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# Expression of the alternative oxidase mitigates beta-amyloid production and toxicity in model systems

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ABSTRACT

Mitochondrial dysfunction has been widely associated with the pathology of Alzheimer's disease, but there is no consensus on whether it is a cause or consequence of disease, nor on the precise mechanism (s). We addressed these issues by testing the effects of expressing the alternative oxidase AOX from *Ciona intestinalis*, in different models of AD pathology. AOX can restore respiratory electron flow when the cytochrome segment of the mitochondrial respiratory chain is inhibited, supporting ATP synthesis, maintaining cellular redox homeostasis and mitigating excess superoxide production at respiratory complexes I and III. In human HEK293-derived cells, AOX expression decreased the production of beta-amyloid peptide resulting from antimycin inhibition of respiratory complex III. Because hydrogen peroxide was neither a direct product nor substrate of AOX, the ability of AOX to mimic antioxidants in this assay must be indirect. In addition, AOX expression was able to partially alleviate the short lifespan of *Drosophila* models neuronally expressing human beta-amyloid peptides, whilst abrogating the induction of markers of oxidative stress. Our findings support the idea of respiratory chain dysfunction and excess ROS production as both an early step and as a pathologically meaningful target in Alzheimer's disease pathogenesis, supporting the concept of a mitochondrial vicious cycle underlying the disease.



## 1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder whose hallmark is the appearance and progressive accumulation of amyloid-β (Aβ)-containing plaques and hyperphosphorylated tau-containing neurofibrillary tangles. Aβ peptide arises from the aberrant processing of the amyloid precursor protein APP. Thus far, no treatments for AD have succeeded, beyond producing marginal symptomatic benefits. Understanding the molecular and cellular basis of the disease is therefore crucial to developing an effective therapy.

Genetic causes of familial, early-onset AD that have been identified include dominant mutations in the APP gene itself, as well as in genes for two proteases involved in its processing, presenilins 1 and 2 [1]. This supports the widely held view that Aβ-associated proteotoxicity is a key step in pathogenesis. However, the exact roles of plaques (and tangles) in pathogenesis is the subject of much debate, as is the primary underlying cause of their production. Although genetic predisposition to the 'sporadic', late-onset form of the disease is well established, the APOE (apolipoprotein E) locus is the only one that appears to contribute substantially thereto [1].

A role for mitochondria in the aetiology of AD has often been invoked. However, convincing proof of this is lacking. Many contradictory hypotheses have been put forward, in which different types of mitochondrial dysfunction are considered a fundamental cause, a necessary step, a useful marker, a relevant consequence or a pure epi-phenomenon, in regard to pathogenesis. Respiratory chain deficiency in itself is far too inconsistent to be considered a common pathological mechanism, e.g. see [2,3]. However, there is more coherent evidence that oxidant species derived from mitochondria are instrumental factors in the cellular dysfunction underlying the disease [4]. Accordingly, the mitochondrially targeted antioxidant MitoQ has been shown to enhance both healthspan and lifespan in a transgenic *Caenorhabditis elegans*

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model of AD [5], in which human Aβ is expressed in the worm.

Hydrogen peroxide ($H_2O_2$) is the major, stable and freely diffusible oxidant species present in cells. As well as giving rise to even more potent oxidants, such as the hydroxyl radical via the Fenton reaction, $H_2O_2$ is itself derived from the highly reactive oxygen species (ROS) superoxide, produced in side-reactions by the mitochondrial respiratory chain [6]. Indeed, the primary cellular defence against superoxide is the enzyme superoxide dismutase, which converts superoxide to peroxide. The ability to limit mitochondrial $H_2O_2$ production would be of value in helping to assess whether it is an underlying cause of Aβ deposition, a consequence thereof, an exacerbating factor in Aβ-related AD pathology, or mechanistically unrelated to the disease. Potentially it could also be of value in designing future therapeutic approaches.

The alternative oxidase, AOX, is a non proton-motive, homodimeric respiratory enzyme found in the mitochondria of plants, fungi, microbes and some animal phyla [7–10]. By catalysing the direct oxidation of ubiquinol by molecular oxygen, it is able to bypass the two terminal steps in the standard respiratory chain or oxidative phosphorylation (OXPHOS) system, namely respiratory complexes III (ubiquinol:cytochrome c oxidoreductase) and IV (cytochrome c oxidase). The gene for AOX is absent from vertebrates and arthropods. However, transgenic expression of the *Ciona intestinalis* alternative oxidase (AOX) in human cells, *Drosophila* or the mouse, which appears to be benign, supports mitochondrial respiration in the presence of inhibitors of complexes III or IV, such as antimycin or cyanide, respectively [11–14]. Under such blockade, we observed that AOX expression greatly decreased the otherwise substantial excess production of ROS. Furthermore, even under non-inhibited conditions, mitochondrial ROS production was diminished, the reasons for which are unclear, but may indicate an inherent antioxidant activity of the enzyme.

The AOX is assumed to have a poor affinity for its substrate, ubiquinol, based on the fact that it is only activated when the mitochondrial quinone pool is highly reduced [15]. Thus, under most physiological conditions, AOX does not substantially compete with respiratory complex III for ubiquinol oxidation. In addition, by maintaining the mitochondrial quinone pool in a relatively oxidized condition, AOX prevents excessive reduction of the respiratory chain, which in turn minimizes the production of excessive superoxide by the mitochondria [16]. In plants, AOX is therefore active in respiration whenever the cytochrome pathway (complexes III and IV) is constrained by metabolic control [17,18], or by the action of toxins [19]. In both cases, it acts as a molecular safety-valve, allowing the plant to resist adverse environmental conditions. Oxidative stress is often triggered under such conditions, and AOX has been shown to play a central role in ROS and NO homeostasis [20,21]. These properties should account for the effects we observed in model organisms expressing *Ciona* AOX. Confusingly, however, it has also been suggested that $H_2O_2$ rather than water is the reaction product of AOX [22,23], which would add to, rather than limit, the burden of oxidative stress. However, under some conditions, AOX endowed with such properties might also be postulated to use $H_2O_2$ as a substrate.

