral cortex. *Annu Rev Cell Dev Biol,* 31: 699-720, 2015.

49. Knapp LT, Klann E: Role of reactive oxygen species in hippocampal long-term potentiation: contributory or inhibitory? *J Neurosci Res,* 70: 1-7, 2002.

50. Serrano F, Klann E: Reactive oxygen species and synaptic plasticity in the aging hippocampus. *Ageing Res Rev,* 3: 431-443, 2004.

51. Silva RE, Larsson NG, Mourier A: Bioenergetic roles of mitochondrial fusion. *Biochim Biophys Acta,* 1857: 1277-1283, 2016.

52. Chen H, McCaffery JM, Chan DC: Mitochondrial fusion protects against neurodegeneration in the cerebellum. *Cell,* 130: 548-562, 2007.

53. O'Keefe J, Nadel L: The hippocampus as a cognitve map. *Oxford University Press, New York*, 1978.

54. Andersen P, Lomo T: Control of hippocampal output by afferent volley frequency. *Prog Brain Res,* 27: 400-412, 1967.

55. Bliss TV, Collingridge GL: A synaptic model of memory: long-term potentiation in the hippocampus. *Nature,* 361: 31-39, 1993.

56. Kumar A: Long-Term Potentiation at CA3-CA1 Hippocampal Synapses with Special Emphasis on Aging, Disease, and Stress. *Front Aging Neurosci,* 3: 7, 2011.

57. Agnati LF, Zoli M, Biagini G, Fuxe K: Neuronal plasticity and ageing processes in the frame of the 'Red Queen Theory'. *Acta Physiol Scand,* 145: 301-309, 1992.

58. Lynch MA: Long-term potentiation and memory. *Physiol Rev,* 84: 87-136, 2004.

59. Huang Y, Hu Z, Liu G, Zhou W, Zhang Y: Cytokines induced by long-term potentiation (LTP) recording: a potential explanation for the lack of correspondence between learning/memory performance and LTP. *Neuroscience,* 231: 432-443, 2013.

60. Radford NB, Wan B, Richman A, Szczepaniak LS, Li JL, Li K, Pfeiffer K, Schagger H, Garry DJ, Moreadith RW: Cardiac dysfunction in mice lacking cytochrome-c oxidase subunit VIaH. *Am J Physiol Heart Circ Physiol,* 282: H726-H733, 2002.

61. Dell'agnello C, Leo S, Agostino A, Szabadkai G, Tiveron C, Zulian A, Prelle A, Roubertoux P, Rizzuto R, Zeviani M: Increased longevity and refractoriness to Ca(2+)-dependent neurodegeneration in Surf1 knockout mice. *Hum Mol Genet,* 16: 431-444, 2007.

62. Yang H, Brosel S, Acin-Perez R, Slavkovich V, Nishino I, Khan R, Goldberg IJ, Graziano J, Manfredi G, Schon EA: Analysis of mouse models of cytochrome c oxidase deficiency owing to mutations in Sco2. *Hum Mol Genet,* 19: 170-180, 2010.

63. Diaz F, Thomas CK, Garcia S, Hernandez D, Moraes CT: Mice lacking COX10 in skeletal muscle recapitulate the phenotype of progressive mitochondrial myopathies associated with cytochrome c oxidase deficiency. *Hum Mol Genet,* 14: 2737-2748, 2005.

64. Diaz F, Garcia S, Padgett KR, Moraes CT: A defect in the mitochondrial complex III, but not complex IV, triggers early ROS-dependent damage in defined brain regions. *Hum Mol Genet,* 21: 5066-5077, 2012.

65. Yu X, Gimsa U, Wester-Rosenlöf L, Kanitz E, Otten W, Kunz M, Ibrahim S. Dissecting the effects of mtDNA variations on complex traits using mouse conplastic strains. *Genome Res,* 19: 159-165, 2009.

66. Cohen LB, Salzberg BM, Davila HV, Ross WN, Landowne D, Waggoner AS, Wang CH: Changes in axon fluorescence during activity: molecular probes of membrane potential. *J Membrane Biol,* 19: 1-36, 1974.

67. Sachadyn P, Zhang XM, Clark LD, Naviaux RK, Heber-Katz E: Naturally occurring mitochondrial DNA heteroplasmy in the MRL mouse. *Mitochondrion,* 8: 358-366, 2008.

