Mapping pathogenic processes contributing to neurodegeneration in Drosophila models of Alzheimer’s disease

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Alzheimer’s disease (AD) is the most common form of dementia, affecting millions of people and currently lacking available disease-modifying treatments. Appropriate disease models are necessary to investigate disease mechanisms and potential treatments. Drosophila melanogaster models of AD include the Aβ fly model and the AβPP-BACE1 fly model. In the Aβ fly model, the Aβ peptide is fused to a secretion sequence and directly overexpressed. In the AβPP-BACE1 model, human AβPP and human BACE1 are expressed in the fly, resulting in in vivo production of Aβ peptides and other AβPP cleavage products. Although these two models have been used for almost two decades, the underlying mechanisms resulting in neurodegeneration are not yet clearly understood. In this study, we have characterized toxic mechanisms in these two AD fly models. We detected neuronal cell death and increased protein carbonylation (indicative of oxidative stress) in both AD fly models. In the Aβ fly model, this correlates with high Aβ1–42 levels and down-regulation of the levels of mRNA encoding lysosomal-associated membrane protein 1, lamp1 (a lysosomal marker), while in the AβPP-BACE1 fly model, neuronal cell death correlates with low Aβ1–42 levels, up-regulation of lamp1 mRNA levels and increased levels of C-terminal fragments. In addition, a significant amount of AβPP/Aβ antibody (4G8)-positive species, located close to the endosomal marker rab5, was detected in the AβPP-BACE1 model. Taken together, this study highlights the similarities and differences in the toxic mechanisms which result in neuronal death in two different AD fly models. Such information is important to consider when utilizing these models to study AD pathogenesis or screening for potential treatments.

Alzheimer’s disease (AD) is a neurodegenerative disorder that leads to progressive cognitive decline. It is the most prevalent form of dementia, affecting 11% of the population over the age of 65, and it is the sixth leading cause of death in the United States [1]. A hallmark of the disease is the aggregation of the amyloid β (Aβ) peptide into fibrillar deposits known as amyloid plaques [2]. However, research in the AD field

Abbreviations
AD, Alzheimer’s disease; Aβ, amyloid beta; AβPP, amyloid beta precursor protein; BACE1, beta-site AβPP-cleaving enzyme; CTFs, C-terminal fragments; MCI, mild cognitive impairment; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.
points towards the soluble Aβ species, rather than the fibrillar deposits, as playing a key pathogenic role in the disease [3]. The generation of Aβ peptides occurs through proteolytic processing of the transmembrane Aβ precursor protein (AβPP) by the β-site AβPP-cleaving enzyme (BACE1) followed by the intramembranous enzyme complex γ-secretase [4–6]. Depending on the site of cleavage, different-sized Aβ peptides are generated, with Aβ1-40 and Aβ1-42 being the most frequent isoforms. Aβ1-42 has a higher propensity to form prefibrillar aggregates compared to Aβ1-40, and it has also been reported to be more toxic than Aβ1-40 [7]. The Aβ peptides are not the only cleavage products from AβPP processing; when AβPP is first cleaved by BACE1, a C-terminal fragment (CTF) consisting of 99 amino acids (C99) is produced. The level of C99 is higher in AD brains, and C99 from BACE1 cleavage of AβPP has been shown to overactivate rab5, leading to endosomal dysfunction [8].

To increase the understanding of the different pathways and mechanisms involved in AD, appropriate disease models are necessary. Drosophila melanogaster, the fruit fly, is one of the most well-studied eukaryotes. The entire genome of the fruit fly was sequenced in 2000, and around 76% of human disease genes have homologues in the fly genome [9]. For almost two decades, the fly has been used to study AD and Aβ proteotoxicity. The more commonly used Aβ fly model has the gene encoding the AβPP sequence cloned into the fly genome; the peptide is expressed fused to a signal sequence, resulting in secretion of the peptide to the extracellular space [10–12]. In the other models, human AβPP is co-expressed with human BACE1 allowing the production of C99 and different isoforms of the Aβ peptide (including post-translationally modified Aβ variants) through the processing of human AβPP by human BACE1 and by endogenous fly γ-secretase (the AβPP-BACE1 fly model) [13,14]. AD fly models have been frequently used during the last two decades to investigate Aβ toxicity, cell-specific vulnerability and aggregation [15–22]. However, potential differences in the toxic mechanisms between the two different AD fly models have not been thoroughly investigated. Recently, we published a study where the toxic effects in these two AD fly models were studied in parallel [14]. We found that the proteotoxic effect, defined as the reduction in median survival time divided by total amount of Aβ1-42, is considerably higher for the AβPP-BACE1 flies compared to the Aβ1-42 flies, implying that the mechanisms of toxicity are different between these two AD fly models. In this study, we further investigate toxicity and disease mechanisms relevant in the context of AD for the Aβ1-42 and AβPP-BACE1 flies by performing immunohistological and biochemical assays to probe: (a) the extent of neuronal death and protein carbonylation, (b) the gene expression level and distribution of markers of early endosomes and lysosomes and (c) the location of AβPP (and its cleavage products including Aβ1-42) and early endosomes and lysosomes in the fly CNS. Here, we present data which reveal that neuronal cell death is present in both AD fly models. The cell death was significantly higher in the Aβ1-42 flies compared to the AβPP-BACE1 flies. However, the extent of cell death found in the AβPP-BACE1 flies was remarkably high considering the low level of Aβ1-42 peptide detected in these flies (about 200 times lower than the Aβ1-42 flies). Therefore, to probe the pathological processes contributing to neuronal cell death in these two fly models, two cellular events that have been closely connected to AD, protein carbonylation and changes in the endo-lysosomal system machinery were investigated [8,23–26].

