**Alternative oxidase rescues mitochondria-mediated dopaminergic cell loss in Drosophila**

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Mitochondrial dysfunction is commonly observed in degenerative disorders, including Alzheimer’s and Parkinson’s disease that are characterized by the progressive and selective loss of neuronal subpopulations. It is currently unclear, however, whether mitochondrial dysfunction is primary or secondary to other pathogenic processes that eventually lead to age-related neurodegeneration. Here we establish an in vivo Drosophila model of mitochondrial dysfunction by downregulating the catalytic subunit of mitochondrial DNA (mtDNA) polymerase in cholinergic, serotoninergic and dopaminergic neurons. The resulting flies are characterized by lowered respiratory chain activity, premature aging, age-related motor deficits as well as adult onset, progressive and cell-type-specific, dopaminergic neurodegeneration. Using this model, we find that associated lethality can be partially rescued by targeting PINK1/parkin signaling or Drp1, both of which have been implicated in mitochondrial dynamics and Parkinson’s disease. Bypassing mitochondrial complex III/IV deficiencies with Alternative oxidase (AOX), however, fully restores ATP levels and prevents dopaminergic neurodegeneration. In contrast, ATP levels and neurodegeneration are not rescued when mitochondrial complex I deficiencies are bypassed with NADH-Q oxidoreductase. Our results demonstrate that mtDNA-mediated mitochondrial dysfunction can cause age-related and cell-type-specific neurodegeneration which AOX is able to alleviate and indicate that AOX or its surrogates may prove useful as a therapeutic tool for limiting respiratory chain deficiencies caused by mtDNA decline in healthy aging and neurodegenerative disease.

INTRODUCTION

Adult-onset neurodegenerative disorders are characterized by the accumulation of protein-based deposits and the progressive loss of neuronal subtypes in specific regions of the nervous system (1). Of individuals over 65 years of age, typically 1–2% are diagnosed with Parkinson’s disease, whereas 13% suffer from Alzheimer’s disease (2). Heritable forms of the diseases, which account for only ~5–10% of cases, are often associated with mutations of pleiotropic genes that are expressed ubiquitously but only affect specific cell populations. The majority of neurodegenerative disease cases are sporadic where the cause of pathogenesis remains elusive. Environmental factors, for example pesticides in Parkinson’s disease (3), and genetic risk factors such as apolipoprotein E in Alzheimer’s disease (2), have been implicated in idiopathic cases.

Despite differences in disease symptoms, comorbidities and affected neuronal sub-populations, a large number of neurodegenerative disorders are characterized by defective mitochondrial remodeling and/or mitochondrial dysfunction (4,5), including Alzheimer’s disease (6), Parkinson’s disease (7) as well as Huntington’s disease (8) and amyotrophic lateral

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sclerosis (9). For example, in the case of Parkinson’s disease, several lines of experimental evidence suggest that defective mitochondrial dynamics is one of the pathogenic mechanisms leading to motor deficits and dopaminergic neurodegeneration (7). Significantly, mutations in Parkinson’s disease-related genes parkin, PINK1 and DJ-1 are all associated with mitochondrial alterations and/or deficits (10), suggesting that mitochondrial dysfunction is a major contributing factor to neurodegeneration and disease progression.

Functional mitochondria are critical for cell homeostasis and survival which is exemplified by animal models. These models reveal that targeted mutation or knock-out of genes involved in mitochondrial DNA (mtDNA) replication and repair phenocopy mitochondrial dysfunction and other physiological features of neurodegenerative disorders (11). For example, knockout of mitochondrial transcription factor TFAM in mice abolishes mtDNA expression, causing a severe respiratory chain deficiency which is embryonic lethal (12), and can result in progressive parkinsonism when targeted to dopaminergic neurons (13).

Expression of a proof-reading-deficient catalytic subunit of DNA polymerase γ (POLGα) can lead to accumulation of mtDNA alterations and premature aging and death (14,15). However, these mice are also characterized by unusually high levels of mtDNA mutations that are neither seen in normal aged wild-type mice (16) nor in patients with recognized mtDNA diseases (17). While illustrating the importance of mtDNA for correct mitochondrial function and cell survival, it is currently unclear whether mitochondrial alterations and dysfunction are a direct cause or consequence of age-related neurodegeneration.

Here we establish a Drosophila model of mitochondrial dysfunction using a combination of genetics, behavioral analysis and integrative physiology and show that downregulation of the catalytic subunit of mtDNA polymerase results in mitochondrial dysfunction and causes progressive motor deficits as well as adult onset, age-related neurodegeneration specific to dopaminergic cells. Moreover, we provide evidence that Alternative oxidase (AOX) is able to compensate reduced adenosine triphosphate (ATP) levels and mitochondria-mediated neurodegeneration in Drosophila. Our data suggest that AOX may prove useful for limiting respiratory chain deficiencies in healthy aging and age-related neurodegeneration.

RESULTS

Reduced mitochondrial gene expression impairs respiration and causes premature aging

To model senecescence-related mitochondrial dysfunction, we used Gal4/UAS-mediated RNA interference (RNAi) to knock down the catalytic subunit of the Drosophila mtDNA polymerase γ (POLGα or tamas) utilizing two separate
hairpin-loop UAS-RNAi lines that either target exon 2 or exon 3 of the POLGα transcript (Fig. 1A). POLGα-IR driven by the daughterless-Gal4 driver, which shows ubiquitous but moderate Gal4 activity during development, caused ∼80% knockdown of POLGα transcript level when compared with controls (Fig. 1B). This reduction in POLGα transcript levels was independent of the POLGα-RNAi strain used (Supplementary Material, Fig. S1A) and hence interchangeable. For subsequent experiments and analysis, we used line POLGα-IR unless stated otherwise.