In this study, we used AOX as a tool to study the involvement of mitochondrial pathways in AD pathology, notably the contribution of ROS overproduction to the accumulation of Aβ and to the downstream pathological consequences thereof. We found that AOX expression significantly decreased Aβ deposition by human cells treated with the complex III inhibitor antimycin, and that this correlated with decreased ROS production. As a corollary to this, we confirmed that water, not $H_2O_2$ is the reaction product of *Ciona* AOX. Our findings support the concept that increased mitochondrial $H_2O_2$ production, resulting from chronic inhibition of the cytochrome chain, is a key step in the pathogenesis of AD. In addition, AOX expression conferred a substantial increase in the short lifespan of *Drosophila* strains also expressing Aβ, with concomitant abrogation of the induction of oxidative stress markers at the RNA level. These findings imply that increased ROS production due to inhibition of the cytochrome chain is also a physiologically meaningful aspect of the pathological process unleashed by Aβ, and place mitochondrial dysfunction as both an early and a late step in AD pathogenesis, consistent with the operation of a vicious cycle.

## 2. Materials and methods

### 2.1. Human cell culture and transfection

Human Embryonic Kidney (HEK293) Flp-In cells (Invitrogen, St. Quentin en Yvelines, France) were cultured in RPMI medium containing 2 g/l glucose supplemented by 2 mM glutamine (as Glutamax™), 10% foetal calf serum, 1 mM pyruvate, 100 μg/ml each penicillin, streptomycin and zeocin (Invitrogen, St. Quentin en Yvelines, France). HEK293 Flp-In cells were seeded in 12-well plates to obtain 70% confluence the following day, then co-transfected in 1 ml of OptiMEM medium containing 4 μl Lipofectamine 2000, 1.2 μg of pOG44 Flp recombinase expression plasmid, and 0.13 μg pEF-1α/AOX plasmid containing the *Ciona intestinalis* AOX cDNA [11]. After 5 h incubation, the medium was replaced by DMEM medium devoid of zeocin. Two days later 150 μg/ml hygromycin B were added in order to select for appropriate targeting of constructs. Recombined cells bearing the AOX transgene insertion were subsequently cloned by serial dilution.

### 2.2. Drosophila strains and maintenance

The *Drosophila* strains transgenic for Gal4-inducible *Ciona intestinalis* AOX ($AOX^{F6}$) in the $w^{1118}$ background [24] and for different human Aβ variants, also in the $w^{1118}$ background [25] were as described previously. The X-chromosomal *elav-GAL4* driver line (C155, Bloomington strain 458), as well as $w^{1118}$ and standard balancers for stock maintenance, were obtained from Bloomington stock centre. Flies were maintained at 25 °C on standard medium [26], except for lifespan measurements which were conducted as described [25] at 29 °C, in vials containing batches of ~20 flies of a given sex and genotype. Lifespan curves were based on combined data from 4 to 8 such vials per sex and genotype, with flies tipped to fresh vials every 3 days, or on any day when dead flies were observed.

### 2.3. Cell growth assay

Cells were grown in DMEM containing 4.5 g/l glucose supplemented with 2 mM glutamine (as Glutamax™, Gibco Invitrogen, Cergy Pontoise, France), 10% foetal calf serum, 2 mM pyruvate and 100 μg/ml penicillin/streptomycin. One million cells were seeded in six-well plates for cell growth analysis. 24 h after seeding, 100 μM antimycin with or without 25 μM propyl gallate (PG) was added and cell growth monitored at 0, 24 and 48 h. At least three replicates of each treatment were used to calculate viability using the Trypan blue exclusion assay.

### 2.4. Mitochondrial isolation

Enriched mitochondrial fractions from wild-type and AOX-transgenic mouse [13] brains were prepared by differential centrifugation. Briefly, brains from three-month old mice were isolated after dislocation. Blunt-ended scissors were used to cut the brains into small pieces. These were transferred to a glass homogenizer with Teflon pestle and homogenized in five volumes of

extraction buffer (250 mM sucrose, 40 mM KCl, 2 mM EGTA, 20 mM Tris/HCl, pH 7.2). The homogenate was centrifuged at $600g_{max}$ for 5 min. The supernatant was carefully recovered and centrifuged at $10{,}000g_{max}$ for 10 min to pellet the mitochondria. After discarding the supernatant, the pellet was resuspended in 1 ml of extraction buffer and re-centrifuged at 10,000 $g_{max}$ for 10 min.

### 2.5. Polarography

Cell respiration and mitochondrial substrate oxidation were polarographically estimated using a Clark oxygen electrode (Hansatech Instruments, Norfolk, England) in a magnetically-stirred chamber maintained at 37 °C in 250 µl of a respiratory medium consisting of 0.3 M mannitol, 5 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate buffer (pH 7.2) and 1 mg/ml bovine serum albumin, plus substrates or inhibitors as described [27]. Substrate and inhibitor concentrations were as follows: 0.004% digitonin, 1 mM ADP, 10 mM succinate, 50 mM PG, 1 or 10 mM KCN. Purified catalase from beef liver (2 mg/ml; Sigma) was prepared in 10 mM phosphate buffer (pH 7.2) and used at 3 IU final concentration in 500 µl of assay medium, consisting of 1 mM phosphate buffer (pH 7.2), 5 mM $MgCl_2$, 10 mM KCl and 0.3 M mannitol.

### 2.6. Western blotting

Western blot analyses were performed as indicated [12]. In addition, blots were re-probed with an antibody against ATP synthase subunit β (1:5000, rabbit polyclonal antibody, kindly provided by A. Tzagoloff) as a standardization control. Peroxidase-conjugated anti-rabbit secondary antibody (1:5000, Amersham, Buckinghamshire, UK) and Western Lightning Ultra from PerkinElmer were used for detection.

