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RESEARCH ARTICLE

Effects of Ageing on the Mitochondrial Genome in Rat Vestibular Organs

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Abstract: ***Background*:** Deterioration in vestibular function occurs with ageing and is linked to age-related falls. Sensory hair cells located in the inner ear vestibular labyrinth are critical to vestibular function. Vestibular hair cells rely predominantly on oxidative phosphorylation (OXPHOS) for energy production and contain numerous mitochondria. Mitochondrial DNA (mtDNA) mutations and perturbed energy production are associated with the ageing process.

***Objective*:** We investigated the effects of ageing on mtDNA in vestibular hair and support cells, and vestibular organ gene expression, to better understand mechanisms of age-related vestibular deficits.

***Methods*:** Vestibular hair and supporting cell layers were microdissected from young and old rats, and mtDNA was quantified by qPCR. Additionally, vestibular organ gene expression was analysed by microarray and gene set enrichment analyses.

***Results*:** In contrast to most other studies, we found no evidence of age-related mtDNA deletion mutations. However, we found an increase in abundance of major arc genes near the mtDNA control region. There was also a marked age-related reduction in mtDNA copy number in both cell types. Vestibular organ gene expression, gene set enrichment analysis showed the OXPHOS pathway was down regulated in old animals.

***Conclusion*:** Given the importance of mtDNA to mitochondrial OXPHOS and hair cell function, our findings suggest the vestibular organs are potentially on the brink of an energy crisis in old animals.

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1. INTRODUCTION

Hair cells of the peripheral vestibular end organs are specialised neuroepithelial sensory cells that detect head movement and transduce this movement into electrochemical signals that are transmitted to the Central Nervous System (CNS) via the eighth cranial nerve. This head movement information is then used to help maintain posture, stabilise vision, and control movement. Thus, hair cells are the primary sensory unit of the vestibular system, a vital system that impacts the majority of, if not all, CNS functions [1]. Vestibular deficits causing dizziness, vertigo, and disequilibrium are considered one of the most common complaints in medicine, affecting around 30% of the general population [2]. Moreover, vestibular dysfunction disproportionately affects the elderly, with around 75% of people over the age of 65 being hospitalised due to falls [3] that are thought to be due, in part, to vestibular system dysfunction [2, 4]. The functional decline in the vestibular system, as assessed using video Head Impulse Testing (vHIT) of semicircular canal function, is apparent in humans by the eighth decade [5-7]. Although, the decline has been reported to occur as early as the sixth decade when dynamic visual acuity-based vestibular function tests were used [8, 9]. One plausible hypothesis to explain the age-related deficits in vestibular function, proposes that loss of sensory hair cells leads to the functional decline. However, in stark contrast to the auditory system, where Age-related Hearing Loss (AHL) has been shown to be associated with loss of cochlear sensory hair cells [10-12], vestibular hair cells appear remarkably resilient to the effects of ageing, with a significant decline in number not evident until the tenth decade [13], later than the appearance of peripheral functional deficits [8, 9]. An alternative hypothesis is therefore needed to explain the vestibular functional decline with ageing, and it is likely that there are hair cell functional deficits that precede frank hair cell loss in humans.

Many studies have implicated mitochondria in the ageing process [14, 15], and given hair cell function is critically dependant on mitochondrial function [16], it is possible that mitochondrial dysfunction plays a role in vestibular hair cell functional impairment in the elderly. Vestibular hair cells rely on mitochondria primarily for two major roles, energy production and calcium buffering [16, 17]. For example, the

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vestibular neuroepithelium has gene and protein expression profiles indicating a high demand for oxidative phosphorylation (OXPHOS) based ATP-production [16]. Also, it is thought that much of a hair cell's ATP requirements are required to pump Ca^{2+}, which enters the hair cell *via* mechanotransduction channels during hair bundle deflection, back into the surrounding endolymph *via* plasma membrane calcium ATPases [18]. It is well-known that OXPHOS produces the majority of a cell's Reactive Oxygen Species (ROS) and that these can potentially lead to damage of mitochondrial DNA (mtDNA) and other cell structures [19, 20]. The proximity of mtDNA to the sites of ROS production likely contributes to the mutation frequency in the mitochondrial genome being up to 17 times greater than in the nuclear genome [20, 21]. It is also thought a lack of mtDNA histones and lower proofreading capabilities contribute to this increased mutation burden [22]. Given these ATP generation and Ca^{2+} buffering requirements and ROS vulnerabilities, vestibular hair cells are potentially susceptible to mitochondrial dysfunction.

Indeed, there is a precedent for age-related mtDNA changes in inner ear hair cells with reports of mtDNA mutations in the cochlea. For example, the abundance of the "common" mtDNA deletion, one specific type of mtDNA deletion mutation, was correlated with the degree of human age-related hearing loss (presbycusis) [23]. Additionally, using a major versus minor arc comparative qPCR approach, an age-related increase in major arc mtDNA deletions was found in rat cochlear hair cells [24]. Evidence for a link between mtDNA mutation and vestibular function is apparent from patients harbouring mtDNA point mutations and showing peripheral vestibular organ dysfunction [25]. These studies demonstrate that mtDNA mutations have the potential to cause age-related vestibular hair cell dysfunction. In our study, we characterised the effects of ageing on mtDNA deletions, and mtDNA copy number, in rat vestibular cristae.

In summary, as mitochondrial function is crucial to neuronal function and mitochondria are thought to contribute to age-related dysfunction [26], we investigated vestibular cristae mitochondria to determine potential involvement in age-related impairments within the vestibular balance system. We determined mitochondrial genome copy number and quantified mtDNA deletions in the inner ear vestibular cristae of young and old rats. We used laser microdissection to separately enrich for the neuroepithelial sensory hair cells and supporting cell layers of the cristae, and then used a comparative qPCR approach to determine the effects of ageing on mtDNA in these layers. Finally, to determine the potential effects of mtDNA changes on mitochondrial OXPHOS function, we carried out a gene set enrichment analysis (GSEA) [27]. We evaluated the degree to which a gene set comprising mitochondrial and nuclear-encoded genes that encode the proteins of the five mitochondrial respiratory complexes, is enriched in the microarray gene expression profiles of the vestibular organs from young and old animals.