Results

In both AD fly models, apoptosis leads to neuronal death

Alzheimer’s disease is the most common neurodegenerative disease; thus, neuronal cell death is a crucial feature of any potential AD animal model. By using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, the presence of apoptotic cells in brain sections from Drosophila was investigated for control w1118 (only expressing Gal4), AβPP (human AβPP695), Aβ1-42 × 2 (fly line with two copies of Aβ1-42) and AβPP-BACE1 (human AβPP695 and human BACE1) flies (Fig. 1A). Flies were analysed at day 21, a time point corresponding to the median survival time previously observed for AβPP-BACE1 flies [14]. The majority of all TUNEL-positive cells were observed in the medulla and the lamina (Fig. 1B). By scoring the presence of TUNEL-positive cells in a blind fashion, a significant increase in the number of TUNEL-positive cells was observed for both the Aβ1-42 × 2 (P ≤ 0.0001) and the AβPP-BACE1 (P ≤ 0.05) flies relative to their control flies (w1118 and AβPP flies, respectively), demonstrating the presence of apoptotic cells in both model systems (Fig. 1C). The increase in TUNEL-positive cells was significantly higher (P ≤ 0.05) for the Aβ1-42 × 2 flies compared to the AβPP-BACE1 flies, revealing a higher level of neuronal apoptosis in the Aβ1-42 × 2 flies at the selected time point.
Fig. 1. Both AD fly models demonstrate apoptotic cell death and protein carbonylation. (A) Apoptotic cells in control, Aβ<sub>1-42</sub> × 2, AβPP and AβPP-BACE1 flies at day 21 identified by TUNEL (green) staining. Image inset highlights TUNEL-positive cells. Micrographs were taken at 40x magnification, scale bar = 50 μm, n = 4–5 brains. DAPI was used to visualize cell nuclei (blue). (B) Schematic image of a fly brain where the red box indicates which areas were analysed for TUNEL-positive cells; this corresponds to the medulla and the lamina. (C) Nonbiased scoring of the presence of TUNEL-positive cells, n = 4–5, data represented as mean ± SD. * represents P ≤ 0.05 and **** represents P ≤ 0.0001 as determined by a one-way ANOVA followed by Tukey’s post hoc test. (D) Quantification of Aβ<sub>1-42</sub> in the different fly genotypes at day 21, n = 3 (20 flies in each repeat). Data represented as mean ± SD. (E) Representative western blot showing the bands corresponding to full-length AβPP and the CTFs for AβPP and AβPP-BACE1 flies at day 21. Tubulin is used as a protein loading control, n = 4 (20 flies in each repeat). (F) Densitometry for full-length AβPP and CTFs correlated to tubulin, data represented as mean ± SEM (n = 4). * represents P ≤ 0.05 as determined by the Mann–Whitney U test. (G) Representative immunoblot showing the total protein carbonylation in control, Aβ<sub>1-42</sub> × 2, AβPP and AβPP-BACE1 flies at day 21, n = 4 (20 flies in each repeat). Nonspecific band in nonderivatized negative control sample found in all sample preparations was used as a protein loading control.
The $\text{A}\beta_{1-42}$ load is significantly higher in the $\text{A}\beta_{1-42} \times 2$ flies compared to the $\text{A}\beta\text{PP}-\text{BACE}1$ flies

As the $\text{A}\beta_{1-42}$ peptide is closely linked to AD and neurodegeneration [24,27,28], the total level of $\text{A}\beta_{1-42}$ present in the different fly genotypes was determined (Fig. 1D). The highest level of $\text{A}\beta_{1-42}$ was detected in the $\text{A}\beta_{1-42} \times 2$ flies ($40 \pm 2.6$ pg per fly), which was approximately 200 times higher than the level detected in the $\text{A}\beta\text{PP}-\text{BACE}1$ flies ($0.20 \pm 0.04$ pg per fly). Thus, a significantly higher level of $\text{A}\beta_{1-42}$ is present in the $\text{A}\beta_{1-42} \times 2$ flies compared to the $\text{A}\beta\text{PP}-\text{BACE}1$ flies and this correlates with the higher level of neuronal apoptosis observed in the $\text{A}\beta_{1-42} \times 2$ flies compared to the $\text{A}\beta\text{PP}-\text{BACE}1$ flies.