### 2.7. Immunocytochemistry

HEK293-Flp-In cells expressing the AOX were seeded at 15,000 cells/cm$^2$ and grown for 2 d in standard conditions on Lab-Tek® chambered coverglass (Nunc, Rochester, NY, USA). Cells were then washed with phosphate buffer saline (PBS; Invitrogen, Saint Quentin en Yvelines, France) and fixed with 4% paraformaldehyde for 20 min. They were permeabilized with 0.1% Triton X-100 (w/v) for 5 min, washed 3 times with PBS and blocked with 5% goat serum in PBS/0.05% Tween (w/v) for 30 min. After blocking, cells were incubated for 2 h with a polyclonal antibody raised against *Ciona intestinalis* AOX peptides (21st Century Biochemicals, Marlboro, MA, USA; [22]). Cells were then washed 3 times with PBS/Tween, treated for 1 h with Alexa 562-conjugated anti-rabbit secondary antibody (1000-fold dilution, Invitrogen, Cergy-Pontoise, France), washed again 3 times and mounted using Fluorescent mounting medium (Dako, Trappes, France) after 5 min incubation with DAPI stain (Dako, Trappes, France). Fluorescence was visualized using an eclipse TE300 microscope (Nikon, Champigny sur Marne, France). For simultaneous mitochondrial staining, 100 nM of Mitotracker® Red CMXRos dye (Life Technologies™) was added for 30 min at 37 °C prior to cell fixation. Cells were then washed 3 times with PBS. This step was followed by the aforementioned procedures.

### 2.8. Elisa

In order to detect the secreted soluble human Aβ40 peptide a specific solid-phase sandwich enzyme-linked immuno-sorbent assay (ELISA) was employed according to the instructions given in the Abeta-ELISA kit by Invitrogen. Briefly, cells were grown to 80% confluence, then for a further 24 h with or without additional treatment with antimycin (100 µM), rotenone (25 µM), ascorbic acid (1 mM), tBQH (25 µM) and PG (50 mM), alone or in combination (see ). Cell supernatants were collected after a centrifugation step ($400g_{max}$ for 5 min) after which ELISA was conducted, whilst cell pellets were used for protein estimation.

### 2.9. RNA analysis

RNA extraction from 5 day-old *Drosophila* adult males cultured at 29 °C and QRTPCR normalized to RpL32 as reference gene were carried out as previously [24], using the following additional primer pairs (all sequences shown 5′ to 3′): Keap1-TGCGTCGGCGGTTATTTATC and AGCATTCCTTTCCCCGTTGT, GstD1 – TCAGCGCCTGTACTTCGACA and TCTTGAAGGCCTCTGGATCG, Hsp22 – CAATGCGTTCCTTACCGATG and GGTAGCGCCACACTCCAAAC, Sod2 – GCCCGTAAAATTTCGCAAAC and CGGCAGATGATAGGCTCCAG.

### 2.10. Statistical treatment

Except where indicated, data were analysed by one way ANOVA using SigmaStat software (Sigma, St. Louis, USA). Data shown are means ± 1 SD. $p < 0.001$, 0.01 or 0.05 are denoted by ***, ** and *, respectively. Non-significant differences are denoted ns.

## 3. Results

### 3.1. AOX mitigates the enhanced formation of Aβ40 by mitochondrial ROS

It has been previously shown that increased mitochondrial ROS production resulting from antimycin treatment leads to enhanced Aβ formation in HEK293 cells [28]. Antimycin maintains the mitochondrial ubiquinone pool in a highly reduced state by specifically inhibiting respiratory complex III [29]. This condition favours the formation of superoxide from oxygen-reactive semiquinone free radicals. We first established conditions of antimycin treatment under which both AOX-expressing and control cells survived. Flp-In™ T-REx™-0293 cells induced to express the *Ciona intestinalis* AOX (Figs. 1A, 1B) showed approximately 60% cyanide-resistance of succinate oxidation, which was abolished by further treatment with propyl gallate (Fig. 1C). Both these cells and control Flp-In™ T-REx™-293 cells survived 24 h of antimycin treatment (Fig. 1D), although control cells were killed by a further 24 h of such exposure, whilst AOX-expressing cells continued to grow (Fig. 1D). After 24 h of antimycin treatment, control cells showed a ~2-fold increase in the production of the 40 amino-acid Aβ peptide Aβ40, compared with untreated cells (Figs. 2A, 2B), whereas the corresponding increase seen in AOX-expressing cells was only ~20%. Concomitant treatment with propyl gallate restored the Aβ40 increase seen in control cells (Fig. 2A). AOX expression was more effective in limiting Aβ40 production than either of two chemical antioxidants (Fig. 2B), ascorbic acid or *tert*-Butylhydroquinone (food additive E319). It thus appears that the AOX potently decreases Aβ40 production enhanced by respiratory chain blockade at complex III.

### 3.2. The AOX product from oxygen reduction is water, not hydrogen peroxide

The fact that AOX expression produced a similar effect as antioxidant treatments appears inconsistent with the notion that hydrogen peroxide could be its reaction product. However, it does not exclude the possibility that the attenuation of Aβ40 production was a hormetic effect, resulting from increased output of $H_2O_2$ triggering an increase in ROS detoxification. Nor does it exclude