2. MATERIALS AND METHODS

2.1. Animals and Tissue Preparation

Male Fisher 344 (F344) rats were group housed and maintained on a standard 12-hour light/dark cycle in a temperature and humidity controlled holding room, with *ad libitum* access to standard rat food and water. The use and monitoring of animals were performed in accordance with the National Health and Medical Research Council's Australian Code of Practice for the Care and Use of Animals for Scientific Purpose, with approval from the University of Newcastle Animal Care and Ethics Committee, Australia (Approval number A-2006-902). At young (4-6 months; n = 7-14) and old (23-26 months; n = 7) ages, animals were euthanised with lethabarb (Virbac; 1mg/kg) and then transcardially perfused with 50 mls of ice-cold PBS, followed by 200 mls of freshly prepared, ice-cold 4% paraformaldehyde. Whole inner ears containing the vestibular labyrinths were removed from the skull and immersion post-fixed in 4% paraformaldehyde overnight, before being transferred to PBS. The three cristae were dissected from each inner ear bone and oriented in Optimum Cutting Temperature (OCT) embedding compound and frozen. Each crista was serially cryosectioned at 10 µm thickness and sections were thawed onto clean glass microscope slides and stored at -20 ˚C until further use.

2.2. Nissl Staining

In preparation for Nissl staining, sections were rinsed in ice-cold PBS and then placed in a 10% cresyl violet solution for 1 minute. Excess dye solution was removed with a 1 minute PBS wash and sections were dehydrated by 15-second immersions in 70% and then in 100% ethanol.

2.3. Laser Microdissection

Nissl stained, dehydrated sections were mounted on a PALM Microbeam laser microdissection system (Zeiss) and cristae hair and supporting cell layers were separately microdissected and collected into DNA lysis buffer (Qiagen). The hair cells were separately collected from the central (CZ) and Peripheral Zones (PZ), as designated previously [28], for each of the horizontal, anterior, and posterior cristae. Supporting cell layers were collected without partitioning crista into zones and samples from each of the 3 cristae were pooled for each animal.

2.4. DNA Extraction and qPCR

DNA was extracted from enriched hair cell and supporting cell layers using QIAGEN DNeasy Blood and Tissue kits, following the manufacturer's instructions. The relative abundance of mtDNA genes was determined using quantitative PCR (qPCR) as previously described [29]. Additionally, mtDNA copy number was also estimated by comparing mtDNA and nuclear DNA (nDNA) levels. For the latter, we used pseudo-*Gapdh* (ps*Gapdh*, Table **1**), a multicopy pseudogene that improves qPCR reproducibility for the nuclear reference gene and therefore permits a more accurate determination of copy number. There is an estimated 364 *Gapdh* pseudogenes in the rat genome [30] and in preliminary qPCR studies, we found that pseudo-*Gapdh* was reproducibly detected 6.9 cycles earlier than standard *Gapdh*. This equates to approximately 240 total *Gapdh* copies, or 66% of the predicted *Gapdh* pseudogene complement. Primers were designed using primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences are listed in Table **1**. qPCR reactions were done in triplicate for each gene using

Table 1. Gene primer sequences.

Genome	Primer	Sequence 5' - 3' (bp)
Mitochondrial	*Nd1* F	TGACCAACTAATGCACCTCCTA (3585- 3606)
	Nd1 R	GAAAATTGGCAGGGAAATGT (3650- 3669)
	12s F	TAGGACCTAAGCCCAATAACGA (420-441)
	12s R	TGGGGTATCTAATCCCAGTTTG (484-505)
	Nd4 F	TTACACGATGAGGCAACCAA (10557 – 10576)
	Nd4 R	GAGTGGGATGGAGCCATTA (10619-10638)
	Cytb F	TGACAAACATCCGAAAATCTCA (14137- 14158)
	Cytb R	AGGTGGCTGGCACGAAATTTACCAA (14176-14197)
Nuclear	*psGapdh* F	GGCATCCTGGGCTACACTGAGGA
	psGapdh R	GTTGCTGTTGAAGTCACAGGAGACA

1x SensiMix SYBR Lo-Rox qPCR mix (Bioline, Australia), 200nM of each forward and reverse primer, and 5µL of DNA sample, in a total volume of 12 µL per reaction. Amplification was performed using an Applied Biosystems 7500 Real-Time PCR machine with initial denaturation and activation step at 95°C for 10 min, followed by 45 cycles of 95°C for 15s and 60°C for 60s.

2.5. Microarray Gene Expression Analysis

Three young and 3 old F344 rats were euthanised and perfused as described above for the DNA work, except that the PBS was diethylpyrocarbonate (DEPC) treated to inactivate RNAses, and there was no paraformaldehyde perfusion. Vestibular organs were dissected out on ice, placed in RNA Later to maintain RNA integrity, and then stored at -80˚C until further use. Total RNA was extracted from the vestibular organs and processed for microarray analysis as we have previously described [31]. Briefly, approximately 100 ng of total RNA was DNAse I (Invitrogen) treated. RNA integrity analysis, cRNA amplification, and hybridization on Affymetrix GeneChIP Rat Gene 1.0 ST microarrays (probe for an estimated 27,342 genes), were then carried out at the Ramaciotti Centre for Genomics (University of New South Wales, Australia). RNA integrity was assessed by Bioanalyser (Agilent Technologies) and the average RIN number was 7.8 (range, 7.4-8.2). Affymetrix CEL files were imported into GenePattern for GSEA and analysed as described below.

2.6. Data Analysis

mtDNA Analyses: For each gene the average threshold cycle (Ct) value was calculated using 7500 SDS software v2.0.6 (Applied Biosystems). In mtDNA with no deletions/rearrangements, all genes should be in equal abundance and, therefore, any change in relative abundance will indicate the presence of mtDNA deletions or rearrangements. To compare relative mtDNA gene abundances the delta Ct (ΔCt) method was used, where ΔCt = Ct[mtDNA GeneA] – Ct[mtDNA GeneB], and then the average ΔCt for each gene pairing for each age was compared. The comparisons included; *Nd1* to *Nd4, Nd1* to *12s, Nd1* to *Cytb, 12s* to *Cytb, 12s* to *Nd4, Cytb* to *Nd4*. The relative mtDNA copy number was calculated in a similar manner, using ps*Gapdh* as the nuclear genome reference and *Nd1* for the mitochondrial gene. Statistical analyses were done using two-tailed T-tests with Hohm-Bonferroni correction applied as necessary for multiple testing [32]. Significance was set at $p < 0.05$ for all analyses. Data are presented as mean ± standard error of the mean (SEM).