Increased level of the C-terminal fragments in the $\text{A}\beta\text{PP}-\text{BACE}1$ flies compared to the $\text{A}\beta\text{PP}$ flies

After the first cleavage of full-length $\text{A}\beta\text{PP}$ by BACE1 or by fly intrinsic $\alpha$-secretase, two different CTFs are produced (C99 and C83, respectively), and C99 from BACE1 cleavage of $\text{A}\beta\text{PP}$ may be involved in neurotoxic events [8]. To specifically investigate the presence of full-length $\text{A}\beta\text{PP}$ and CTFs in the $\text{A}\beta\text{PP}$ flies and the $\text{A}\beta\text{PP}-\text{BACE}1$ flies, a western blot was performed using a C-terminal $\text{A}\beta\text{PP}$ antibody from Sigma-Aldrich (St. Louis, MO, USA) (Fig. 1E – entire blot in Fig. S1). The result revealed a significant decrease in the level of full-length $\text{A}\beta\text{PP}$ and a significant increase in the level of CTFs (C99) in the $\text{A}\beta\text{PP}-\text{BACE}1$ flies compared to the $\text{A}\beta\text{PP}$ flies (Fig. 1F).

Increased protein carbonylation in both AD fly models

Mitochondrial dysfunction and subsequent increased oxidative stress have been connected with neurodegeneration and AD [23]. Protein carbonylation, an indicator of oxidative stress [29], was investigated in the fly models. Protein carbonylation was detected in all four genotypes (Fig. 1G); however, an increase in protein carbonylation was detected for both the $\text{A}\beta_{1-42} \times 2$ flies and the $\text{A}\beta\text{PP}-\text{BACE}1$ flies compared to their respective controls ($\text{w}^{1118}$ and $\text{A}\beta\text{PP}$ flies). Interestingly, the proteins that were carbonylated differed between the $\text{A}\beta_{1-42} \times 2$ and $\text{A}\beta\text{PP}-\text{BACE}1$ flies. In the $\text{A}\beta_{1-42} \times 2$ flies, two carbonylated protein bands were detected, one band above 188 kDa and one band around 62 kDa. These two bands were essentially absent in the $\text{A}\beta\text{PP}-\text{BACE}1$ flies, and the carbonylation detected in the $\text{A}\beta\text{PP}-\text{BACE}1$ flies occurred for proteins with lower molecular weights compared to the $\text{A}\beta_{1-42} \times 2$ flies ($< 62$ kDa).

Distribution of early endosomes and lysosomes in the two AD fly models

Endosomal and lysosomal dysfunctions can be observed in the early stages of AD, and with time, it progresses to a widespread failure of intraneuronal waste clearance and eventually neuronal death [26,30–32]. To investigate the distribution of early endosomes and lysosomes in the AD flies, Drosophila brain sections for control $\text{w}^{1118}$, $\text{A}\beta\text{PP}$, $\text{A}\beta_{1-42} \times 2$ and $\text{A}\beta\text{PP}-\text{BACE}1$ flies were stained with a Drosophila anti-rab5 antibody, investigating the presence of early endosomes (Fig. 2A), or with a Drosophila anti-LAMP1 antibody, investigating the presence of lysosomes (Fig. 2B). The area of the brain analysed is the same as for the TUNEL analysis, highlighted in Fig. 1B.

The immunohistochemistry analysis showed that early endosomes were located perinuclear as well as separated from the cell bodies in all fly genotypes (Fig. 2A). Staining control $\text{w}^{1118}$ flies with a Drosophila anti-axon antibody reveals a network of axons separated from the cell bodies (Fig. 2C). This staining pattern of axons is very similar to the staining pattern of early endosomes separated from the cell nuclei. Thus, the early endosomes detected separated from the cell bodies are likely located in this network of axons, indicating that early endosomes are present both around the cell nuclei, in the cell body and in the axons of the fly neurons. No significant differences in the rab5 mRNA levels were observed between the four genotypes (Fig. 2D).

The immunohistochemistry analysis of the distribution of lysosomes showed both perinuclear staining and staining separated from the cell bodies in all fly genotypes (Fig. 2B). Looking at the mRNA level of the lysosomal marker, LAMP1, a small but significant ($P \leq 0.05$) up-regulation of lamp1 was detected for the $\text{A}\beta\text{PP}-\text{BACE}1$ flies compared to $\text{A}\beta\text{PP}$ flies while a small but significant ($P \leq 0.05$) down-regulation was detected for lamp1 mRNA in the $\text{A}\beta_{1-42} \times 2$ flies compared to control $\text{w}^{1118}$ flies (Fig. 2E).