Knockdown of POLGα decreased DNA levels of the mitochondrial encoded genes cytochrome c oxidase subunits I and III (COX1, COXIII) and cytochrome b (Cytb) (Fig. 1C) and protein levels of the mitochondrial encoded genes NADH-ubiquinone oxidoreductase chain subunit 5 (ND5) and COXI (Fig. 1D and E) and COXII (Supplementary Material, Fig. S1B). In all cases, a significant decrease in protein abundance was detected in adult POLGα-RNAi flies when compared with controls (Fig. 1D and E; Supplementary Material, Fig. S1B). Nuclear-encoded ATP synthase, subunit α (CytV), however, was unaffected (Fig. 1D and E). Together, these data show that partial reduction of POLGα is sufficient to decrease mitochondrial gene expression.

To assess the physiological effect of decreased mitochondrial gene expression, we measured the respiratory activity and total ATP content of POLGα-RNAi flies. In Act->POLGα-IR flies, complex I activity (Fig. 1F) and ATP levels (Fig. 1G) were significantly decreased when compared with controls. These data confirm that POLGα-RNAi directly affects mitochondrial function by decreasing expression of mtDNA-encoded respiratory chain subunits.

Next we determined whether decreased mitochondrial gene expression, and in turn decreased mitochondrial function, could directly affect aging. We studied the effects of POLGα-RNAi on survival using either Tubulin-Gal4 or Actin-Gal4, drivers that are ubiquitously and constitutively active during development, adulthood and aging. Tub->POLGα-IR mimicked tamas mutations (18) and caused larval/pupal lethality (Fig. 2A), whereas Act->POLGα-IR caused partial larval/pupal lethality (Fig. 2B) with adult escapers that were characterized by reduced longevity (Fig. 2C; Table 1). To exclude developmental effects, we knocked down POLG only in adult flies using the Gal80 system (19). These flies (Tub->TubP-Gal80°->POLGα-IR) were also characterized by a significantly shortened lifespan, with a median lifespan of 59 days and a maximum lifespan of 70 days, compared with controls that showed a median lifespan of 70 days and a maximum lifespan of 87 days (Fig. 2D; Table 1). These data demonstrate that adult-specific knockdown of POLGα is sufficient to decrease mitochondrial gene expression and to shorten lifespan in Drosophila.

Cell-type-specific mitochondrial dysfunction causes progressive behavioral deficits

Alzheimer’s disease is associated with age-related degeneration of cholinergic neurons, whereas loss of dopaminergic neurons is observed in Parkinson’s disease (3). To assess the effect of POLGα knockdown on these neuronal subpopulations in aging flies, we used choline acetyltransferase-Gal4 (Cha-Gal4) and tyrosine-hydroxylase-Gal4 (TH-Gal4) to drive POLGα-RNAi and measured climbing as a basic readout of neuronal function. Cha->POLGα-IR flies showed an age-related decline in climbing performance similar to controls (Fig. 3A), with a steep decline that we attribute to the presence of Dcr2 which is used to enhance RNAi efficacy (20). In contrast, TH->POLGα-IR flies showed a significant

![Figure 2. POLGα-RNAi affects development and survival dependent on phenotypic threshold effects. (A) Tubulin-Gal4-driven POLGα-RNAi (Tub->POLGα-IR) mimics tamas mutations and causes larval/pupal lethality. (B) Actin-Gal4-driven POLGα-RNAi (Act->POLGα-IR) causes larval/pupal lethality but also leads to adult flies. (C) Actin-Gal4-driven POLGα-RNAi (Act->POLGα-IR) results in substantially shorter lifespan compared with act+/+ controls. Kaplan–Meier data fitted to a Weibull distribution (dashed lines). Mean lifespans: Act->POLGα-IR = 25.4, Act+/+ = 69.7. (D) POLGα-IR knockdown in adult flies (Tub->TubP-Gal80°->POLGα-IR) results in significantly reduced lifespan compared with Tub->TubP-Gal80°/+ controls. Kaplan–Meier data fitted to a Weibull distribution (dashed lines). Mean lifespans: Tub->TubP-Gal80°->POLGα-IR = 56.9, Tub->TubP-Gal80°/+ = 69.3.](image-url)
age-related decline in climbing performance when compared with controls (Fig. 3B) which was 4.3-fold greater than controls ($P < 0.01$; Fig. 4A; Supplementary Material, Table S1); a second, independent $POLG\alpha$-IR strain showed a 2.4-fold greater decline than controls ($P < 0.001$; Fig. 4C; Supplementary Material, Table S1). These effects were not due to
premature aging, as adult TH>POLGa-IR flies showed normal lifespan (Fig. 4B and D; Table 1).

We reasoned, however, that climbing could be specifically sensitive to neuronal dysfunction of dopaminergic but not cholinergic cells. To address this, we used an open-field paradigm (21). When compared with controls, analysis of Day 62 Cha>POLGa-IR flies (Supplementary Material, Fig. S2A–D) did not reveal any differences in spontaneous activity, speed of walking or total distance traveled. In contrast, TH>POLGa-IR flies showed decreased activity (Fig. 3D and E) and, when moving, they walked at a slower speed (Fig. 3F) and covered less total distance (Fig. 3G) compared with controls, consistent with the observed decline in climbing performance of TH>POLGa-IR flies.

To address the possibility that cell-type-specific phenotypes might be due to neurotransmitter-related effects, we used tryptophan-hydroxylase-Gal4 (TRH-Gal4) to target POLGa-RNAi to serotonergic neurons and analyzed both climbing and open field behavior in aging TRH>POLGa-IR flies. When compared with age-matched controls, TRH>POLGa-IR flies did not show decreased climbing performance (Fig. 3C); they moved as fast as control flies and covered much distance as controls (Supplementary Material, Fig. S2E–H). However, a small but significantly decreased activity was detectable in aged TRH>POLGa-IR flies (Supplementary Material, Fig. S2F), suggesting that serotonergic neurons of old-aged flies become vulnerable to decreased mitochondrial function. These data demonstrate that decreasing mitochondrial gene expression can cause age-related and cell-type-specific behavioral deficits, indicating that dopaminergic neurons, but not cholinergic or serotonergic neurons, are specifically vulnerable to POLGa-RNAi-mediated decrease in mitochondrial gene expression.