Oxidative Phosphorylation Related Gene Expression: Data files for GSEA were prepared according to the GSEA User Guide. Files were uploaded to and GSEA was run via the GenePattern portal. We used an OXPHOS gene set curated from the Kyoto Encyclopedia of Genes and Genomes (KEGG) with additional genes as indicated in the Rat Genome Database (RGD; http://rgd.mcw.edu/wg/home) for the enrichment analysis. The gene set was based on the KEGG Pathway – 1.2 Energy metabolism – 00190 – Oxidative phosphorylation, set for Rattus Norvegicus (http://www.genome.jp/kegg-bin/show_pathway?org_name=rno&mapno=00190&mapscale=1.0&show_description=show). The gene set contained 134 nuclear and mitochondrial genome encoded genes (see Supplementary Materials for OXPHOS Gene Set). The Affymetrix Ra_Gene_ST CEL files were imported into AffySTExpressionFileCreator to create the GCT file for importation into GSEA. RMA normalization and background correction was carried out in AffySTExpressionFileCreator. GSEA was run using the following parameters: Number of permutations = 1000, Permutation type = Gene set. All other settings were left as defaults.

3. RESULTS

3.1. Laser Microdissection of Vestibular Crista Hair Cells and Supporting Cells

Isolation of enriched hair cell and supporting cell layers from the vestibular cristae was achieved using laser microdissection. We have previously demonstrated laser microdissection enriches samples for specific cell types [29, 33]. We were able to microdissect and isolate the sensory hair cell

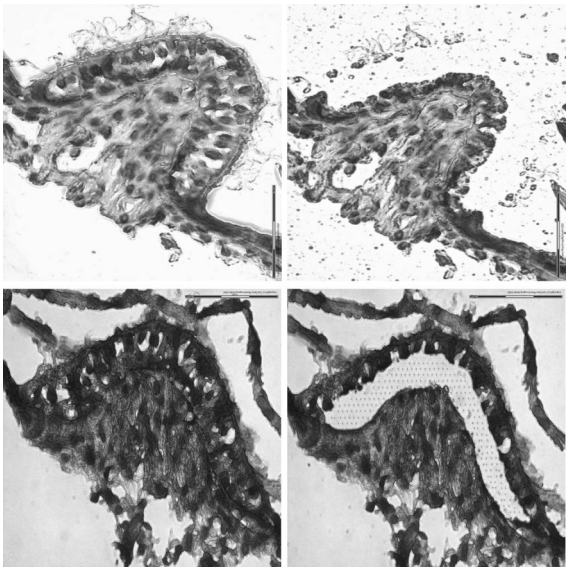

Fig. (1). Semicircular canal crista showing hair cell (top panels) and supporting cell (lower panels) layers. Left panels: cristae as they appear prior to laser microdissection. Right panels: cristae post microdissection, with hair cell (top) and supporting (lower) cell layers removed. Scale bars = 75μm.

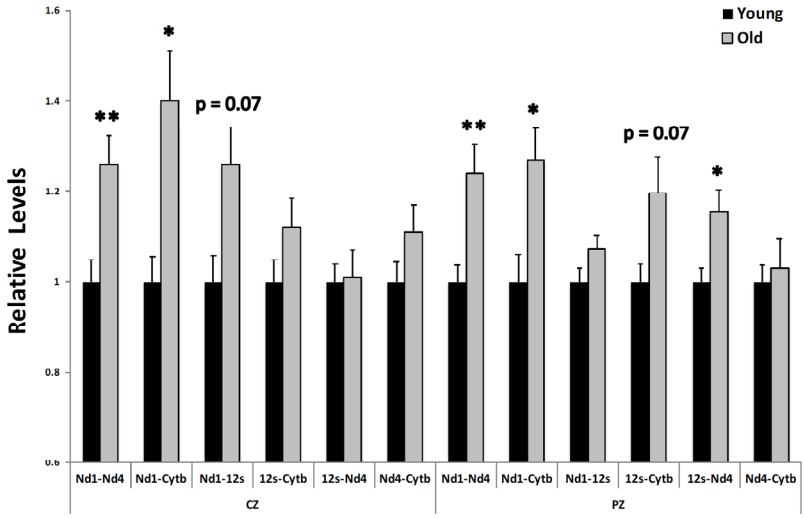

Fig. (2). Effects of ageing on mtDNA in vestibular cristae hair cell layers. The relative levels of four mtDNA-encoded genes were determined for young (Black bars; n=7) and old (Grey bars; n=7) animals. Bars represent the mean (+SEM) of all possible pair-wise comparisons between the four mtDNA genes for the both the CZ and PZ. The major arc genes, *Cytb* and *Nd4*, were significantly more abundant than the two minor arc genes, *Nd1* and *12s*, in old animals. However, we found no difference in the effects of aging between the CZ and PZ. * $p < 0.05$, ** $p < 0.02$.

layers from the underlying supporting cells, and the supporting cell layers from the non-neuroepithelial tissue of the crista, resulting in relatively enriched cell samples, respectively (Fig. **1**).

3.2. Age-Related Changes in mtDNA Genes in Vestibular Cristae Hair Cells

We determined the presence of mtDNA deletions/rearrangements by comparing the relative abundances of individual mtDNA genes from different regions of the mitochondrial genome (*Nd1, Nd4, Cytb,* and *12s* rRNA), as we have previously reported for midbrain dopamine neurons [31]. Two-way ANOVA analysis with age, crista, and crista region as the main effects, showed there were no significant effects of crista for any of the mtDNA comparisons, including copy number. Therefore, the data for individual animals were averaged across the three cristae for each gene. The CZ and PZ were kept as separate regions of interest. There were significant age-related differences in the abundance of *Cytb* and *Nd4* relative to *Nd1* in the CZ, with these two genes being approximately 40% and 30% more abundant in old animals, respectively, compared to *Nd1* (Fig. **2**). In the PZ, a similar relative abundance pattern was found, with both *Nd4* and *Cytb* being approximately 30% more abundant than *Nd1* in the old animals (Fig. **2**). *Nd4* was also found to be significantly more abundant than *12s* in the PZ of old animals (Fig. **2**). The relative levels of all other mtDNA genes tested were not significantly different between young and old. There was no evidence for mtDNA deletions.

3.3. Effects of Ageing on mtDNA Copy Number in Vestibular Cristae Hair Cells

The relative abundances of mtDNA and nDNA genes were used to determine the effects of ageing on mtDNA copy number. There were no significant effects of crista on mtDNA copy number, so the values for the three separate cristae were averaged for each animal, while still maintaining CZ and PZ separation. There was a significant reduction in mtDNA copy number in crista hair cell layers of old animals, with an approximate 50% decrease in both the CZ and PZ (Fig. **3**). However, there was no significant difference in the reduction between the CZ and PZ.

3.4. Age-Related Changes in mtDNA Genes in Vestibular Cristae Supporting Cells

There were significant increases in *Cytb* relative to *Nd1*, *Nd4*, and *12s*, and a trend towards an increase of *12s* relative to *Nd1* (Fig. **4**). There were no significant differences in relative mtDNA gene levels between *Nd1* and *Nd4*, or *Nd4* and *12s*. These data indicate the only effect of ageing was to increase the *Cytb* region relative to other mtDNA genes. Again, there was no evidence for mtDNA deletions.