Taken together, the distribution of endosomes and lysosomes was found both perinuclear and separated from the cell bodies. No differences in the mRNA levels of the rab5 endosomal marker were detected, but an up-regulation of lamp1 was observed in the $\text{A}\beta\text{PP}-\text{BACE}1$ flies compared to $\text{A}\beta\text{PP}$ flies, whereas there was a down-regulation in lamp1 mRNA in the $\text{A}\beta_{1-42} \times 2$ flies compared to control $\text{w}^{1118}$ flies.
The AβPP/Aβ antibody 4G8 signal occurs in close vicinity to the staining pattern of early endosomes in the AβPP-BACE1 flies

To compare the location of AβPP and/or Aβ with early endosomes, Drosophila brain sections were costained with the Drosophila anti-rab5 antibody (marker for early endosomes, green) or with a Drosophila anti-LAMP1 antibody (marker for lysosomes, green). DAPI (blue) was used to visualize cell nuclei. White arrowheads indicate perinuclear rab5 staining in Aβ1-42x2 and AβPP-BACE1 flies in panel (A). Micrographs were taken at 100x magnification, scale bar = 20 μm and n = 6 in (A) and (B). (C) Drosophila brain sections of control flies stained with a Drosophila anti-axon antibody, n = 3. mRNA levels of rab5 (D) and lamp1 (E) were analysed, n = 3 (20 flies in each repeat). * represent P ≤ 0.05 as determined by Wilcoxon signed-rank test. The final data presented as 2DDCmin to 2DDCmax with SE.

Fig. 2. Lysosomal alterations in AD fly models. (A) Drosophila brain sections, day 21, of control, Aβ1-42x2, AβPP and AβPP-BACE1 flies were stained with a Drosophila anti-rab5 antibody (marker for early endosomes, green) or (B) with a Drosophila anti-LAMP1 antibody (marker for lysosomes, green). DAPI (blue) was used to visualize cell nuclei. White arrowheads indicate perinuclear rab5 staining in Aβ1-42x2 and AβPP-BACE1 flies in panel (A). Micrographs were taken at 100x magnification, scale bar = 20 μm and n = 6 in (A) and (B). (C) Drosophila brain sections of control flies stained with a Drosophila anti-axon antibody, n = 3. mRNA levels of rab5 (D) and lamp1 (E) were analysed, n = 3 (20 flies in each repeat). * represent P ≤ 0.05 as determined by Wilcoxon signed-rank test. The final data presented as 2DDCmin to 2DDCmax with SE.
Next, Drosophila brain sections were costained with the Drosophila anti-LAMP1 antibody and 4G8 or the Mabtech antibody to compare the locations of AβPP and/or Aβ and lysosomes in the fly brain (Fig. 4). Control w1118 flies showed no 4G8 or Mabtech staining (Fig. 4A,E). As observed in the previous staining (Fig. 3B,F), the 4G8 and Mabtech signals were located around the cell nuclei in the Aβ1-42 × 2 flies (Fig. 4B, F). In the AβPP flies, a 4G8 signal was located in the axons, separated from the cell bodies, but no lysosome staining occurred at this location (Fig. 4C). No Mabtech signal was detected in the AβPP flies (Fig. 4G). In the AβPP-BACE1 flies, an intense 4G8 signal was present around the cell nuclei and in the axons but the signal did not coincide with the lysosome staining (Fig. 4D). A Mabtech signal was observed in the AβPP-BACE1 flies around the cell nuclei (Fig. 4H).

Taken together, a signal from the 4G8 antibody was detected around the cell nuclei for the Aβ1-42 × 2 flies, in the axons for the AβPP flies and in both places for the AβPP-BACE1 flies. The staining pattern of 4G8 and endosomes coincided in the AβPP flies and the AβPP-BACE1 flies, while the 4G8 signal in the Aβ1-42 × 2 did not coincide with the endosome signal. The staining pattern of lysosomes did not coincide with the 4G8 signal in any of the flies. Signals from the Mabtech antibody were observed around the cell nuclei for the Aβ1-42 × 2 and for the AβPP-BACE1 flies but did not coincide with the lysosome or endosome signals.

**Discussion**

Understanding the underlying mechanisms of AD toxicity is a key requirement to developing mechanism-based therapeutic strategies, and the use of Drosophila to investigate the pathogenesis of AD has allowed scientists to achieve important goals in this research field [34]. AD research using Drosophila frequently implies one of two approaches; either the Aβ peptides are fused to a secretion sequence and directly produced from transgenes (the Aβ fly model) or the Aβ peptides are produced by the processing of human AβPP (the AβPP-BACE1 fly model) [10,14,35–38]. In this paper, we have looked, in detail, at the pathways leading to toxicity within the two AD fly models and have highlighted differences in the underlying mechanisms of the AD-related toxicity observed in these systems.

In our previous study, longevity and locomotor analyses showed significant toxic effects for both the Aβ42 flies and AβPP-BACE1 flies [14]. The time frame selected for this study was 21 days, corresponding to the median survival time for the AβPP-BACE1 flies. Around this age, the flies in both AD models start to

![Image](https://example.com/image.png)
display dysfunctional locomotor behaviour. Studies have shown that dysfunctional locomotor behaviour in Drosophila is associated with neurodegeneration [39].