**Decreased mitochondrial gene expression sensitizes specific neurons to degeneration**

We next investigated whether POLGa-RNAi can lead to age-related neurodegeneration. We examined mid-aged (Day 31) and old-aged (Day 70) brains of flies with or without POLGa-RNAi targeted to dopaminergic, serotonergic or cholinergic neurons. To visualize and number targeted neurons, we expressed a membrane-bound form of green fluorescent protein (GFP) (UAS-mCD8::GFP) alone or together with UAS-POLGa-RNAi in a cell-type-specific manner (Supplementary Material, Fig. S3). The number of GFP-positive cells was counted to determine the neuron survival at Day 31 and Day 70 (Fig. 5A–D).

Analysis of Cha>POLGa-IR flies did not show any neuron loss (Fig. 5A), whereas TRH>POLGa-IR flies revealed serotonergic neuron loss only in old-aged flies (Fig. 5B), suggesting that serotonergic neurons are partially vulnerable to decreased mitochondrial gene expression but only at old age in Drosophila. Conversely, TH>POLGa-IR flies showed a

**Figure 4.** POLGa knockdown in dopaminergic cells causes progressive motor deficits but does not affect lifespan. (A) Linear regression fit of TH>Dcr2, GFP, POLGa-IR compared with control (TH>Dcr2, GFP) shows significant and progressive decline in climbing performance. (B) Lifespan analysis of TH>Dcr2, GFP, POLGa-IR reveals no significant changes versus control (Gehan–Wilcoxon test, \( P = 0.105 \), Table 1). (C) A second, independent POLGa-RNAi strain (TH>Dcr2, GFP, POLGa-IR') shows similar age-related decline in climbing performance compared with control (TH>Dcr2, GFP). (D) Lifespan comparison of flies expressing POLGa-IR in dopaminergic neurons shows no alterations compared with controls (Gehan–Wilcoxon test, \( P = 0.107 \), Table 1). (A, C) Linear regression slopes compared using ANOVA. **\( P < 0.01 \), ***\( P < 0.001 \); see also Supplementary Material, Table S1 for statistical analysis.
significant and age-related decrease in dopaminergic neurons. When compared with age-matched controls, knockdown of POLGa caused an average loss of 14/80 dopaminergic neurons by Day 31 and an average loss of 20/80 dopaminergic cells by Day 70 (Fig. 5C). Two-way analysis of variance (ANOVA) and post-hoc analysis of Day 31 and Day 70 data revealed that TH>POLGa-IR caused a significant 16.5% loss of dopaminergic neurons at Day 31 and a larger 23.4% loss of dopaminergic neurons at Day 70 compared with controls. This phenotype was confirmed by significantly increased dopaminergic neuron loss between Day 31 and Day 70 (Fig. 5C; Supplementary Material, Fig. S4), suggesting progressive neurodegeneration. In comparison, controls (TH>Dcr2, mCD8::GFP) showed no cell loss between age groups (Fig. 5C), as observed previously (21). Moreover, neuron numbers were unaltered in 5-day-old TH>POLGa-IR flies (Fig. 5C). Together, these data demonstrate that POLGa-RNAi-mediated decrease in mitochondrial gene expression can cause adult-onset and progressive dopaminergic neurodegeneration.

Dopaminergic neurons in the adult fly brain can be grouped into clusters that show stereotypical axonal projections to target regions that represent distinct functional areas (21) (Supplementary Material, Fig. S3A–D). We therefore characterized cluster-specific dopaminergic neuron loss in TH>POLGa-IR flies (Fig. 5D). In controls, neuron numbers at mid-age and late-life remained the same in each cluster analyzed, as observed previously (21). In contrast, TH>POLGa-IR flies showed cluster-specific and age-related neuron loss. A significantly increased loss of dopaminergic neurons was seen in the PPL1 and PPM1/2 clusters when comparing mid-aged and old-aged TH>POLGa-IR flies, whereas neuron loss occurred early in PPM3 and PAL clusters, but did not increase significantly with age (Fig. 5D).

Figure 5. Mitochondrial dysfunction causes age-related and cell-type-specific neurodegeneration. (A) Cholinergic neuron numbers with or without POLGa-IR are unaltered at Day 31 and Day 70. (B) POLGa knockdown in serotonergic neurons only affects cell number at Day 70. (C) TH-Gal4-driven POLGa-IR affects neuron numbers at Day 31, with progressive loss until Day 70. Analysis of TH>POLGa-IR at Day 5 revealed no significant neuron loss compared with Day 30 \((P = 0.45)\), demonstrating adult-onset neurodegeneration. (D) Progressive neurodegeneration affects individual dopaminergic cell clusters. For all three genotypes, the numbers of GFP-labeled cells were recorded per brain hemisphere. \(^* P < 0.05, \quad ** P < 0.01, \quad *** P < 0.001\). Error bars, SEM.
Figure 6. Mitochondria-mediated neurodegeneration is cell-type-specific. (A, B) Confocal images of whole-mount adult brain immunolabeled with anti-TH visualizing dopaminergic neurons. (C, D) Anti-5HT immunolabeling of serotonergic neurons whole-mount adult brain. (E–G) Pan-neuronal Elav-Gal4-driven UAS-POLGα-RNAi knockdown specifically affects dopaminergic neurons (E) but not serotonergic neurons (F). (G) Loss of anti-TH labeled cells indicates loss of dopaminergic neurons in aging Elav>_POLGα-IR flies; the observed degenerative cell loss affects all dopaminergic cell clusters except PAL. For neuron counts, numbers of anti-5HT and anti-TH labeled cells were recorded per brain hemisphere, respectively. Scale bars: 100 μm. ∗∗∗P < 0.001. Error bars, SEM.