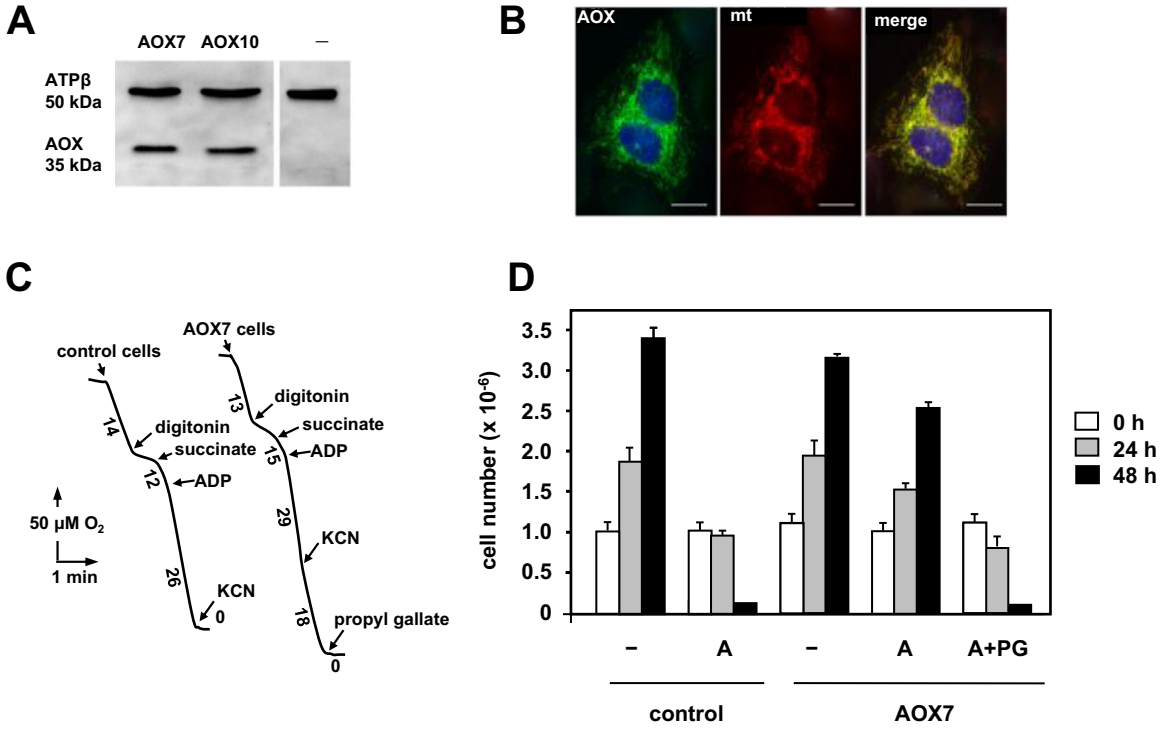


**Fig. 1.** The effect of expressing AOX in Flp-IN™ T-REx™-293 cells on cell growth and mitochondrial properties. (A) Western blot analysis of protein extracts from two AOX-expressing clones (AOX7 and AOX10) and control cells ( – ). Blots were probed with antibodies against AOX and, as loading control, ATP synthase β-subunit. Gels cropped to remove irrelevant space (band sizes are well established from the literature). (B) Immunocytochemistry of AOX-expressing cells (AOX7), stained for AOX (green) and counter-stained with Mitotracker® Red CMXRos. Scale bar 50 μm. (C) Polarography of AOX-expressing (AOX7) and control cells, with additions of detergent, substrates and inhibitors, as shown. (D) Survival of AOX-expressing (AOX7) and control cells seeded in the absence ( – ) or presence of antimycin (A) or propyl gallate (PG), as indicated. Cells were counted after the indicated times.

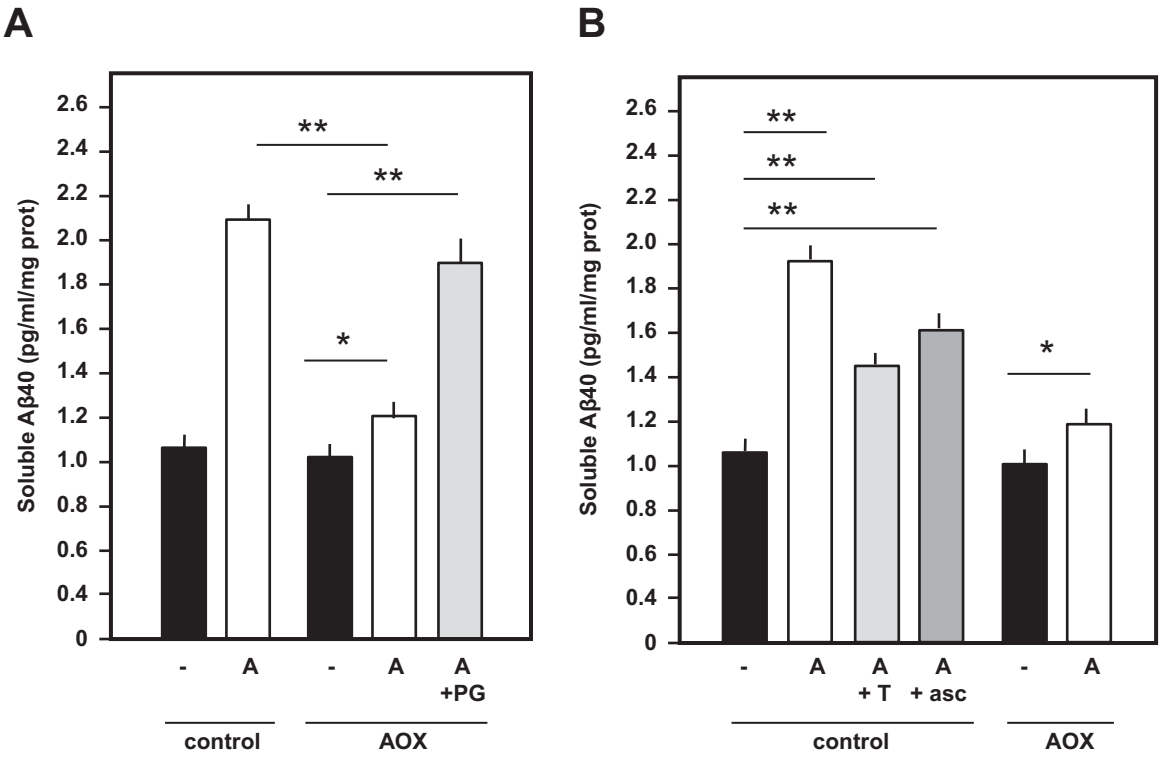


**Fig. 2.** The effect of expressing AOX on Aβ production in Flp-IN™ T-REx™-293 cells. Soluble human Aβ40 production in AOX-expressing and control cells treated with inhibitors and antioxidants as indicated. A – antimycin, PG – propyl gallate, T – *tert*-Butylhydroquinone, ascorbic acid (asc), compared to antimycin-treated cells (B).