The results from the TUNEL assay revealed the presence of apoptotic cells in both AD models albeit to a higher extent in the Aβ₁₋₄₂ 92 flies compared to the AβPP-BACE1 flies. This difference in apoptotic cell death was found to correlate with the dramatically higher level of Aβ₁₋₄₂ present in the Aβ₁₋₄₂ 92 flies, where 200 times more Aβ₁₋₄₂ accumulated as compared to the AβPP-BACE1 flies at day 21. This difference in the Aβ₁₋₄₂ level is in concordance with previous data demonstrating a ratio of 1:40 of the Aβ₁₋₄₂ level between the AβPP-BACE1 and the Aβ₁₋₄₂ 92 flies at day 7 [14]. Hence, Aβ₁₋₄₂ accumulates to an even higher degree in the Aβ₁₋₄₂ 92 flies compared to the AβPP-BACE1 flies with subsequent ageing.

The Aβ₁₋₄₂ peptide is more hydrophobic than the shorter isoforms and is therefore more prone to aggregating and forming toxic species [7,40,41]. It can form large amyloid aggregates which can sequester other proteins, leading to toxicity due to loss of function [42]. Aβ₁₋₄₂ oligomers of different sizes have been found to impair memory in AD rodent models and the peptide itself has been shown to interact with other proteins, such as cell surface receptors, leading to downstream signalling which may contribute to neurodegeneration [43–46]. Thus, it is likely that the neuronal death observed in the Aβ₁₋₄₂ 92 flies is due to high accumulation of toxic Aβ₁₋₄₂ species. Indeed, this is supported by several other studies where high levels of Aβ₁₋₄₂ have been shown to cause neurodegeneration in Drosophila models of AD [12,47,48].

An early event in AD pathology is an increase in oxidative stress, which can be observed in patients with mild cognitive impairment (MCI) before any significant increase in amyloid plaques or neurofibrillary tangles can be detected [23]. Oxidative stress is an indicator of mitochondrial dysfunction, causing a rise in reactive oxygen species which results in an increase in protein carbonylation [29]. Interestingly, both the Aβ₁₋₄₂ 92 flies and the AβPP-BACE1 flies showed an increase in protein carbonylation compared to control w1118 and AβPP flies. This implies that oxidative stress is a possible contributor to neurodegeneration in both AD fly models. The Aβ peptide has been shown to impair degradation of mitochondrial proteins and to change mitochondrial membrane potential, which may trigger the release of cytochrome c and thus induce apoptosis [25,49,50]. Therefore, a noticeable contribution to the neuronal death in the AβPP-BACE1 flies

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**Fig. 4.** Signals for the AβPP/Aβ antibody 4G8 nor Aβ antibody Mabtech do not coincide with lysosomes in the AD fly models. Drosophila brain sections (day 21), of control, Aβ₁₋₄₂ × 2, AβPP and AβPP-BACE1 flies were costained with a Drosophila anti-LAMP1 antibody (green; lysosomal marker), to investigate the presence of lysosomes, and the AβPP/Aβ antibody 4G8 (red) (A–D) or with the N-terminal Aβ antibody from Mabtech (red) (E–H). DAPI was used to visualize cell nuclei (blue). Micrographs were taken at 100× magnification, scale bar = 20 μm and n = 6.
could be due to intracellular Aβ that disrupts mitochondrial function, leading to increased oxidative stress and eventually apoptosis. This can explain how a relatively low level of Aβ_{1–42} may induce neurodegeneration.

Another early event in AD pathology includes abnormalities in the endo-lysosomal pathway [30] where increased levels of rab5 and rab7 proteins, markers for early and late endosomes, respectively, have been found to be up-regulated in individuals with MCI as well as in AD patients [32]. Aβ has been shown to accumulate in lysosomes, a pathogenic event indicating a loss of lysosomal integrity and the ability to degrade its material [51–54]. Endo-lysosomal pathways are essential in maintaining cellular homeostasis. Dysfunction of this intriguing system has been suggested to represent a converging mechanism for many diseases involving neurodegeneration, including AD [55]. Investigation of the endo-lysosomal system in the two AD fly models revealed that Lamp1 mRNA was increased in the AβPP-BACE1 flies and decreased in the Aβ_{1–42} × 2 flies. The increased Lamp1 mRNA level in the AβPP-BACE1 flies is in line with previous studies where increased lamp1 mRNA expression in AβPPSl transgenic mice expressing AβPP with Swedish and London mutations has been found [56]. These data suggest abnormalities in the endo-lysosomal system for both fly models that might contribute to the toxicity in these flies. For the AβPP-BACE1 flies, abnormality in the endo-lysosomal system may explain toxicity despite the low level of Aβ_{1–42} in these flies. Indeed, small amounts of intracellular accumulation of Aβ in endocytic vesicles can trigger Aβ oligomerization [57], disrupting the vesicles’ ability to mature and leading to a decrease in protein degradation and eventually inducing toxicity. For the Aβ_{1–42} flies, the toxicity may be caused by the down-regulation of lysosomes resulting in the lysosome machinery being overwhelmed by Aβ species and consequently leading to neuronal death.