Table 2. Genetic rescue of lethality caused by POLGα knockdown

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<th>Responder</th>
<th>Actin-Gal4</th>
<th>CyO</th>
<th>Total</th>
<th>% with Actin-Gal4</th>
<th>% Expected versus w^{118}</th>
<th>% Expected versus genetic control</th>
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<td>156</td>
<td>2.56</td>
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Number of flies eclosed with Actin-Gal4-driven UAS-POLGα-IR either alone or in combination with selected genes (responder). Actin-Gal4/CyO crossed to w^{118} served as control. ‘Actin-Gal4’ shows the number of flies eclosed with Actin-Gal4 driver; ‘CyO’ shows the number of flies eclosed with CyO balancer; ‘Total’ shows the total number of flies eclosed; ‘% with Actin-Gal4’ shows the number of Actin-Gal4 flies divided by the total number of eclosed flies. The 48.3% eclosed flies from control cross Actin-Gal4/CyO to w^{118} without CyO were set as 100% expected w^{118} eclosion. ‘% Expected versus w^{118}’ shows the percentage of Actin-Gal4 flies eclosed divided by 48.3%. ‘% Expected versus genetic control’ shows percentage expected versus w^{118} divided by the respective genetic control data in % Expected versus w^{118}. TH, tyrosine hydroxylase; Drp1, dynamin-related protein 1; dhd, deadhead; Ndi1, yeast-derived NADH dehydrogenase Ndi1; AOX, C. intestinalis-derived Alternative oxidase.

To exclude the possibility that differences in neuron loss were dependent on Gal4 drivers used, we utilized the pan-neuronal Elav-Gal4 driver. We counted the number of neurons using antibodies specific to tyrosine hydroxylase (anti-TH) (Fig. 6A and B; Supplementary Material, Fig. S3A and C) or serotonin (anti-5HT) (Fig. 6C and D; Supplementary Material, Fig. S3E and G) and observed a significant loss of dopaminergic neurons (Fig. 6E), but not serotonergic neurons (Fig. 6F) in 60-day-old Elav> POLGα-IR flies when compared with age-matched controls. In addition, we also observed cluster-specific dopaminergic neuron loss in Elav> POLGα-IR brains (Fig. 6G). These data confirm that decreased mitochondrial gene expression can trigger age-related and cell-type-specific neurodegeneration in Drosophila.

Parkinson’s disease-related genes partially rescue POLGα knockdown effects

In Parkinson’s disease, mitochondrial dysfunction and age-related loss of dopaminergic neurons has been linked to pathogenic PINK1/Parkin signaling and altered fission/fusion (10). We therefore wondered whether targeting Parkinson’s disease-related genes implicated in PINK1/Parkin signaling or mitochondrial fission/fusion could ameliorate Act> POLGα-IR-induced lethality (Fig. 2B, Table 2) and co-expressed in an Act> POLGα-IR genetic background either parkin that promotes mitophagy or dynamin related protein 1 (Drp1) which induces mitochondrial fission (10).

Analysis of Act> POLGα-IR, park flies revealed that parkin was able to partially rescue POLGα-RNAi-mediated lethality.
Co-expression of Drp1 with POLG a-RNAi (Act>POLG a-IR, Drp1) rescued lethality by 64.0% when compared with control (Act>Drp1; Fig. 7C). These data suggest that the Parkinson’s disease-related genes parkin and Drp1 partially rescue POLG a-RNAi-mediated lethality.

Oxidative stress has been related to mitochondrial dysfunction and dopamine-specific neurodegeneration (4). We therefore wondered whether targeting the antioxidative response might be able to rescue Act>POLG a-IR-induced lethality. We chose to target thioredoxin, an evolutionarily conserved antioxidant and redox enzyme (22), that is able to block Parkinson’s disease-related MPP + -toxicity affecting mitochondrial complex I activity in human cells (23) and can rescue behavioral deficits and selective loss of dopaminergic neurons in Drosophila (24). Expression of the Drosophila thioredoxin homolog deadhead (dhd) alone was benign and had no effect on viability (Table 2). Co-expression of dhd with POLG a-RNAi (Act>POLG a-IR, dhd) rescued lethality by 67.7% when compared with control (Act>dhd; Fig. 7D). These data show that dhd partially rescues POLG a-RNAi-mediated lethality.

Bypassing respiratory chain deficiency rescues cell-type-specific neurodegeneration

Knockdown of POLG a causes decreased mitochondrial gene expression and in turn decreases both respiratory activity and ATP levels (Fig. 1). We therefore tested whether components of the respiratory chain have impact on Act>POLG a-IR-induced lethality. For this, we used the NADH dehydrogenase Nd1 from Saccharomyces cerevisiae and AOX from the sea squirt, Ciona intestinalis. Nd1 consists of a single polypeptide chain that acts as the main entry point to the respiratory chain similar to mammalian mitochondrial complex I (25). Nd1 can bypass Complex I deficiencies and, when expressed in Drosophila, is able to mitigate the age-related decline in respiratory capacity and the

Figure 7. AOX restores ATP levels and rescues dopaminergic neurodegeneration. (A) Strong knockdown of POLG a causes lethality, also in the presence of tyrosine hydroxylase; compare with Act>POLG a-IR control in Figure 2B. Act>POLG a-IR-induced lethality can be partially rectified by simultaneous expression of (B) Parkin involved in mitophagy; (C) dynamin-related protein 1, Drp1, involved in mitochondrial remodeling; (D) the thioredoxin homolog and antioxidant deadhead, dhd; and (E) yeast-derived NADH dehydrogenase Nd1. (F) In contrast, sea squirt-derived AOX fully rescued Act>POLG a-IR-induced lethality. (G) POLG a-RNAi-driven dopaminergic neurodegeneration at Day 31 can be rescued with AOX but not Nd1 expression. Control and POLG a-IR (Day 31) neuron counts from Figure 5B and D are shown for clarity. (H) AOX expression restores ATP production in adult POLG a knockdown flies (n = 5 in duplicate). *P < 0.05, **P < 0.01, ***P < 0.001. Error bars, SEM.
accompanying increase in mitochondrial reactive oxygen species (ROS) (26). AOX is a single-subunit ubiquinol oxidase found in plant mitochondria, as well as in the mitochondria of fungi, protists and animals, but not in arthropods or vertebrates (27). AOX from C. intestinalis is able to complement cytochrome c oxidase deficiency in human cells (28) and in Drosophila can bypass Complex III and IV deficiencies (29).