3.5. Effects of Ageing on mtDNA Copy Number in Vestibular Cristae Supporting Cells

There was a significant and marked effect of ageing on mtDNA copy number in crista supporting cells, with an approximate 75% reduction in old animals compared to young animals (Fig. **5**).

3.6. Effects of Ageing on Oxidative Phosphorylation Related Gene Expression in the Vestibular Organs

Microarray profiling and GSEA detected the vast majority of annotated genes, with 14,101 listed in the ranked genes list. Overall, of the 134 nuclear and mitochondrial encoded genes in the OXPHOS gene set, 106 were reported in the ranked list as determined by GSEA, and therefore included in the enrichment analysis. Six of the 13 mtDNA en-

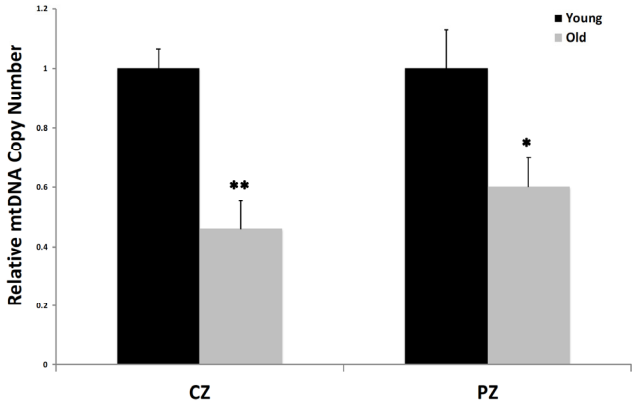

Fig. (3). Effects of ageing on mtDNA copy number in vestibular cristae hair cells. The relative abundances of mtDNA (*Nd1*) and nDNA (ps*Gapdh*) were compared between young (Black bars; n=7) and old (Grey bars; n=7) for both CZ and PZ regions. There was a significant reduction in mtDNA copy number in old animals for both regions. * $p < 0.05$, ** $p < 0.01$.

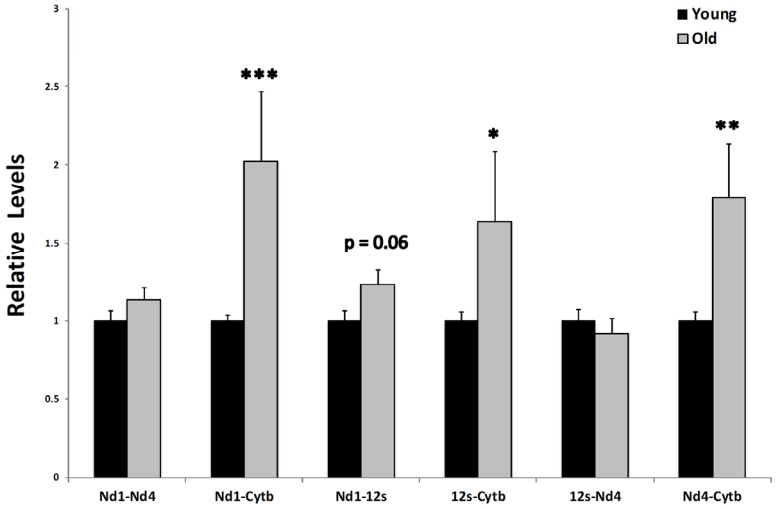

Fig. (4). Effects of ageing on mtDNA in vestibular cristae supporting cells. The relative levels of four mtDNA-encoded genes were determined for young (Black bars; n=14) and old (Grey bars; n=7) animals. Bars represent the mean (+SEM) of all possible pair-wise comparisons between the four mtDNA genes. The major arc gene, *Cytb* was significantly more abundant than *Nd1*, *Nd4*, and *12s*. A trend toward an increase in *12s* abundance relative to *Nd1* was found ($p = 0.06$ after correction for multiple comparisons). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

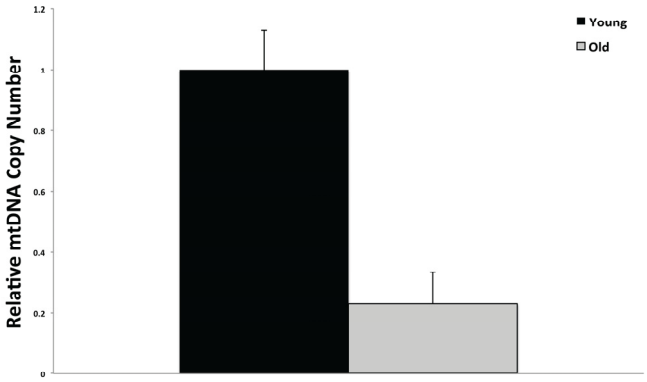

Fig. (5). Effects of ageing on mtDNA copy number in vestibular cristae supporting cells. The relative abundances of mtDNA (*Nd1*) and nDNA (ps*Gapdh*) were compared between young (Black bars; n=14) and old (Grey bars; n=7). There was a significant reduction in mtDNA copy number in old animals. ** $p < 0.01$.

coded genes were not listed (*Nd3*, *Nd4*, *Nd4l*, *Cox3*, *Atp6*, *Atp8*). GSEA revealed there was a significant enrichment of OXPHOS genes only for the young age group ($p = 0.023$), indicating ageing has significantly impacted this pathway. Core enrichment genes are those genes that make the largest contribution to the enrichment score and, importantly, 86% (6/7) of mtDNA encoded genes that were in the ranked gene list, were core enrichment genes (Table **2**). Ageing also impacted the expression of nuclear genome encoded OXPHOS genes. Eighteen complex I, 1 complex II, 2 complex III, 9 complex IV, and 19 complex V, nuclear-encoded genes were in the 55 core enrichment genes (Table **2**). Overall, combining mitochondrial and nuclear-encoded OXPHOS genes, 55% of complex I genes (22 of 40 GSEA ranked genes), 25% of complex II genes (all 4 complex II genes are nuclear encoded and were ranked by GSEA), 33% of complex III genes (2 of 6 GSEA ranked genes), 55% of complex IV genes (7 of 20 GSEA ranked genes), and 53% of complex V genes (19 of 36 GSEA ranked genes), were in the core enrichment list (Table **2**).

4. DISCUSSION

Mitochondrial DNA deletions are known to accumulate with ageing and are a characteristic of tissues from aged animals, particularly post-mitotic tissues such as the nervous system, and skeletal and cardiac muscle [34-40]. Increased abun-

Table 2. Core enrichment genes within the 106 gene OXPHOS gene set.