BACE1 is able to cleave AβPP at the plasma membrane, but more frequently, BACE1 cleavage occurs in the early endosomes resulting in the production of C99 [58]. Interestingly, Aβ is not the only cleavage product from AβPP processing known to cause endosomal dysfunction; C99 produced from BACE1 cleavage of AβPP has been shown to pathologically activate rab5, leading to an accumulation of swollen endosomes [8]. In both the AβPP and AβPP-BACE1 flies, the signal for the AβPP/Aβ antibody (4G8) was detected in close vicinity with Drosophila endosomes. Interestingly, the coincidence of these signals was distributed in different areas within the two flies. In the AβPP flies, the area where the 4G8 and endosome signals coincide is located distinctly from the cell nuclei in the axons, while in the AβPP-BACE1 flies, the 4G8 and endosome signals were strongly clustered around the cell nuclei as well as in the axons. The Mabtech signal (specific for the Aβ peptide) in the AβPP-BACE1 flies did not coincide with the endosome staining, suggesting that the 4G8 signal in the AβPP-BACE1 flies corresponds to either full-length AβPP or C99. The increase in the C99 level detected for the AβPP-BACE1 flies compared to the AβPP flies suggests that the 4G8 staining around the cell nuclei in the AβPP-BACE1 flies corresponds to accumulation of C99 while the 4G8 staining visible in the axons of the AβPP-BACE1 flies and the AβPP flies corresponds to full-length AβPP. Thus, the high level of C99 detected for the AβPP-BACE1 flies that coincided with endosomes, together with the increased amount of apoptotic cells identified in these flies, compared to the AβPP flies, suggests that a possible contributor to the apoptosis in the AβPP-BACE1 flies is the accumulation of C99 in endosomal vesicles. This may lead to a disruption in the endosomal pathway that will decrease the ability of the neurons to degrade or recycle proteins, thereby leading to apoptosis [26]. In the Aβ_{1–42} × 2 flies, the 4G8 and Mabtech signals did not coincide with either endosome or lysosome markers, despite being in close proximity to the cell nuclei. Hence, if these species, detected by 4G8 and Mabtech antibodies, are located intracellularly, they are generally not associated with endosomes or lysosomes. Another possibility is that the 4G8 and Mabtech signals in the Aβ_{1–42} × 2 flies are detecting aggregated extracellular Aβ species. Indeed, both the 4G8 and Mabtech antibodies have been documented to detect not only monomeric Aβ but also oligomers and larger aggregated species [33].

Taken together, in this study we have identified possible toxic mechanisms in two distinct AD fly models; high levels of Aβ_{1–42} correlate with a high number of apoptotic cells in the Aβ_{1–42} × 2 flies, which also displays increased protein carbonylation indicating oxidative stress. In addition, the lysosomal machinery was found to be slightly down-regulated in the Aβ_{1–42} × 2 flies which can contribute to the pathological events detected in this model. In the AβPP-BACE1 flies, a considerable amount of apoptotic cells was detected, and these flies also display increase in protein carbonylation, representative of oxidative stress. However, it is unlikely that the small amount of Aβ_{1–42} detected is solely responsible for the cell death in these flies. Possible contributors to the toxicity in the AβPP-BACE1
flies are an increased level of intracellular C99 and abnormalities in the endo-lysosomal system.

Notably, this study highlights the versatility of these fly models and how they can be used to increase our understanding of the mechanisms underlying AD. Furthermore, taken together, these AD fly models present a possibility to investigate potential treatment strategies that target Aβ production and Aβ aggregation but also other cellular events closely linked to the disease, for example oxidative stress and dysfunction in the endo-lysosomal pathway.

Materials and methods

Drosophila stocks

The Gal4/UAS system was used to achieve a tissue-specific protein expression in UAS transgenic D. melanogaster [59]. Elav-Gal4 flies were used as the driver line. This allows expression in the CNS and the PNS, in developing neuronal cells and in early glial cells of the flies. Control w1118 flies (only expressing Gal4) were used as a control for the Aβ1-42 × 2 flies, and a fly line expressing Gal4 and human AβPP was used as a control for the AβPP-BACE1 flies. The AβPP-BACE1 fly model has previously been described [14]. Aβ1-42 flies were kindly provided by D. Crowther (AstraZeneca, Floceleris, Oxbridge Solutions Ltd.). These Aβ flies produce an aberrant Aβ42 peptide with additional N-terminal glutamine residue [19]. A fly line containing double copies of signal peptide Aβ1-42 (Aβ1-42 × 2 flies) was generated as previously described [48]. The fly lines were not backcrossed prior to the experiment. Fly crosses were set up at 18 °C at 60% humidity with 12:12-h light:dark cycles. For all biochemical assays, flies were aged for 21 days at 29 °C and then snap-frozen or embedded in Tissue-Tek OCT Compound (25608-930; VWR, Stockholm, Sweden).