68. Chomyn A, Martinuzzi A, Yoneda M, Daga A, Hurko O, Johns D, Lai ST, Nonaka I, Angelini C, Attardi G: MELAS mutation in mtDNA binding site for transcription termination factor causes defects in protein synthesis and in respiration but no change in levels of upstream and downstream mature transcripts. *Proc Natl Acad Sci USA,* 89: 4221-4225, 1992.

69. Mayer J, Reichart G, Tokay T, Lange F, Baltrusch S, Junghanss C, Wolkenhauer O, Jaster R, Kunz M, Tiedge M, Ibrahim S, Fuellen G, Köhling R: Reduced adolescent-age spatial learning ability associated with elevated juvenile-age superoxide levels in complex I mouse mutants. *PLOS ONE,* 10: e0123863, 2015.

70. Gil-Pages M, Stiles RJ, Parks CA, Neier SC, Radulovic M, Oliveros A, Ferrer A, Reed BK, Wilton KM, Schrum AG: Slow angled-descent forepaw grasping (SLAG): an innate behavioral task for identification of individual experimental mice possessing functional vision. *Behav Brain Funct,* 9: 35, 2013.

71. Posimo JM, Titler AM, Choi HJ, Unnithan AS, Leak RK: Neocortex and allocortex respond differentially to cellular stress in vitro and aging in vivo. *PLOS ONE,* 8: e58596, 2013.

TABLES

Table 1. Conplastic mouse models used in this study.

Name	mtNOD	Control
mt-tRNA(Arg)	10 Adenines	10-12 Adenines
Mutation	Complex IV mt-Co3, nt9348 G>A = Val-Ile	-
Strain	C57BL6J-mt$^{NOD/LtJ}$	C57BL6J-mt$^{MRL/MpJ}$

Table 2. Quantitative real-time PCR probes.

Gene category	Protein name	Assay (Applied Biosystems)
Cytosolic ROS defense	Catalase (CAT)	Mm01340247_m1
	Superoxide dismutase 1 (SOD1)	Mm01700393_g1
	glutathione peroxidase 1 (GPX1)	Mm00656767_g1
Mitochondrial ROS defense	Superoxide dismutase 2 (SOD2)	Mm00690588_m1
	Uncoupling protein 2 (UCP2)	Mm00627599_m1
	Parkinson deglycase (PARK7 = DJ-1)	Mm00498538_m1
	Peroxiredoxin 3 (PRDX3)	Mm00545848_m1
Mitochondrial fusion	Dynamin-related GTPase Optic atrophy 1 (OPA1)	Mm01349716_m1
	Mitofusin 1 (MFN1)	Mm01289369_m1
	Mitofusin 2 (MFN2)	Mm01255785_m1
Mitochondrial fission	Dynamin-1-like protein (DNM1L)	Mm01342903_m1
	Fission 1 protein (FIS1)	Mm00481580_m1
Mitochondrial transcription	Transcription factor A, mitochondrial (TFAM)	Mm00447485_m1
Mitochondrial post-translational modifications	PTEN-induced kinase 1 (PINK1)	Mm00550827_m1
	NAD-dependent deacetylase sirtuin-3 (SIRT3)	Mm00452131_m1
Housekeeping gene	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Mm99999915_g1

Table 3. Lifespan analysis.

Strain	n	Median survival (95% CI) [days]	Percentiles		Mean age ± SEM [days]
			75% [days]	25% [days]	
mtNOD	39	803 (759 – 847)	701	851	786 ± 17
control	43	885 (790 – 920)	802	955	877 ± 17

FIGURE LEGENDS

Figure 1. Mitochondrial superoxide levels in brain.

Mitochondrial superoxide production in brain slices was stained by MitoSOX Red solution (shown in red). Nuclei were counterstained with DAPI (shown in blue). **(A)** Box plots illustrate comparison of mitochondrial superoxide levels between control and mtNOD mutant strains. Values represent mean fluorescence intensity (MFI) of MitoSOX Red as percentage to MFI of DAPI. Values are pooled from neocortex and hippocampus and shown with n = 5 (5 animals per strain per age group, 10 sections per animal, 6 ROIs each slice), Mann-Whitney-U test, † $p < 0.01$, * $p < 0.05$. **(B-C)** Representative ROI pictures taken from the neocortex of 24-month-old control (B) and mtNOD (C) mice (120 x magnification).