Complex I		Complex II	Complex III	Complex IV	Complex V	
Nd1 (-0.049)	*Ndufb10* (0.032)	*Sdha* (0.005)	*Cyc1* (0.014)	***Cox1*** (-0.025)	***Atp5a1*** (-0.032)	***Atp6v0d2*** (-0.061)
Nd2 (-0.030)	*Ndufb11* (0.017)	*Sdhb* (0.006)	*Cytb* (-0.008)	***Cox11*** (-0.022)	***Atp5b*** (-0.016)	*Atp6v0e1* (0.021)
Nd5 (-0.022)	***Ndufb2*** (-0.019)	*Sdhc* (0.031)	*Uqcrc1* (0.033)	***Cox15*** (-0.013)	***Atp5c1*** (-0.035)	***Atp6v0e2*** (-0.038)
Nd6 (-0.018)	***Ndufb3*** (-0.026)	***Sdhd*** (-0.033)	***Uqcrc2*** (-0.026)	***Cox17*** (-0.026)	*Atp5d* (0.054)	***Atp6v1a*** (-0.053)
Ndufa1 (0.016)	***Ndufb5*** (-0.018)	-	*Uqcrfs1* (0.021)	***Cox18*** (-0.026)	***Atp5f1*** (-0.016)	*Atp6v1b1* (0.024)
Ndufa12 (-0.012)	*Ndufb6* (0.032)	-	***Uqcrq*** (-0.027)	*Cox19* (0.022)	***Atp5g2*** (-0.065)	***Atp6v1b2*** (-0.115)
Ndufa13 (0.024)	***Ndufb7*** (-0.018)	-	-	***Cox2*** (-0.023)	***Atp5g3*** (-0.023)	***Atp6v1c1*** (-0.025)
Ndufa2 (-0.007)	***Ndufb8*** (-0.012)	-	-	*Cox4i1* (0.012)	***Atp5h*** (-0.022)	*Atp6v1d* (0.009)
Ndufa3 (0.022)	*Ndufb9* (0.007)	-	-	*Cox4i2* (0.045)	***Atp5i*** (-0.012)	*Atp6v1e1* (-0.005)
Ndufa4 (-0.036)	***Ndufc2*** (-0.049)	-	-	*Cox5a* (-0.006)	***Atp5j*** (-0.039)	*Atp6v1e2* (0.006)
Ndufa5 (-0.034)	***Ndufs1*** (-0.048)	-	-	***Cox6a1*** (-0.014)	***Atp5j2*** (-0.022)	*Atp6v1f* (0.023)
Ndufa6 (0.031)	*Ndufs2* (-0.006)	-	-	***Cox6a2*** (-0.016)	***Atp5l*** (-0.071)	***Atp6v1g1*** (-0.029)
Ndufa7 (-0.003)	***Ndufs3*** (-0.072)	-	-	***Cox6b1*** (-0.032)	*Atp5s* (0.004)	***Atp6v1g2*** (-0.034)
Ndufa8 (-0.048)	***Ndufs4*** (-0.023)	-	-	*Cox6b2* (0.034)	*Atp5sl* (0.033)	*Atp6v1g3* (-0.001)
Ndufa9 (-0.015)	*Ndufs5* (0.008)	-	-	***Cox6c*** (-0.020)	***Atp6ap1*** (-0.018)	*Atp6v1h* (0.006)
Ndufab1 (-0.031)	***Ndufs7*** (-0.013)	-	-	*Cox7a2* (0.000)	*Atp6ap1l* (0.028)	*Tcirg1* (0.016)
Ndufaf1 (0.020)	*Ndufs8* (0.031)	-	-	*Cox7a2l* (-0.009)	*Atp6v0a1* (0.009)	-
Ndufaf2 (-0.067)	***Ndufv1*** (-0.011)	-	-	***Cox7b*** (-0.070)	*Atp6v0a4* (0.001)	-
Ndufaf3 (0.007)	*Ndufv2* (0.016)	-	-	*Cox8a* (0.037)	*Atp6v0b* (0.027)	-
Ndufaf4 (0.028)	-	-	-	*Cox8c* (0.011)	*Atp6v0d1* (0.004)	-

Note: **bolding** denotes core enrichment, underlined indicates mtDNA encoded. Numbers in brackets are GSEA's Signal2Noise ranking metric score for each gene, which is the difference in mean expression of the young and old groups divided by the sum of their standard deviations. A negative score indicates the average expression of the gene was lower in the old group.

dance of mtDNA deletions is associated with mitochondrial dysfunction in the brain [41, 42] and skeletal muscle [35, 40]. Age-related accumulation of mtDNA deletions can therefore compromise cell activity through compromised mitochondrial function. Indeed, it has even been argued that mtDNA deletions drive the ageing process [43]. The vestibular neuroepithelium (hair cells and supporting cells) is thought to rely predominantly on mitochondrial oxidative phosphorylation (as opposed to glycolysis) for its ATP requirements [16]. As mtDNA encodes for 13 subunits of the mitochondrial respiratory chain, deletion mutations in sufficient abundance would affect mitochondrial and thus hair cell/supporting cell function. Notably, mtDNA deletions have been detected in the other neurosensory structure of the inner ear, namely the cochlea, in both aged humans and rats [24, 44]. Since we have reported increased mtDNA deletion abundance in CNS regions, and substantia nigra dopamine neurons in aged F344 rats, the strain used in this study [29, 36], we therefore hypothesised that ageing would result in increased levels of mtDNA deletions in the vestibular neuroepithelium.

Somewhat surprisingly, our current data however, indicate ageing does not result in an increase in mtDNA deletions in either hair cells or supporting cells of the vestibular neuroepithelium. On the contrary, using the same primer sequences and qPCR analysis methods with which we previously demonstrated age-related mtDNA deletions [29], we detected increased relative levels of the major arc region genes, *Cytb* and *Nd4*, in the hair cell layer, and *Cytb* in the supporting cell layer (Figs. **2** and **4**). The effects of ageing on these mtDNA major arc regions were similar for hair cells from both the central and peripheral zones of the neuroepithelium, two zones thought to differ in the response dynamics of their respective hair cell populations [45]. One possible explanation for our results is mtDNA duplication. Ageing is associated with a variety of mtDNA rearrangements including point [46-49] and deletion mutations [34, 36, 39, 41, 50], and tandem duplications [22, 51, 52]. The reasons for age-related increases in mtDNA duplication abundance are not known, but it has been suggested that they may be protective against age-related deletion mutations [52], a suggestion that is consistent with the importance of vestibular function to survival.