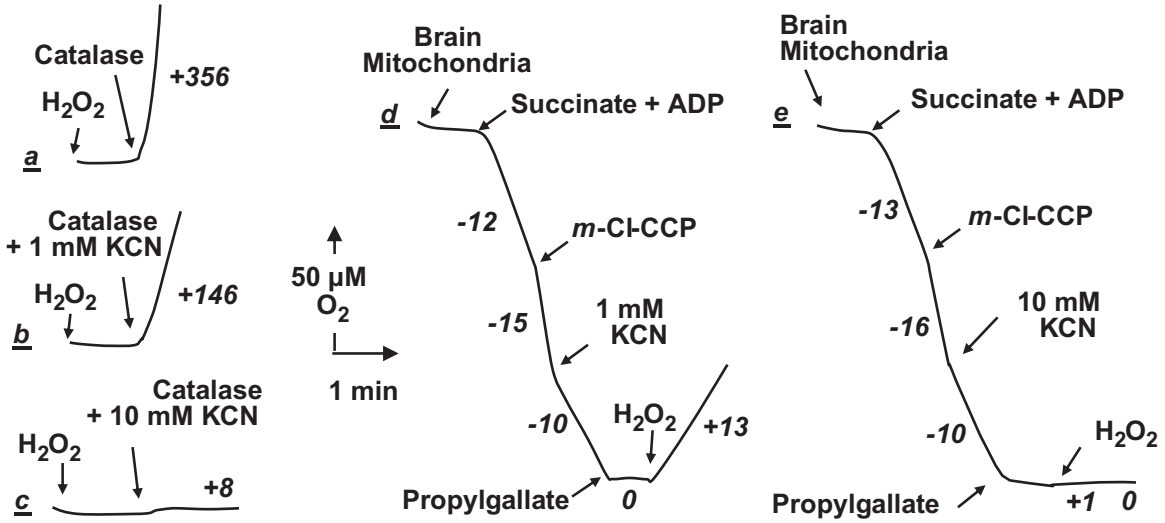


**Fig. 3.** Water, not hydrogen peroxide, is the product of oxygen reduction by *Ciona* AOX. Polarography on (traces A-C) 150 µM hydrogen peroxide triggered by purified catalase (3 IU), with addition of different KCN concentrations, as shown; (traces D, E) brain mitochondria from AOX-expressing mice, with addition of substrates and inhibitors as indicated. *m*-Cl-CCP, *m*-chlorophenylhydrazone carbonyl cyanide. Note that for purified mitochondria, as here, there is almost no measurable oxygen consumption prior to the addition of substrate, whereas for whole cells (Fig. 1C) there is a significant initial rate due to substrates already present within the cells, which drops as a result of leakage, upon permeabilization by digitonin, and is then maximized by the addition of exogenous substrates.

the possibility that AOX may be able to deplete $H_2O_2$ by catalysing a reverse reaction. To exclude completely the possibility that AOX metabolism involves $H_2O_2$, we took advantage of the fact that cyanide also inhibits catalase, but in a very different concentration range than it does cytochrome c oxidase.

This is important, since catalase activity may mask the hypothetical production or use of $H_2O_2$ by AOX. Any hydrogen peroxide produced by the AOX would be readily broken down by catalase into oxygen and water [30,31], creating the illusion that water is the real product of oxygen reduction by AOX, as well as decreasing the amount of oxygen that appears to be consumed when AOX is functioning [32]. The differential sensitivity to cyanide of cytochrome *c* oxidase (Ki $<0.3$ µM; [32]) and of catalase (Ki about 10 µM; [30,31]) allowed us to rule out this possibility.

In a test assay, the activity of purified bovine liver catalase was only ~60% inhibited by 1 mM KCN (Fig. 3, traces A, B), whereas 10 mM KCN brought about almost complete inhibition of the enzyme (Fig. 3, trace C). In contrast, 1 mM KCN was sufficient to fully inhibit succinate oxidation by wild-type mouse brain mitochondria, as expected (not shown). Oxygen consumption by brain mitochondria from mice expressing AOX [13] was then tested in the presence of various substrates and inhibitors. After initially supplying succinate+ADP as substrate, the rate of oxygen consumption was maximized by the addition of the uncoupler *m*-Cl-CCP (Fig. 3, traces D, E). Inhibition of cytochrome oxidase with 1 mM KCN (Fig. 3, trace D) decreased oxygen uptake by ~30%, while a subsequent addition of propyl gallate fully abolished it, confirming that the residual oxygen consumption was AOX-dependent. Under this condition (1 mM KCN), the subsequent addition of hydrogen peroxide resulted in a rapid oxygen release attributable to catalase activity. A similar experiment was next carried out, but using 10 mM KCN (Fig. 3, trace E). The same sequence of additions produced almost identical results, except for the oxygen release after hydrogen peroxide addition, which was totally abolished due to catalase inhibition. Most importantly, no difference in oxygen uptake was seen in the presence of 1 or 10 mM cyanide, indicating that hydrogen peroxide is neither a product nor a substrate for *C. intestinalis* AOX. Its anti-oxidant function must therefore be indirect, as is generally accepted also to be the case in plants [33–35].

### 3.3. AOX expression increases the short lifespan of Drosophila expressing human Aβ in neurons

The fact that AOX expression mitigates the increase in Aβ production due to respiratory chain inhibition leaves open the question of whether Aβ production may also have a detrimental effect on mitochondrial functions. This would potentially initiate a vicious cycle of damage, as suggested by previous studies indicating that oxidative stress [36] and mitochondrial dysfunction [37] are consequences of Aβ deposition. To test this, we made use of a set of *Drosophila* models expressing human Aβ peptides in neurons [25] under the control of the pan-neuronal driver *elav-GAL4* at elevated temperature (29 °C). These models exhibit a degenerative phenotype, with drastically shortened life-span and mitochondrial dysfunction [38]. Neuronal toxicity is believed to be mediated by intracellular accumulation of Aβ aggregates [25].