Ectopic expression of Ndi1 partially rescued POLGα-RNAi-mediated lethality as 73.0% of Act>POLGα-IR, Ndi1 flies eclosed (Fig. 7E) when compared with controls (Act>Ndi1, P = 0.003). The proportion of eclosed Act>POLGα-IR, Ndi1 flies was significantly greater than Act>POLGα-IR flies (Fig. 2B). Analysis of Act>POLGα-IR, AOX flies showed that ubiquitous co-expression of AOX with POLGα-RIR completely rescued POLGα-RNAi lethality (Fig. 7F). Significantly, all Act>POLGα-IR, AOX flies eclosed when compared with control. This AOX-mediated rescue of POLGα-RNAi-induced lethality was unlikely to be caused by Gal4 dilution as ubiquitous co-expression of TH with POLGα-IR (11.5%, Fig. 7A) closely resembled Act>POLGα-IR (8.4%, Fig. 2B).

Next we determined whether AOX and Ndi1 were able to rescue dopamine-specific neurodegeneration caused by POLGα knockdown. As a control, we ectopically expressed Ndi1 or AOX by TH-Gal4, and neither resulted in any loss of dopaminergic neurons (Supplementary Material, Fig. S4). When expressed together with POLGα-RNAi, analysis of TH>POLGα-IR, Ndi1 flies revealed that yeast Ndi1 was not able to prevent neurodegeneration in any of the dopaminergic clusters (Fig. 7G). In contrast, analysis of 31-day-old TH>POLGα-IR, AOX flies showed that expression of AOX was sufficient to protect against neurodegeneration in all dopaminergic neuron clusters that are normally affected in age-matched TH>POLGα-IR flies (Fig. 7G).

When expressed in Drosophila, AOX can complement defects in mitochondrial oxidative phosphorylation (29). We therefore wanted to know whether the neuroprotective potential of AOX expression (Fig. 7G) might be mediated by a restoration of mitochondrial metabolism in POLGα knockdown flies. Hence we measured total ATP content of adult Act>POLGα-IR, AOX flies and compared them with adult age-matched Act>POLGα-IR and Act>AOX flies. Although AOX expression on its own had no significant effect on ATP levels (P = 0.307), co-expression of AOX with POLGα-RNAi significantly improved ATP levels and returned them to control levels when compared with Act>POLGα-IR flies (Fig. 7H). Together, these data show that AOX expression in Drosophila is able to compensate for POLGα-RNAi-mediated lethality, decreased ATP levels and cell-type-specific neurodegeneration.

**DISCUSSION**

Our results demonstrate that decreasing levels of mitochondrial gene expression in Drosophila results in premature aging and age-related as well as progressive and cell-type-specific neurodegeneration which AOX is able to alleviate. These findings have implications for understanding mitochondria-mediated mechanisms of aging and cell-type-specific neurodegeneration and they identify AOX as a potential therapeutic tool for respiratory chain deficiencies in mtDNA disorders and neurodegenerative diseases.

**A Drosophila model of mtDNA-mediated mitochondrial dysfunction**

We used targeted knockdown of POLGα to model mitochondrial dysfunction in Drosophila. POLGα encodes the catalytic subunit of mtDNA POLGα which is the sole mitochondrial polymerase required for mtDNA transcription (18). In Drosophila, as in other eukaryotes, mtDNA encodes several components of mitochondrial complexes I, III, IV and V of the electron transport chain. Our Drosophila model of mitochondrial dysfunction decreases the availability of functional wild-type POLGα and in turn mtDNA-encoded proteins required for mitochondrial respiration. Previous studies suggested that a minimal threshold level of mitochondrial damage or decline is required to cause respiratory chain deficiency and mitochondrial dysfunction (17) that can impact on aging (30). In our model, the resulting phenotypic effects of mitochondrial dysfunction depend on transgene activity and hence Gal4 driver and UAS responder efficacy. Ubiquitous POLGα knockdown causes either larval/pupal lethality or shortened lifespan. Our data therefore provide experimental evidence that phenotypic threshold effects of mitochondrial dysfunction (31) impact on aging and survival in Drosophila. Aging in Caenorhabditis elegans and Drosophila (32,33), fish (34) as well as in mice (35), rats (36) and humans (37) has been characterized by downregulation of mitochondrial genes, including those encoded by mtDNA (38). Our results demonstrate that adult-onset knockdown of Drosophila POLGα is sufficient to shorten lifespan, indicating that decreasing mitochondrial gene expression can recapitulate an evolutionarily conserved molecular aging mechanism (35,39).

In addition to an aging phenotype, our POLGα-IR experiments establish that cell-type-specific decreases in mitochondrial gene expression can trigger progressive motor deficits and dopaminergic neurodegeneration that resemble major manifestations of familial and sporadic Parkinson’s disease (40). These progressive phenotypes do not occur in young flies, but require advancing age before cell loss is observed. It is conceivable that the progressive phenotypes observed in our fly model are the combined consequence of POLGα-IR-induced low base-line levels of mitochondrial gene expression together with the natural age-related decline. We previously showed that under normal wild-type conditions, at least in Drosophila, age-related dopaminergic neurodegeneration is not typically observed (21), suggesting that senescence-related decrease in mitochondrial gene expression of wild-type Drosophila is insufficient to cause neurodegeneration. Together with our previous data, our POLGα-IR findings therefore suggest that phenotypic manifestation of mitochondrial dysfunction occurs only when a threshold level is exceeded which in turn can cause age-related and cell-type-specific neurodegeneration.
Dopaminergic neurons are selectively vulnerable to mitochondrial dysfunction

Mitochondrial dysfunction is commonly observed in neurodegenerative disorders that target different neuronal subpopulations (4,5). For example, cholinergic neurons preferentially degenerate in Alzheimer’s disease (41), whereas loss of dopaminergic neurons is a pathological hallmark of Parkinson’s disease (7). This selective, disease and cell-type-specific neurodegeneration raises the question how particular neuronal sub-populations are selectively vulnerable to disease formation. We show that in aging flies, decreasing mitochondrial gene expression specifically targets dopaminergic neurons for progressive neurodegeneration. POLGα-IR did not affect the survival of cholinergic neurons when targeted to this neuronal subpopulation, and when POLGα-IR was targeted to serotonergic neurons, phenotypic defects were only observed in old aged flies. These data suggest that serotonergic and especially dopaminergic neurons are specifically vulnerable to defective mitochondria.