Mitochondrial copy number was significantly reduced in both the hair cell and supporting cell layers (Figs. **3** and **5**). We have previously reported a modest age-related decrease in mtDNA copy number in the brain of the F344 strain of rat [53], although others using the same animal model have found no effect [54], the latter finding is consistent with human data [55]. However, other peripheral tissues have shown age-related mtDNA copy number changes. For example, decreases have been reported in liver and skeletal muscle [54, 56-58]. Although it should be noted, there appears to be species- and/or gender-dependency for some of these effects [55, 57, 59]. Indeed, the effects are very likely tissue, subtissue (*e.g.* different types of skeletal muscle, see [60]), and cell-specific. To date the effects of ageing on mtDNA copy number in the cochlea have not been reported, so our present findings constitute the first report on the effects of ageing on mtDNA copy number in the inner ear. The number of mtDNA copies and OXPHOS capacity are tightly linked [61], and as the vestibular organs are thought to rely heavily on OXPHOS for ATP [16], the ~50-70% reductions in mtDNA copy number reported here indicate the aged peripheral vestibular system has significantly reduced energy producing capacity and therefore is likely to be on the brink of an energy crisis. For example, sensitivity of vestibular hair cells to the ototoxic actions of gentamicin could be increased in the elderly due to this potential energy crisis [62]. Indeed, our OXPHOS GSEA indicates the oxidative phosphorylation pathway in the vestibular organs is affected by ageing. The OXPHOS gene set, which comprised 106 genes detected by microarray and GSEA, was significantly enriched in the expression profile of the young animals. In other words, across the OXPHOS gene set the expression levels were higher in the young vestibular organs than in the old. This was true for both mitochondrial and nuclear genome encoded OXPHOS genes. Reduced mitochondrial OXPHOS gene expression would be expected based on our mtDNA results, and have been reported for other tissues with ageing [56]. There is also mito-nuclear interaction that ensures the two cellular compartments are coupled with regards OXPHOS function [63]. For example, mitochondrial and nuclear-encoded OXPHOS subunits assemble in precise stoichiometric ratios and if these are perturbed mitonuclear imbalance can occur, triggering proteostasis restoration mechanisms such as the mitochondrial unfolded protein response [64]. In the present study, we found a marked drop in mtDNA copy number, which would be expected to cause a drop in OXPHOS gene expression for both mitochondrial and nuclear-encoded subunits, in order to maintain mito-nuclear balance. In support of this notion, we found ageing affected all five OXPHOS complexes, including succinate dehydrogenase (Complex II), which is completely nuclear encoded, as well as complexes with contributions from both genomes (See Table **2**).

The age-related reduction in mtDNA copy number could be due to a number of factors. For example, there may be a decrease in mtDNA copies per mitochondrion and/or a reduction in the number of mitochondrial organelles. Given that we normalised mtDNA to the nuclear genome, the drop cannot be explained by an age-related decrease in cell number. Furthermore, ageing has a relatively modest effect on vestibular hair cell degeneration (for discussion, see [65]. Decreased mitochondrial biogenesis or increased mitophagy will result in a drop of mitochondrial organelles in a cell. Maintenance of cell function requires quality control of mitochondria, and this is achieved through balancing autophagic destruction (mitophagy) of damaged mitochondria and organelle biogenesis [66]. In fact, maintenance of cellular homeostasis requires the elimination of damaged old mitochondria, often with low membrane potential and high ROS production, and concomitant replacement by new and more efficient mitochondria [67]. Whether there is a biogenesis-mitophagy imbalance in the inner ear vestibular apparatus (or cochlea) with ageing is not known.

The reasons for the relative increase in a subset of mtDNA genes with age are not clear from the present study. Intriguingly, these increased levels of mtDNA in the *Cytb* and *Nd4* regions are, in part, similar to the multimers (~200-800 bp long) found in the brains and other post-mitotic tissues of "mutator" mice, an experimental model of acceler-

ated ageing [68]. Notably, it has been suggested these mtDNA multimers may detrimentally affect mtDNA replication [68], which may explain the reduced mtDNA copy number we see in the vestibular crista neuroepithelium. Another, not necessarily mutually exclusive possibility, is the occurrence of partial mtDNA fragments that arise due to incomplete genome replication. Replication of mtDNA starts at the heavy strand origin (O_H) located in the control region [69], and proceeds towards the *Cytb* and *Nd4* genes, which are significantly elevated in cristae hair cell and supporting cell layers of old animals. Oxidative damage is considered a ubiquitous aspect of the ageing process, and the mtDNA replisome stalls at sites of oxidative DNA damage [70], potentially resulting in truncated mtDNA fragments. We speculate it is these fragments that we have detected in our old vestibular cristae. Whatever the cause of the elevated mitochondrial genome fragments, further work is required to confirm the nature of the fragments, determine the underlying reasons for their existence, and evaluate their impacts on mtDNA replication.

CONCLUSION

Ageing is associated with a significant reduction in mtDNA copies in the vestibular crista neuroepithelium, and with an overall reduction in the expression of oxidation phosphorylation genes. Also, there was an increase in the relative abundance of mtDNA major arc genes *Cytb* and *Nd4*, which we speculate could reflect stalled mtDNA replication and potentially cause the mtDNA decline. Whatever the underlying mechanisms, our mtDNA and oxidative phosphorylation gene expression data indicate the aged peripheral vestibular system is energetically vulnerable and susceptible to mechanical and chemical damage.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved from the University of Newcastle Animal Care and Ethics Committee, Australia (Approval numbers A-2006-902).

HUMAN AND ANIMAL RIGHTS

No humans were involved in this study. The use and monitoring of animals were performed in accordance with the National Health and Medical Research Council's Australian Code of Practice for the Care and Use of Animals for Scientific Purpose.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

REFERENCES

[1] Goldberg JV, Wilson KC, *The Vestibular System: a sixth sense*. 2012, New York: Oxford University Press.

[2] Neuhauser H, Lempert T. Vertigo: epidemiologic aspects. 2009.

[3] Kreisfeld R, Harrison JE. Hospital separations due to injury and poisoning, Australia 2005-06. 2010: Australian Institute of Health and Welfare.

[4] Agrawal Y, Carey J, Santina CD, *et al*. Disorders of balance and vestibular function in US adults: Data from the National Health and Nutrition Examination Survey, 2001-2004. Arch Int Med 2009 169(10): 938-44.

[5] Li C, Layman AJ, Geary R, *et al*. Epidemiology of vestibulo-ocular reflex function: Data from the Baltimore Longitudinal Study of Aging. Otology & neurotology: Official publication of the American Otological Society, American Neurotology Society [and] European. Acad Otol Neurotol 2015; 36(2): p. 267.

[6] Matiño-Soler EE, Esteller-More JC, Martin-Sanchez JM, *et al.* Normative data on angular vestibulo-ocular responses in the yaw axis measured using the video head impulse test. Otol Neurotol 2015; 36(3): p. 466-71.