As noted previously [25] these models exhibit different degrees of phenotypic severity (Fig. 4), in descending order, as follows: the Arctic E22G variant of Aβ42 (AlzArc2) > wild-type Aβ42 on each of chromosomes 2 and 3 (Alz42.2, Alz42.3) > one copy of Aβ42 (Alz42.3 only) > Aβ40 (Alz40.3) > no transgene ($w^{1118}$ background only). Flies expressing the Arctic Aβ42 variant died within 2 weeks, whereas Aβ40 produced hardly any decrease in lifespan compared with the background strain. Males showed slightly shorter lifespans than females in each case, with some experiment-to-experiment variation due to the temperature-sensitivity of the phenotype.

Co-expression of AOX produced a clear improvement in Aβ mutant phenotypes (Fig. 4), although the magnitude of this effect again varied between experiments, was stronger in females than males, and was more pronounced for the severe mutants AlzArc2 and Alz42.2/Alz42.3. AOX alone on otherwise wild-type flies in the $w^{1118}$ background produced no effect on lifespan.

### 3.4. AOX attenuates the expression of oxidative stress markers elicited by human Aβ expression in Drosophila neurons

The observed AOX-driven rescue in *Drosophila* Aβ-expressing models raises the question as to mechanism. In order to test

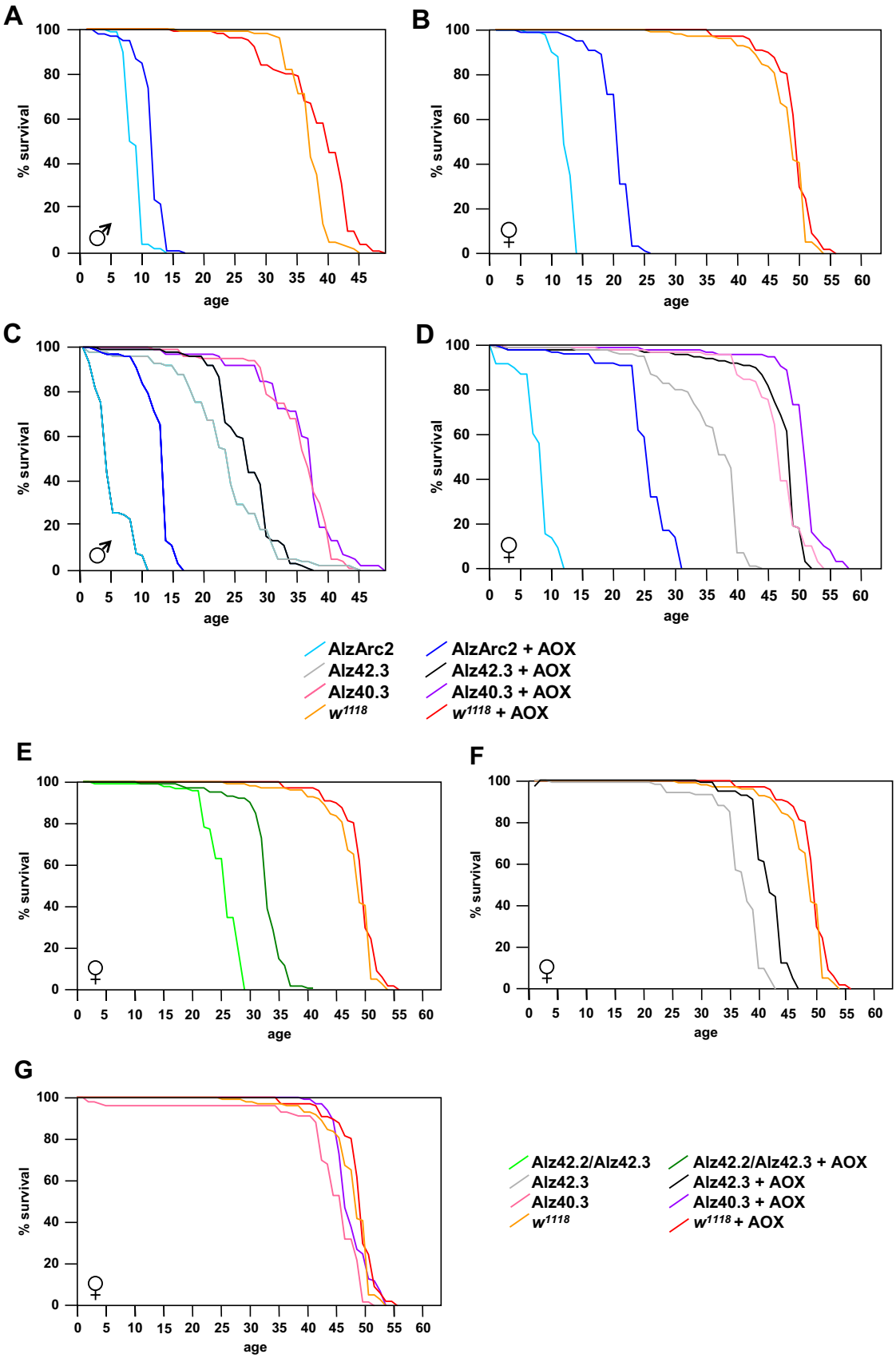


**Fig. 4.** AOX expression partially alleviates the short lifespan of *Drosophila* AD models. Lifespan curves for flies of the indicated sexes and genotypes, with and without the co-expression of hemizygous $AOX^{F6}$, on chromosome 2. AlzArc2, Alz42.3, Alz42.2 and Alz40.3 denote hemizygosity, respectively, for the following transgenic insertions of Gal4-dependent Aβ transgenes: Aβ42 with the E22G 'Arctic' mutation (chromosome 3), wild-type Aβ42 on chromosomes 3 and 2, respectively, and wild-type Aβ40 (chromosome 3). Panels A, B, E, F and G are all from a single experiment, with data for different genotypes (except background strain $w^{1118}$) shown separately, for clarity. Panels C and D are from a separate experiment.