Dopaminergic neurons are characterized by high-intracellular calcium levels and high-energy demands that are partly due to widely ramified axonal branches and dense dendritic arborizations (40)—also seen in flies (21)—and require effective axonal transport of mitochondria (42). Defective sequestration of dopamine into vesicles can lead to cytoplasmic accumulation and auto-oxidation which ultimately leads to the generation of reactive oxygen and other toxic species that can damage nucleic acid, proteins and lipids (40). Our data show that POLGα-IR-mediated mitochondrial dysfunction specifically sensitizes dopaminergic neurons to degenerative cell death in aging flies. Time-of-onset and progression of dopaminergic neurodegeneration suggest that the pathological process depends on a combination of age-related mitochondrial dysfunction and at least another cell-type-specific stressor (43). Lowering levels of tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis, has been shown to ameliorate α-synuclein- or rotenone-induced phenotypes in Drosophila models of Parkinson’s disease (44). This suggests that in combination with POLGα-RNAi, dopamine or dopamine-related stressors might be sufficient to trigger phenotypic threshold effects in aging Drosophila that cause age-related and progressive degeneration of dopaminergic neurons.

Respiratory chain deficiency can cause age-related neurodegeneration

Respiratory chain deficiencies are frequently observed in neurodegenerative diseases (4,5) and several lines of evidence suggest that defective respiration specifically contributes to dopaminergic cell loss in Parkinson’s disease. For example, mutation of the Parkinson’s disease-associated gene PINK1 has been associated with complex I deficiency (45–48) and genetically induced respiratory chain deficiencies in mice are associated with fragmentation of the mitochondrial network and dopaminergic cell loss (49). We used targeted knockdown of POLGα to model mitochondrial dysfunction and our data show that POLGα-IR results in decreased expression of mtDNA-encoded genes and proteins. The resulting phenotypic effects include respiratory chain deficiency, lowered ATP levels and age-related dopaminergic neurodegeneration, suggesting that defective respiration is causally related to adult-onset and cell-type-specific neurodegeneration in Drosophila.

Previous studies identified Ndi1 and AOX as potential tools to counteract respiratory chain deficiencies in human cells and animals (28,50,51). We have used both enzymes for heterologous expression in Drosophila in an attempt to counteract phenotypic effects of POLGα-IR. Yeast Ndi1 is a single-subunit enzyme of the mitochondrial matrix catalyzing NADH-quinone oxidoreduction similar to complex I but without proton pumping function. Application of Ndi1 is able to ameliorate phenotypes in acute models of Parkinsonism where MPP+ or rotenone treatment directly affect mitochondrial complex I (51), and recent data suggest that it can rescue, at least to some extent, altered mitochondrial morphology and synaptic dysfunction in PINK1-mutant flies (52). Interestingly, however, Ndi1 was not able to rescue lethality and dopaminergic neurodegeneration in our POLGα-IR-mediated model of mitochondrial dysfunction. POLGα-IR affects the expression not only of mitochondrial complex I components, but also of components of complexes III, IV and V that are encoded by mtDNA, indicating that bypassing complex I with Ndi1 is insufficient to compensate for the phenotypic effects of mtDNA-mediated mitochondrial dysfunction. Moreover, this also indicates that respiratory chain deficiencies other than complex I, such as complex IV deficiencies also seen in Parkinson’s disease (53), significantly contribute to age-related neurodegeneration.

We identified AOX as a potent alternative enzyme to counteract mtDNA-mediated respiratory chain deficiency and mitochondrial dysfunction. Previous studies have shown that AOX can partially replace the electron transfer chain by directly receiving electrons from ubiquinol to reduce molecular oxygen to water, thus bypassing complexes III and IV of the oxidative phosphorylation system; it can prevent metabolic acidosis and overproduction of the quinone pool and complements respiratory deficiencies in human cells (28,50). When expressed in Drosophila, AOX decreases mitochondrial ROS production and compensates phenotypes caused by mutations affecting complex IV or the Parkinson’s disease-related gene DJ-1B (29,54). Our findings demonstrate that AOX expression in Drosophila is able to restore POLGα-IR-mediated phenotypes, including reduced ATP levels and adult-onset, age-related dopaminergic neurodegeneration in the presence of defective mitochondria. Together with previous studies, this suggests that AOX may prove useful for limiting respiratory chain deficiencies caused by mtDNA decline as seen in healthy aging (38,55) and age-related neurodegenerative diseases (7,56).

MATERIALS AND METHODS

Drosophila strains and genetics

Flies were obtained from the Bloomington Stock Centre and raised at 25°C in a 12 h light/dark cycle using a standard/agar diet unless stated otherwise. For POLGα knockdown, we used UAS-POLGα-IR lines from the VDRC (#106955; mRNA: 2176 → 2852; and #3133; mRNA: 3075 → 3359).
Reverse transcription-polymerase chain reaction

Four flies per genotype were homogenized using a pestle (Fisher) in 125 μl of Trizol (Invitrogen) per fly until no body structures were identifiable. RNA extraction using Trizol was performed following the manufacturer’s instructions. RNA was resuspended in nuclease-free (diethylpyrocarbonate-treated) H₂O using 1 μl of water per 10 μl of Trizol. RNA content was measured using a NanoDrop (Thermo Scientific); concentrations were typically 150–270 μg/ml.