[7] McGarvie LA, MacDougall HG, Halmagyi GM, *et al.* The video head impulse test (vHIT) of semicircular canal function– Age-dependent normative values of VOR gain in healthy subjects. Front Neurol 2015; 6: 154.

[8] Agrawal Y, Zuniga M, Davalos-Bichara M, *et al*. Decline in semicircular canal and otolith function with age. Otol Neurotol 2012; 33(5): p. 832-9.

[9] Dong Y, Li M, Liu P, *et al*. Genes involved in immunity and apoptosis are associated with human presbycusis based on microarray analysis. Acta otolaryngol 2014; 134(6): p. 601-8.

[10] Engle JR, Tinling S, Recanzone GH. Age-related hearing loss in rhesus monkeys is correlated with cochlear histopathologies. PLoS One 2013; 8(2): p. e55092.

[11] Nelson EG, Hinojosa R. Presbycusis: A human temporal bone study of individuals with downward sloping audiometric patterns of hearing loss and review of the literature. The Laryngoscope, 2006; 116(S112): p. 1-12.

[12] Perez P, Bao J. Why do hair cells and spiral ganglion neurons in the cochlea die during aging? Aging Dis 2011; 2(3): p. 231.

[13] Lopez I, Ishiyama G, Tang Y, *et al.* Regional estimates of hair cells and supporting cells in the human crista ampullaris. J Neurosci Res 2005; 82(3): p. 421-31.

[14] Boumezbeur F, Mason GF, De Graaf RA, *et al.* Altered brain mitochondrial metabolism in healthy aging as assessed by in vivo magnetic resonance spectroscopy. J Cereb Blood Flow Metab 2010; 30(1): p. 211-21.

[15] Martin LJ. Biology of mitochondria in neurodegenerative diseases. Prog Mol Biol Transl Sci 2012; 107: p. 355.

[16] Spinelli KJ, Klimek JE, Wilmarth PA, *et al.* Distinct energy metabolism of auditory and vestibular sensory epithelia revealed by quantitative mass spectrometry using MS2 intensity. Proc Nat Acad Sci 2012; 109(5): p. E268-77.

[17] Lenzi D, Roberts WM. Calcium signalling in hair cells: Multiple roles in a compact cell. Curr Opin Neurobiol 1994; 4(4): p. 496-502.

[18] Shin JB, Streijger F, Beynon A, *et al.* Hair bundles are specialized for ATP delivery via creatine kinase. Neuron 2007; 53(3): p. 371-86.