Figure 2. GFAP to NeuN ratio in hippocampal cell layers.

GFAP to NeuN ratio was measured by antibody staining of hippocampal slices. Box plots show comparison of GFAP to NeuN ratio in hippocampal cell layers from 12- and 24-month-old mice. Each bar represents mean fluorescence intensity (MFI) of GFAP as percentage to MFI of NeuN. All data shown with n = 5 (5 animals per strain per age group, 2 slices per animal, 3 pictures per slice). Mann-Whitney-U test, † $p < 0.01$, * $p < 0.05$.

Figure 3. Gene expression analysis of proteins involved in ROS defense.

Gene expression analysis of proteins in cytosolic (A) and mitochondrial (B) antioxidative response. Gene expression is shown as the relative expression normalized to mean value of 12-month-old control mice. Note that expression of catalase in aged animals was significantly elevated compared to their 12-month-old littermates (indicated by asterisks). Mitochondrial Sod2 is significantly lower in 24-month-old mutated mtNOD mice compared to age-matched controls. All data are shown with n = 5, Mann-Whitney-U test, *$p < 0.05$, †$p < 0.01$.

Figure 4. Analysis of mitochondrial dynamic network formation.

(A-B) Gene expression analysis of proteins involved in fusion (A) and fission (B) of mitochondrial network. Gene expression is shown as relative expression normalized to mean value of 12 months old mice. Expression of fission 1 (Fis1) is significantly reduced in 24 months old mutated mtNOD mice. All data are shown with n = 5. Mann-Whitney-U test, *$p < 0.05$. **(C-E)** Mitochondrial network homogeneity measured with MitoTracker Deep Red in brain slices from 24 months old mice. **(C)** Box plot illustrates MitoTracker Deep Red fluorescence pixels over threshold. Values are pooled from neocortex and hippocampus and shown with n = 5 (5 animals per strain, 4 slices per animal, 3 ROIs each slice). **(D-E)** Sample pictures of neocortical cells from 24-month-old control (D) and mtNOD (E) mice. Pictures were taken with 120x magnification. MitoTracker Deep Red fluorescence pixels over threshold are shown in red, nuclei were counterstained with DAPI (shown in blue).

Figure 5. Analysis of learning and memory in vitro (LTP).

(A) Time course of field excitatory postsynaptic potentials (fEPSP) measured in Schaffer collateral-CA1 synapses from 24-months-old mtNOD (n = 27 slices / 10 animals) and control mice (n = 8 slices / 5 animals). Each circle represents the percentage of fEPSP slope relative to mean baseline value. Following a 10 min baseline recording, three times of theta-burst stimulation protocol (TBS) was delivered at time point 0. **(B)** Box plots of LTP, calculated between minute 55 and minute 60 after theta-burst stimulation (TBS) relative to baseline. **(C-D)** Input/Output curves of 12 (C) and 24 (D) months age groups. In both ages mutated mtNOD mice significantly differed from control mice and showed increased excitability. ANOVA, † $p < 0.01$.

Figure 6. Analysis of learning and memory in vivo (Morris Water Maze).

(A) Morris Water Maze performance of 12-months-old mice shown as learning curves of seven consecutive days (spatial acquisition). Data points represent mean values of swimming distances of cohorts each day (± SEM). Control mice (n = 10) in light gray, mtNOD mice (n = 10) in blue. **(B)**

Swimming speed in cm/s of 12-months-old mice shown as box plots. **(C)** Results of 12-months-old mice passing probe trial 1 day after MWM. Both strains showed spatial memory retention by spending more time in the target quadrant. **(D)** Proportion of 24-months-old animals that passed respectively failed in finishing Morris Water Maze task. Only 1 out of 10 mtNOD mutated mice finished MWM. Fisher's exact test, † $p < 0.01$.

Figure 7. Survival analysis of mtNOD mutated mice compared to control mice.

(A) Kaplan-Meier survival curves, Log-rank test (Mantel-Cox), †$p < 0.01$; Control (light gray) n = 43 (21 male, 22 female) median survival 855 days; mtNOD (blue) n = 39 (19 male, 20 female) median survival 803 days. **(B)** Body weight as % of age-matched control group weight. While young (3-6 months) and old (24 months) mice did not differ, 12-months-old mtNOD mice had significantly reduced body weights. Mann-Whitney-U test, *$p < 0.05$