For the reverse transcription reaction, 1 μg of DNase-treated RNA was amplified for 60 min at 37°C using mouse megavirus reverse transcriptase (M-MLV RT; Promega) and random hexamer oligonucleotide primers (Fermentas) following the manufacturer’s instructions. M-MLV RT was inactivated by heating to 70°C for 15 min. cDNA was stored at −20°C for later use.

To measure POLGα RNA levels, cDNA was amplified using polymerase chain reaction (PCR) in a series of increasing cycle numbers to obtain the linear phase of amplification. PCR reactions were carried out to compare POLGα transcripts in experimental conditions; as a control, equal amounts of cDNA present in the starting reaction was confirmed by measuring d-actin.

Primers used were:

**polymerase γ subunit α (POLGα)**
- forward primer: 5′-TCCATAACGGCACAAGGCGGTCG-3′
- reverse primer: 5′-TGCGGAGACACAAAGGAAGCG-3′

**d-actin**
- forward primer: 5′-ACTTTCTGCTGAAAGTGGAC-3′
- reverse primer: 5′-AATCCGCAAGGATCTGATGC-3′

Amplified DNA was separated by electrophoresis in 1.1% agarose. DNA was visualized using ethidium bromide and digitally imaged by a computer-mounted camera. Grey value of the DNA bands captured in the digital gel images were measured using plot profile in FIJI image processing package (http://pacific.mpi-cbg.de).

mtDNA analysis

Total DNA was extracted from experimental and control flies using lithium chloride/potassium acetate. Ten whole bodies per extraction were homogenized in 400 μl of buffer [100 mM Tris–HCl, pH 7.5, 100 mM NaCl, 100 mM ethylene-diaminetetraacetic acid (EDTA), 0.5% w/v sodium dodecyl sulfate (SDS)] using a pellet pestle in a 1.5 ml tube and incubated at 65°C for 30 min to denature protein. Eight hundred microliters of 2.5 5 M KAc 6 M LiCl solution was added, the samples were incubated on ice for 15 min and centrifuged at 16 000g for 15 min. The resultant supernatant was transferred to a fresh tube and 600 μl of ice-cold isopropanol was added, inverted to mix and centrifuged once again for 17 min. Isopropanol was discarded and the pellet was washed twice with 70% ethanol. The DNA pellet was re-suspended in 75 μl of ddH₂O. mtDNA-encoded genes were amplified using PCR. Primers used were:

**cytochrome c oxidase subunit I (COXI):**
- forward primer: 5′-GGTGCTCCTGATATAGCATTCCCA CGA-3′
- reverse primer: 5′-CTCCCTCTCCCAGCCTGGTCA-3′

**cytochrome c oxidase subunit III (COXIII):**
- forward primer: 5′-TGACATTAACAGGAGCTATCGGA GC-3′
- reverse primer: 5′-TGATGCTCCTAATCTCCGCG-3′

**cytochrome b (Cytb)**
- forward primer: 5′-CGAATTTTACATGCTAACGGGTG-3′
- reverse primer: 5′-CGGATTGTCTACTTAAAAGAGTGTG-3′

Subsequent analysis was carried out as for RT-PCR.

Western blotting of mitochondrially encoded proteins

Flies were homogenized into 200 μl of lysis buffer [radioimmunoprecipitation assay (RIPA) buffer: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% v/v NP-40, 5 mM EDTA, 0.5% w/v sodium deoxycholate, 0.1% SDS] with protease and phosphatase inhibitor cocktails (1 cOmplete Mini (Roche) and 1 PhosSTOP (Roche) tablet in 7 ml of RIPA). Particular matter was pelleted by centrifugation, and protein content in the supernatant was measured using the BioRad DC protein assay (BioRad). Supernatant samples were prepared by diluting samples 5:1 in 5× loading buffer (312.5 mM Tris–HCl, pH 6.8, 10% w/v SDS, 250 mM dithiothreitol, 50% v/v glycerol) followed by boiling for 3 min. Samples were electrophoresed using a NuPage 4–12% Bis–Tris Gel and transferred to HyBond ECL nitrocellulose membrane (GE Healthcare). After blocking for 2 h using phosphate-buffered saline supplemented with 0.05% Triton X-100 and 5%
non-fat milk (M-PBST), proteins of interest were detected using anti-ND5 (1:100; Abcam), anti-COXI (1:5000; MitoSciences), anti-ATP synthase subunit α (1:20 000; MitoSciences) and anti-β-tubulin (1:200; Developmental Studies Hybridoma Bank) incubated overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were used at dilutions optimized for each primary antibody and detected using electrochemiluminescence. Images were collected digitally and band intensities were quantified by densitometry using the GeneTools image analysis software (Syngene) and normalized for protein loading using β-tubulin.

**ATP measurements**

Four to five pupae were homogenized in 50 µl of lysis buffer (100 mM Tris, 4 mM EDTA) with 6 M guanidine. Five adult flies were homogenized into 200 µl of lysis buffer without guanidine and snap-frozen in liquid nitrogen. Samples were boiled for 3 min followed by centrifugation for 5 min at 8000g. Supernatant from adult samples was diluted 1:50 using extraction buffer. ATP levels were determined from 5 µl of lysate using ATP Determination Kit (Invitrogen) in a Wallac Victor2 Multilabel photon counter (ThermoFisher) at 28°C as described previously (62) and normalized to protein content using a Bradford assay (Bio-Rad).

**Complex I activity measurements**

Complex I activity was measured using a 96-well colorimetric assay as described previously (62).

**Startle-induced negative geotaxis assay**

Climbing performance was analyzed using a startle-induced negative geotaxis assay as described previously (62).