[19] Ott MV, Gogvadze V, Orrenius S, *et al.* Mitochondria, oxidative stress and cell death. Apoptosis 2007; 12(5): p. 913-22.
[20] Shokolenko I, Venediktova N, Bochkareva A, *et al.* Oxidative stress induces degradation of mitochondrial DNA. Nucleic Acids Res 2009; 37(8): p. 2539-48.
[21] Tuppen HA, Blakely EL, Turnbull DM, *et al*. Mitochondrial DNA mutations and human disease. Biochim Biophys Acta (BBA)-Bioenerget 2010; 1797(2): p. 113-28.
[22] Wei YH, Pang CY, You BJ, *et al*. Tandem Duplications and Large-Scale Deletions of Mitochondrial DNA Are Early Molecular Events of Human Aging Processa. Ann N Y Acad Sci 1996; 786(1): p. 82-101.
[23] Markaryan A, Nelson EG, Hinojosa R. Quantification of the mitochondrial DNA common deletion in presbycusis. Laryngoscope 2009; 119(6): p. 1184-9.
[24] Yin S, Yu Z, Sockalingam R, *et al.* The role of mitochondrial DNA large deletion for the development of presbycusis in Fischer 344 rats. Neurobiol Dis 2007; 27(3): p. 370-7.
[25] Iwasaki S, Egami N, Fujimoto C, *et al.* The mitochondrial A3243G mutation involves the peripheral vestibule as well as the cochlea. Laryngoscope 2011; 121(8): p. 1821-4.
[26] Troulinaki K, Bano D. Mitochondrial deficiency: A double-edged sword for aging and neurodegeneration. Front Genet 2012; 3: 244.
[27] Subramanian A, Tamayo P, Mootha VK, *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Nat Acad Sci 2005; 102(43): p. 15545-50.
[28] Desai SS, Ali H, Lysakowski A. Comparative morphology of rodent vestibular periphery. II. Cristae ampullares. J Neurophysiol 2005; 93(1): p. 267-80.
[29] Parkinson G, Dayas CV, Smith DW. Increased Mitochondrial DNA Deletions in Substantia Nigra Dopamine Neurons of the Aged Rat. Curr Aging Sci 2014; 7(3): p. 155-60.
[30] Liu YJ, Zheng D, Balasubramanian S, *et al.* Comprehensive analysis of the pseudogenes of glycolytic enzymes in vertebrates: The anomalously high number of GAPDH pseudogenes highlights a recent burst of retrotrans-positional activity. BMC Genomics 2009; 10(1): p. 480.
[31] Parkinson GM, Dayas CV, Smith DW. Age-related gene expression changes in substantia nigra dopamine neurons of the rat. Mech Ageing Develop 2015; 149: p. 41-9.
[32] Abdi H. Holm's sequential Bonferroni procedure. Encycloped Res Des 2010; 1(8).
[33] Brown AL, Day TA, Dayas CV, *et al*. Purity and enrichment of laser-microdissected midbrain dopamine neurons. BioMed Res Int 2013; 2013: 747938.
[34] Bender A, Schwarzkopf RM, McMillan A, *et al.* Dopaminergic midbrain neurons are the prime target for mitochondrial DNA deletions. J Neurol 2008; 255(8): p. 1231-5.
[35] Bua E, Johnson J, Herbst A, *et al.* Mitochondrial DNA–deletion mutations accumulate intracellularly to detrimental levels in aged human skeletal muscle fibers. Am J Hum Genet 2006; 79(3): p. 469-80.
[36] Cahif A, Parkinson GM, Dayas CV, *et al*. Characterisation of mitochondrial DNA deletions by long-PCR in central nervous system regions of young, middle-and old-aged rats. Curr Aging Sci 2013; 6(3): p. 232-8.
[37] Cheng Y, Ren X, Gowda AS, *et al.* Interaction of Sirt3 with OGG1 contributes to repair of mitochondrial DNA and protects from apoptotic cell death under oxidative stress. Cell Death Dis 2013; 4(7): p. e731.
[38] Meissner CP, Bruse SA, Mohamed, *et al.* The 4977bp deletion of mitochondrial DNA in human skeletal muscle, heart and different areas of the brain: a useful biomarker or more? Exp Gerontol 2008; 43(7): p. 645-52.
[39] Williams SL, Mash DC, Züchner S, *et al*. Somatic mtDNA mutation spectra in the aging human putamen. PLoS Genet 2013; 9(12): p. e1003990.
[40] Yu-Wai-Man P, Griffiths PG, Gorman G, *et al.* Multi-system neurological disease is common in patients with OPA1 mutations. Brain 2010; 133(3): p. 771-86.
[41] Kraytsberg Y, Kudryavtseva E, McKee AC, *et al.* Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. Nat Genet 2006; 38(5): p. 518-20.
[42] Krishnan KJ, Ratnaike TE, De Gruyter HL, *et al*. Mitochondrial DNA deletions cause the biochemical defect observed in Alzheimer's disease. Neurobiol Aging 2012; 33(9): p. 2210-4.
[43] Vermulst M, Wanagat J, Kujoth GC, *et al.* DNA deletions and clonal mutations drive premature aging in mitochondrial mutator mice. Nat Genet 2008; 40(4): p. 392-4.
[44] Markaryan A, Nelson EG, Hinojosa R. Detection of mitochondrial DNA deletions in the cochlea and its structural elements from archival human temporal bone tissue. Mutat Res 2008; 640(1): p. 38-45.
[45] Eatock RA; Songer JE. Vestibular hair cells and afferents: Two channels for head motion signals. Annu Rev Neurosci 2011; 34: p. 501-34.
[46] Cantuti-Castelvetri I, Lin MT, Zheng K, *et al.* Somatic mitochondrial DNA mutations in single neurons and glia. Neurobiol Aging 2005; 26(10): p. 1343-55.
[47] Kennedy SR, Salk JJ, Schmitt MW, *et al*. Ultra-sensitive sequencing reveals an age-related increase in somatic mitochondrial mutations that are inconsistent with oxidative damage. PLoS Genet 2013; 9(9): p. e1003794.
[48] Michikawa Y, Mazzucchelli F, Bresolin N, *et al*. Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication. Science 1999; 286(5440): p. 774-9.
[49] Vermulst M, Bielas JH, Kujoth GC, *et al.* Mitochondrial point mutations do not limit the natural lifespan of mice. Nat Genet 2007; 39(4): p. 540-3.
[50] Taylor SD, Ericson NG, Burton JN, *et al.* Targeted enrichment and high-resolution digital profiling of mitochondrial DNA deletions in human brain. Aging Cell 2014; 13(1): p. 29-38.
[51] Moore CA, Gudikote J, Van Tuyle GC. Mitochondrial DNA rearrangements, including partial duplications, occur in young and old rat tissues. Mutat Res 1998; 421(2): p. 205-17.
[52] Skujina I, McMahon R, Lenis VPE, *et al*. Duplication of the mitochondrial control region is associated with increased longevity in birds. Aging (Albany NY) 2016; 8(8): p. 1781.
[53] McInerny SC, Brown AL, Smith DW. Region-specific changes in mitochondrial D-loop in aged rat CNS. Mech Ageing Dev 2009; 130(5): p. 343-9.
[54] Cassano P, Sciancalepore A, Lezza A, *et al.* Tissue-specific effect of age and caloric restriction diet on mitochondrial DNA content. Rejuvenation Res 2006; 9(2): p. 211-4.
[55] Frahm T, Mohamed SA, Bruse P, *et al.* Lack of age-related increase of mitochondrial DNA amount in brain, skeletal muscle and human heart. Mech Ageing Dev 2005; 126(11): p. 1192-200.
[56] Gomes AP, Price NL, Ling AJ, *et al.* Declining NAD+ induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging. Cell 2013; 155(7): p. 1624-38.
[57] Wachsmuth M, Huebner A, Li M, *et al*. Age-related and heteroplasmy-related variation in human mtDNA copy number. PLoS Genet 2016; 12(3): p. e1005939.
[58] Welle S, Bhatt K, Shah B, *et al.* Reduced amount of mitochondrial DNA in aged human muscle. J Appl Physiol 2003; 94(4): p. 1479-84.
[59] Miller FJ, Rosenfeldt FL, Zhang C, *et al*. Precise determination of mitochondrial DNA copy number in human skeletal and cardiac muscle by a PCR-based assay: lack of change of copy number with age. Nucleic Acids Res 2003; 31(11): p. e61.
[60] Pesce V, Cormio A, Fracasso F, *et al.* Age-related changes of mitochondrial DNA content and mitochondrial genotypic and phenotypic alterations in rat hind-limb skeletal muscles. J Gerontol A Biol Sci Med Sci 2005; 60(6): p. 715-23.
[61] Rocher C, Taanman JW, Pierron D, *et al.* Influence of mitochondrial DNA level on cellular energy metabolism: Implications for mitochondrial diseases. J Bioenerg Biomembr 2008; 40(2): p. 59.
[62] Huth M, Ricci A, Cheng A. Mechanisms of aminoglycoside ototoxicity and targets of hair cell protection. Int J Otolaryngol 2011; 2011: 937861.
[63] Quirós PM, Mottis A, Auwerx J. Mitonuclear communication in homeostasis and stress. Nat Rev Mol Cell Biol 2016; 17(4): p. 213-26.
[64] Shpilka T, Haynes CM. The mitochondrial UPR: mechanisms, physiological functions and implications in ageing. Nat Rev Mol Cell Biol 2018; 19(2): 109-20.
[65] Brosel S, Laub C, Averdam A, *et al*. Molecular aging of the mammalian vestibular system. Ageing Res Rev 2016; 26: p. 72-80.

[66] Gottlieb RA, Gustafsson AB. Mitochondrial turnover in the heart. Biochim Biophys Acta (BBA)-Mol Cell Res 2011; 1813(7): p. 1295-301.

[67] Kim I, Lemasters JJ. Mitophagy selectively degrades individual damaged mitochondria after photoirradiation. Antioxid Redox Signal 2011; 14(10): p. 1919-28.

[68] Williams SL, Huang J, Edwards YJ, *et al.* The mtDNA mutation spectrum of the progeroid Polg mutator mouse includes abundant control region multimers. Cell Metabol 2010; 12(6): p. 675-82.

[69] Phillips AF, Millet AR, Tigano M, *et al.* Single-Molecule Analysis of mtDNA Replication Uncovers the Basis of the Common Deletion. Mol Cell 2017; 65(3): p. 527-38.

[70] Stojkovič G, Makarova AV, Wanrooij PH, *et al.* Oxidative DNA damage stalls the human mitochondrial replisome. Sci Rep 2016; 6: p. 28942.