Samples preparation and protein quantification of Aβ1-42

For the analysis of total Aβ1-42, a multispot 96-well V-PLEX human Aβ1-42 kit plate (K151LBE-1; Meso Scale Discovery, Rockville, MD, USA) was used. Samples were prepared, and quantification was carried out as previously described in Ref. [14]. In short, approximately 20 fly heads or bodies were homogenized in 25 μL extraction buffer [50 mM HEPES, 5 mM guanidine chloride, 5 mM EDTA, 1 × protease inhibitor (complete EDTA-free Protease Inhibitor Cocktail Tablets; Roche Diagnostics, Basel, Switzerland)], for extraction of both insoluble and soluble Aβ1-42 species. After correcting total protein concentration in each sample due to differences in the homogenization step using the Bio-Rad DC Protein Assay Kit II (500-0112; Bio-Rad, Hercules, CA, USA), protein samples were added to the wells of a multispot 96-well V-PLEX human Aβ1-42 kit plate. The assay was then carried out according to manufacturer’s instructions.

TUNEL assay

OCT blocks with embedded fly heads were sectioned using a Microm HM550 Cryostat (Microm International GmbH, Dreieich, Germany) into 20-μm-thin sections and stored at −20 °C until use. The TUNEL assay was performed using FragEL™ DNA Fragmentation Detection Kit, Fluorescent – TdT Enzyme (QIA39; Merck Millipore, Burlington, MA, USA). The assay was carried out as per the manufacturer’s instructions; however, the incubation time with proteinase K was set to 2 min and the sections were allowed to incubate with the TdT enzyme for 60 min at 37 °C. The slides were analysed using a Zeiss LSM 780 confocal microscope (Zeiss, Oberkochen, Germany). Micrographs were processed in Adobe Photoshop (Adobe Systems, San Jose, CA, USA); background levels were reduced, and the signal levels were enhanced. All images were treated identically. For each genotype, four to five brain sections corresponding to the medulla and lamina were scored in a nonbiased fashion. The scoring system ranged from 0 (no TUNEL-positive cells), 1 (a few TUNEL-positive), 2 (more TUNEL-positive cells, but still a lot of TUNEL-negative cells), 3 (approximately 50% TUNEL-positive cells) to 4 (more TUNEL-positive cells than TUNEL-negative cells). The data were plotted and analysed using GRAPHPAD PRISM 7 (San Diego, CA, USA). To identify any significant difference between the groups, a one-way ANOVA followed by Tukey’s post hoc test was performed.

qPCR analysis

w1118, Aβ1-42 × 2, AβPP and AβPP-BACE1 flies were collected and stored at −80 °C. Total RNA was extracted using the RNeasy Micro Plus Kit (Qiagen, Caldwell, ID, USA). The A260/A280 was determined to be above 2.0 on a NanoDrop ND2000 UV-vis Spectrophotometer (Labtech International Ltd., Uckfield, UK), and the RNA integrity was confirmed on a 1% agarose gel showing a single band ~ 2.0 kbp in size, representative of the 18S rRNA and the 28S rRNA (which, in Drosophila, is cleaved into two fragments that migrate at the same position as the 18S rRNA) [60]. cDNA was synthesized using the RNA samples and the ImProm-II™ Reverse Transcription System (Promega UK Ltd., Southampton, UK). qPCR primer sequences for the Drosophila genes, rab5 and lamp1, and the reference genes, gapdh2 and xTub84B, were previously published [61]. Standard curves for all four genes were generated using cDNA concentrations of 0.04, 0.2, 1, 5 and 25 ng and performing standard qPCRs under the experimental conditions: a 20 μL reaction included 0.2 μM primers (Sigma-Aldrich), Fast SYBR® Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), cDNA (ranging 0.04–25 ng per well).
and dH₂O. Efficiency of all reactions was found to be between 90 and 110%, and therefore, the use of the comparative C₇ method for data analysis was applied [62]. Reactions were performed in a StepOnePlus Real-Time PCR System (Applied Biosystems Ltd., Foster City, CA, USA). Each well included: 0.2 μM primer, 2.5 ng cDNA and 1× Fast SYBR® Green Master Mix; each sample was analysed in duplicate. Reactions were performed with an initial denaturation (95 °C, 10 min), followed by 42 cycles of denaturation (95 °C, 15 s), annealing and extension (60 °C, 1 min). Melting curves were monitored between 60 °C and 95 °C. Products were checked by electrophoresis on a 2% agarose gel to verify the presence of one single band (amplicon) with a correct product size. Data were collected from three independent batches (n = 3) of flies (20 flies in each repeat). The qPCR results from multiple runs were analysed using the comparative C₇ method [62]. The change in expression of the two target genes (rab3 and lamp1) in AβPP-BACE1 (Aβ1-42 × 2) was determined relative to the appropriate control sample, that is AβPP (w¹¹¹⁸), and presented as mRNA fold change. Wilcoxon signed-rank test was used to test statistical significance.