**Motion tracking**

Open-field locomotion was analyzed using an assay described previously (21) with the following modifications: the arena for video motion tracking was constructed from the lid of a 9 cm plastic Petri dish. Twelve flies were briefly anesthetized with CO2 and placed in an arena, which was then placed above an array of white light-emitting diodes within a temperature-controlled incubator (Stuart Scientific). Flies were allowed to recover for 1 h before recordings were made at 25 frames per second for 30 min with Virtaldub (http://www.virtua.ldub.org). Videos were compressed as they were being recorded with an MPEG-4-compatible Xvid codec from ffdshow tryouts (http://ffdshow-tryout.sourceforge.net/). Recorded videos were converted to fly movie format from the motmot package (64) which was loaded into Ctrax software (65) to analyze the walking positions of the flies through the video. Position data for 30 min was exported as a Matlab-compatible (Mathworks) matrix file. Errors in the tracking were fixed using FixErrors GUI (65) in Matlab (Mathworks). Fixed trajectories were loaded into GNU Octave 3.2.4 (http://www.gnu.org/software/octave/) and custom scripts were used to determine mean velocity, mean activity and mean cumulative distance. Activity was defined as a movement per frame above a velocity of 0.5 mm/s. Average activity was the percentage of frames where the fly was active (>0.5 mm/s velocity). Mean velocity was the average of velocities in each frame of the recording only when the fly was active.

**Immunohistochemistry**

Immunolabeling of adult brains was carried out as described previously (21). Primary antibodies were: 1:50 mouse anti-tyrosine hydroxylase (α-TH; Immunostar, 22941) to stain dopaminergic neurons, 1:500 rabbit anti-serotonin (α-5HT; Sigma-Aldrich, S5545) to stain serotoninergic neurons and 1:100 mouse anti-choline acetyltransferase (α-ChAT; Developmental Studies Hybridoma Bank [DSHB]) to stain cholinergic neurons. Secondary antibodies were goat anti-mouse or anti-rabbit 568 Alexa fluorochromes at 1:150 (Invitrogen).

**Confocal microscopy and image processing**

Confocal microscopy and image acquisition were performed with a Leica TCS SP5 laser confocal microscope and Leica Application Suite Advanced Fluorescence (LASAF) version 2.0.2 software. Images were scanned at 1024 × 1024, with a slice thickness of 1.75 µm and a line and frame average of 2. Different channels were scanned sequentially to avoid bleed-through. Confocal stacks were loaded into FIJI image processing package (http://pacific.mpi-cbg.de) using LOCI Plugins for ImageJ version dev-4.2.7503 (http://www.loci.wisc.edu/software). Z-projections were created and analyzed using FIJI image processing package.

**Neuron counting**

For cell counting, ImageJ Cell Counter Plugin (Kurt De Vos, http://rsbweb.nih.gov/ij/plugins/cell-counter.html) was used. For counting of DA neurons, whole-mount heterozygous TH>α-IR females with or without POLGa-IR were analyzed as described previously (21) with the exception of PPL1, where we did not include the more lateral cells in that cluster. For serotoninergic neurons, whole-mount heterozygous TRH>α-IR females with or without POLGa-IR were counterstained for serotonin and those cells overlapping GFP fluorescence with anti-serotonin were counted. For cholinergic neurons, two regions of whole-mount heterozygous Cha>α-IR females with or without POLGa-IR were analyzed. Anteriorly, the cell cluster immediately adjacent to the mushroom body calyx contained within one Z-stack slice was counted. Posteriorly, a 20 µm cube was selected and the cell bodies contained within that region were counted. For all three genotypes, the numbers of cells per hemisphere were recorded. For brains from Elav>α-IR flies with or without POLGa-IR, the number of cell bodies stained with anti-TH for DA neurons or anti-5HT for serotoninergic neurons were counted at Day 61.

**Lifespan analysis**

Lifespan analysis was performed essentially as described previously (21). Virgin females from the driver lines were crossed...
with UAS-POLGα-RNAi males. Synchronized egg collection was carried out on apple agar plates (2.13% w/v agar, 1.25% w/v sugar, 25% v/v apple juice, 0.2% w/v Nipagen), which were swapped twice a day. Lifespans were performed on 15% sugar/yeast medium at a maximum density of 15 flies per tube at 25°C. For temperature-sensitive repression of Gal4 activity using TubP-Gal80ts transgene to measure the effect of adult-only POLGα-IR (Tub>Usp-Gal80ts->POLGα-IR), developmental stages were reared at 18°C. Two days after eclosion, adult flies were transferred to 25°C to carry out lifespans. The probability of surviving to time t, \( S(t) = Pr(T > t) \), was calculated by the Kaplan–Meier survival function. Differences in lifespan were only considered if they were above 5% greater or lower than the median lifespan of the control group.

**Survival assay (fatality/eclosion)**

Female virgin UAS-POLGα-RNAi flies were crossed with male Actin5C-Gal4/CyO driver flies to generate heterozygous Act>POLGα-IR expressor flies or POLGα-IR/CyO controls. Parent flies were left to lay eggs for 2 days and then transferred to a fresh vial. The vial with laid eggs was allowed to develop for 20 days and the number of progeny of each genotype was counted. The proportion of flies eclosing was calculated as the number of flies eclosed divided by the total number of flies eclosed, i.e. including the CyO internal control. For the control genotypes, this was normalized by the total number of flies eclosed, i.e. including the CyO internal control. For additional genotypes tested, e.g. POLGα-IR in combination with UAS-Drp1WT, the experimental condition (Act>POLGα-IR, Drp1) was compared against the control condition without POLGα-IR, e.g. Act>Drp1 (see Table 2).

**Statistical analysis**

For analysis of variance (ANOVA), two-way ANOVA of neuron counts, lifespan analysis and linear regression comparisons R Project 2.9.2 (http://www.r-project.org/) was used. To assess whether the slopes in the climbing experiments were statistically different between TH>POLG-IR and controls flies, the lm() function was used with coincidence. Linear model data were then compared with the anova() function to carry out lifespans. The probability of surviving to time \( t \) is the \( S(t) = Pr(T > t) \), was calculated by the Kaplan–Meier survival function. Differences in lifespan were only considered if they were above 5% greater or lower than the median lifespan of the control group. 

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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