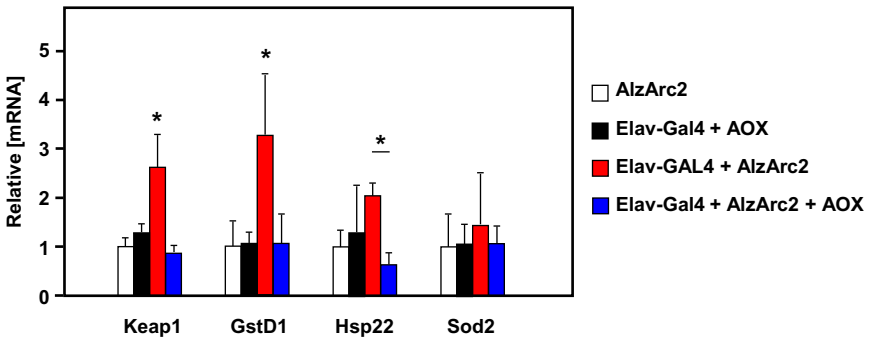


**Fig. 5.** AOX abrogates induction of oxidative stress markers in a *Drosophila* AD model. Relative mRNA levels of genes as shown, based on QRTPCR normalized against RpL32, in adult males of the indicated genotypes. Asterisks indicate data classes significantly different from controls (AlzArc2 flies without driver, white bars; Student's *t* test, $p < 0.05$) except for Hsp22, where the difference from control flies was just outside the border of significance, but the indicated comparison was significant.

whether rescue could be due to the alleviation of oxidative stress produced by respiratory chain inhibition, we measured the expression, at the RNA level, of several markers of oxidative stress, including Hsp22 [39], GstD1 [40,41], Keap1, a negative regulator of Nrf2-driven oxidative stress signaling that is itself induced by Nrf2 [42], and Sod2, an enzyme crucial for responses to oxidative stress, but which is dispensable in the brain [43]. Since the expression of both AOX and human Aβ was targeted uniquely to neurons, i.e. to a minority of the fly's tissues, and to minimize confounding effects from oogenesis (which can affect the levels of housekeeping genes such as Gapdh or RpL32, that are prominently expressed in oocytes), we confined the analysis to males, and to the Aβ variant giving the strongest phenotype, i.e. AlzArc2, in 5 day-old adults. Based on QRTPCR (Fig. 5), three of the four genes tested showed upregulation in the AlzArc2 strain, with a significant protective effect from co-expression of AOX. No such effect was seen with Sod2.

## 4. Discussion

Our findings are consistent with a role for mitochondrial dysfunction in both the early and late steps of AD pathogenesis, namely by enhancing Aβ peptide production, as well as a mechanism of pathological neurotoxicity caused by Aβ. Thus they support the concept of a vicious cycle of mitochondrial damage as a crucial disease process.

Mitochondrial involvement in AD is plagued by contradictory findings, by the use of diverse and not always appropriate models, and by the fact that pathological findings in AD patients typically represent a late stage of the disease where primary and secondary effects are hard to distinguish. As indicated above, the findings linking inhibition of mitochondrial OXPHOS to AD pathology are typical of such inconsistencies, despite the obvious attraction of ATP depletion as a mechanism for impaired proteostasis. A large body of literature indicates decreased activity of cytochrome c oxidase and other mitochondrial enzymes in AD e.g. [2,37,44–47], whereas other studies show an increase in mitochondrial components [48]. These findings can potentially be reconciled if it is assumed that the latter are mitochondrial degradation products, and that mitochondrial turnover might itself be impaired in the disease [48]. However, most such studies do not convincingly distinguish mitochondrial damage from being the cause or the consequence of AD pathology, and do not address the issue of whether ATP deficit and/or increased ROS production and consequent oxidative stress are the underlying mechanism.

In the work reported here we based our initial experiments on the findings of a previous study [28] in which Aβ production in cells was shown to be enhanced by the administration of antimycin, an inhibitor of mitochondrial respiratory complex III, as well as by rotenone, an inhibitor of complex I. Both drugs lead to large increases in mitochondrial ROS production. Mitochondrial ROS has elsewhere been implicated in the pathway leading to increased Aβ production due to hypoxia [49] or treatment with oxidants [50]. In principle, our observation that AOX can block increased Aβ production in the presence of antimycin could reflect one or more of several properties of the enzyme, including the (direct or indirect) alleviation of oxidative stress, an increased capacity for ATP production or the restoration of other aspects of cellular redox and metabolic homeostasis. Although we excluded a direct enzymatic effect on $H_2O_2$ levels, as well as any hormetic effect due to increased $H_2O_2$ output (which was not detectable), an indirect effect of AOX on mitochondrial superoxide production seems to be the most parsimonious interpretation of our findings. By releasing respiratory chain blockade, AOX restores the normal oxidized condition of the mitochondrial ubiquinone pool, as well as upstream redox couples and, as shown previously, thus abolishes the increase in ROS production following antimycin treatment [12]. Previous studies suggested that ROS activates Aβ production in this context by virtue of effects on the expression of proteases involved in APP processing [49,50].

However, we cannot exclude the possibility that AOX may exert its effect at least partially by boosting cellular ATP supply. In plants, AOX activation can be functionally associated with co-activation of the rotenone-resistant non-proton motive NADH dehydrogenase, by-passing ATP production completely [51]. However, when *Ciona* AOX is expressed in animal cells, ATP synthesis is supported by complex I, which performs proton-motive CoQ reduction even in the functional absence of the cytochrome chain [12]. Thus, by allowing electron flow to resume, AOX indirectly supports ATP production via proton pumping at the level of complex I. Many aspects of protein metabolism, including folding/refolding and proteolysis, are ATP-requiring. Thus, increased ATP supply, even just locally, may influence the production or turnover of Aβ.

Note that, because Ciona AOX is here expressed xenotopically in mammalian cells not naturally endowed with AOX, and is active whenever the cytochrome chain is blocked, any physiological post-translational regulation of the enzyme, such as is well established in plants [10], is most unlikely to be operative. However, any loss of post-translational regulation is not crucial for activity under our conditions.