Immunohistochemistry

OCT blocks with embedded fly heads were sectioned as described above. The sections were fixed with 4% (w/v) PFA for 10 min at RT and then washed 3 × 3 min with PBS-T. Additional permeabilization of the sections was carried out using 0.5% Tween-20 for 10 min at RT. The washing step was repeated, and the sections were blocked for 60 min at RT using 10% BSA in PBS-T. After blocking, the sections were incubated with the primary antibodies, 4G8 (SIG-39220; BioLegend, San Diego, CA, USA); anti-human Amyloid-β mAb Abeta (3740-5-250; Mabtech); anti-rab5 antibody (ab31261; Abcam, Cambridge, UK); anti-LAMP1 antibody (ab30687); anti-axons antibody (ab12455), all diluted 1 : 500 in 1% BSA in PBS-T. After repeating the washing step, the sections were incubated with secondary antibodies goat anti-mouse Alexa 594 (R37121; Thermo Fisher Scientific) and goat anti-rabbit Alexa 488 (R37116; Thermo Fisher Scientific), diluted 1 : 500 in 1% BSA for 60 min at RT. After a final washing step, the sections were rinsed with dH₂O and left to dry before mounting them with VECTASHIELD DAPI (H-1200; Vector Laboratories, Burlingame, CA, USA). The slides were analysed using a Zeiss LSM 780 confocal microscope. Micrographs were processed in Adobe Photoshop; background levels were reduced, and the signal levels were enhanced. All images were treated identically.

Protein carbonylation assay

The heads of snap-frozen flies (20 flies/genotype) were homogenized in 25 μL RIPA lysis and extraction buffer (89900; Thermo Fisher Scientific) with 1× Protease Inhibitor (cOmplete EDTA-free Protease Inhibitor Cocktail Tablets; Roche Diagnostics) and 50 mM dithiothreitol. After centrifuging the samples for 10 min at 18 928 g, the supernatant was collected and the total protein level extracted was determined using a Bio-Rad DC Protein Assay Kit II (500–0112; Bio-Rad). Samples were prepared to have a final protein concentration of approx. 30 mg·mL⁻¹. The sample preparation was then divided into two Eppendorf tubes, where derivatization of the carbonyl groups was carried out using the OxyBlot Protein Oxidation Detection Kit (S7150; Merck, Kenilworth, NJ, USA) according to the manufacturer’s instructions on one half of the sample. The other half was used as a negative control, where derivatization-control solution (S7150; Merck) was added instead of DNPH solution (S7150; Merck). Gel electrophoresis was performed using Bolt 4–12% Bis-Tris Plus Gels (NW04120BOX; Life Technologies, Carlsbad, CA, USA). Transfer was performed using an original iBlot Gel Transfer Device from Life Technologies onto PVDF mini membranes (IB401002; Life Technologies). The membrane was blocked using 10% BSA for 1 h at RT. The primary antibody (rabbit anti-DNP antibody, 90451; Merck) was prepared diluted 1 : 150 in 1% BSA and added to the membrane for 1 h, RT. This was followed by a washing step, 3 × 3 min with PBS-T before adding the secondary antibody (goat anti-rabbit, HRP-conjugated, 90452; Merck) for 1 h, RT, diluted 1 : 300 in 1% BSA. The washing step was repeated before incubating the membrane with Clarity Western ECL Substrate (17050605; Bio-Rad) for 5 min before imaging on a ImageQuant LAS 4000 (GE Healthcare Life Sciences, Marlborough, MA, USA). Bands from the nonderivatized negative control sample preparation that appears in all samples were used as a loading control.

Western blot analysis

Protein extract from fly heads was obtained as described above. Samples of approximately 5 μg) were loaded onto a Bolt 12% Bis-Tris Plus Gel and after protein separation by electrophoresis transferred onto a nitrocellulose membrane. The membrane was boiled for 5 min in PBS and thereafter blocked in 5% milk in TBS-Tween. Immunodetection was performed with monoclonal primary antibodies: anti-C-terminal AβPP (A8717, 1 : 8000; Sigma-Aldrich) and anti-tubulin (loading control; ab7291; 1 : 2000; Abcam) followed by HRP-conjugated corresponding secondary antibodies (Dako, Santa Clara, CA, USA). Densitometric analysis was performed on four separate blots using IMAGEJ 1.50i (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Bands corresponding to full-length APP and the C-terminal cleavage fragment (CTF) were normalized to tubulin expression. Statistical analysis was performed using the Mann–Whitney U test. Differences were considered significant when P ≤ 0.05.
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Conflict of interest

The authors declare no conflict of interest.

Author contributions

A-CB and LB conceived and designed the project; LB, ZD, HA and GE acquired the data; LB, A-CB, ZD, JRK, LSI, HA and KK analysed and interpreted the data; A-CB, LB, KK, ZD, HA and JRK and LSI wrote the paper.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Entire blot containing the specific bands for full length AβPP and CTFs shown in Fig. 1E.