Most literature relating mitochondrial dysfunction to AD pathology has focused on mitochondria as a target rather than a source of primary oxidative damage, and/or a mechanism by which Aβ brings about a neurotoxic outcome. Mitochondrial dysfunction has been reported as an early feature of AD-related pathology in various models, such as in mice expressing human Aβ

[52,53]. Our finding that AOX can substantially increase the curtailed lifespan of flies expressing human Aβ in the nervous system supports the proposition that the neurotoxicity of Aβ is mediated by interference with electron flow through the cytochrome segment of the respiratory chain. The effect of AOX appeared to be more substantial in females than males, and in the models experiencing the most damaging effects of Aβ, i.e., the Arctic variant of Aβ42, or the doubly hemizygous strain expressing wild-type Aβ42. Note that the beneficial effect of AOX expression cannot be explained by promoter dilution: the point has already been addressed previously in regard to other phenotypes [14,24]. In the AD models used here, the second Aβ42 transgene in the doubly hemizygous strain produced roughly twice the decrease in lifespan seen in the strain expressing a single copy of Aβ42, indicating that any promoter dilution effect is negligible.

Diminished electron flow through both complex I and III has been previously reported to occur as a result of Aβ expression. The fact that AOX, which by-passes complexes III and IV, but not complex I, can alleviate the pathological phenotype in the fly model, suggests that damage to complexes III and/or IV may be more pathologically relevant. However, it will be interesting to conduct an analogous series of experiments to by-pass cI, using the non proton-motive NADH dehydrogenase from yeast (Ndi1) or *Ciona* (NDX, [54]), both alone or in combination with AOX. This would be able to determine whether Aβ peptides also affect cI activity directly and, if so, whether the effects on cI and on cIII+cIV are additive or synergistic.

Note that, in previous studies, we observed that ubiquitous expression of AOX had no effect on the lifespan of otherwise wild-type flies maintained at 25 °C [55], whereas ubiquitous expression of Ndi1 did extend lifespan [26]. In a separate study, an increased lifespan was reported in the case of Ndi1 expressed specifically in neurons, using the drug-inducible GeneSwitch system, in flies maintained at 25 °C [59]. In the present study, expression of AOX in neurons (without the use of drug induction) had almost no effect on lifespan of control flies at 29 °C, in the $w^{1118}$ background (Fig. 4). Thus, any confounding effect from transgenic expression of the respiratory chain enzymes *per se* can be discounted.

Once again, the physiological mechanism by which AOX rescues the pathological phenotype in the fly AD models remains open: restored redox homeostasis, decreased ROS production and increased ATP supply are all plausible. A recent report found that MitoQ, an agent that quenches mitochondrial ROS, was able partially to rescue pathological phenotypes, including curtailed lifespan, due to human Aβ expression in *C. elegans* [5]. It should be noted that, in the latter study, transgenic Aβ was expressed in the body muscle wall, whereas the *Drosophila* model that we employed targets Aβ to neurons. The pathological mechanism may differ in the two cases, although the *Drosophila* model is arguably more physiologically relevant to AD in humans.

The induction, in the AlzArc2 model, of genes known to mediate and be responsive to oxidative stress, plus its negation when AOX was co-expressed (Fig. 5) provides strong supporting evidence that increased mitochondrial ROS production is the key pathological mechanism, at least in this model, and is consistent with the proposition that AOX acts by alleviating ROS production due to the build-up of over-reduced electron carriers in the respiratory chain. The lack of significant induction of the superoxide dismutase Sod2, whilst superficially surprising, fits to previous literature: tissue-specific RNAi knockdown experiments showed that Sod2 is required in muscle, but non-essential in the brain [43]. Although its ectopic over-expression is beneficial in various stress models, e.g. [56,57], including one of *tau*-induced neurodegeneration, Sod2 has not, to our knowledge, been reported as being transcriptionally induced under stress in *Drosophila*, consistent with the data reported here. Even its enzymatic activity is only slightly induced by oxidative stress in the fly [58]. Two caveats nevertheless apply to our finding. Firstly, the tissue(s) in which oxidative stress markers are induced in response to Aβ expression in neurons, are not yet identified, so the effect could still be indirect. Secondly, an effect on ROS production and its mitigation does not exclude the possibility that contributions from other cellular or metabolic consequences of Aβ and AOX expression are material to the phenotype.

Our findings are consistent with earlier observations that suppressors of the Fenton reaction (over-expression of catalase or of the heavy or light chains of the iron-binding protein ferritin) alleviate the phenotypes of the same set of *Drosophila* AD models [38], with increases in median lifespan of up to 60%. In the latter study, over-expression of the cytosolic Cu/Zn superoxide dismutase (Sod1) potentiated the pathological phenotype, whereas over-expression of Sod2, or Sod1 knockdown by RNAi or expression of a dominant-negative Sod1 were all suppressive. Taken together with these findings, our results strongly implicate excess mitochondrial ROS production, due to dysfunction of the respiratory chain, as a pathological mechanism of Aβ neurotoxicity.

Several recent studies have indicated that the involvement of mitochondria in the pathogenesis of AD may involve altered interactions between the mitochondria and a specific sub-compartment of the endoplasmic reticulum (ER), the mitochondria-associated membranes (MAMs). The resulting ER stress and dysregulated lipid metabolism are proposed to lead to abnormal proteolysis, favouring the generation of the more pathogenic forms of Aβ [60,61]. Oxidative stress due to mitochondrial dysfunction may be the initial trigger for these events. This concept is consistent with our finding that Aβ production is increased by respiratory chain dysfunction in a way that is suppressible by AOX. ER dysfunction has been postulated not only as an underlying cause of increased Aβ production, but also as one of the effects of its accumulation [62–64]. Our data imply a similar vicious cycle of cellular damage operating in the mitochondria. The intimate functional connections between mitochondria and the ER leave open the possibility that both organelles are involved in this process.

The present study employs AOX to address aspects of the molecular mechanisms of AD pathogenesis, rather than to test its possible applications in therapy, which is clearly a distant prospect. Therapeutic delivery of AOX would be a major challenge, requiring a genetic approach. On the other hand, once these issues would be solved, AOX might become attractive as a minimally invasive type of treatment. It requires no externally applied regulation, instead operating as a 'pressure-relief' valve that is only in play when the cytochrome chain is unavailable.

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