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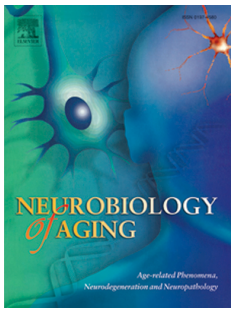

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Metabolic changes may precede proteostatic dysfunction in a *Drosophila* model of amyloid β peptide toxicity

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Abstract

Amyloid beta peptide (Aβ) aggregation is linked to the initiation of Alzheimer's disease; accordingly, aggregation-prone isoforms of Aβ, expressed in the brain, shorten the lifespan of *Drosophila melanogaster*. However, the lethal effects of Aβ are not apparent until after day 15. We used *shibire*^{TS} flies that exhibit a temperature-sensitive paralysis phenotype as a reporter of proteostatic robustness. In this model we found that increasing age, but not Aβ expression, lowered the flies' permissive temperature, suggesting that Aβ did not exert its lethal effects by proteostatic disruption. Instead we observed that chemical challenges, in particular oxidative stressors, discriminated clearly between young (robust) and old (sensitive) flies. Using nuclear magnetic resonance spectroscopy in combination with multivariate analysis, we compared water-soluble metabolite profiles at various ages in flies expressing Aβ in their brains. We observed two genotype-linked metabolomic signals, the first reported the presence of any Aβ isoform and the second the effects of the lethal Arctic Aβ. Lethality was specifically associated with signs of oxidative respiration dysfunction and oxidative stress.

Introduction

Many common disorders of the elderly are linked to the deposition of specific protein aggregates resulting in damage to neurological, vascular and endocrine tissues. The incidence of these conditions increases approximately exponentially with age and consequently the underlying mechanisms have become the focus of much research (Morimoto, 2008,Taylor and Dillin, 2011). Some of the common pathological protein aggregates include plaques (Hardy and Selkoe, 2002) and tangles (Mandelkow, et al., 2007) in Alzheimer's disease (AD) (Blennow, et al., 2006), while other amyloid deposits are associated with disorders such as amyloid angiopathy (Revesz, et al., 2002) and type II diabetes (Moreira, 2012). As the vast majority of these pathological hallmarks only become apparent in old age, it has been natural to question whether a common cause is a failure of the organism to maintain the correct, native conformation of proteins throughout its life. This concept of "proteostatic collapse" occurring in mid-life is supported by *in vivo* data, particularly from experiments conducted with the nematode worm *C. elegans* (reviewed in (Balch, et al., 2008,Taylor and Dillin, 2011)). Studies led by Morimoto and Dillin indicate a concerted loss of stability of a wide range of proteins during mid-life in the worm; moreover this destabilisation can be accentuated by the presence of a protein misfolding stressor such as an aggregation-prone poly-glutamine (polyQ) peptide (Ben-Zvi, et al., 2009,Gidalevitz, et al., 2006).

The underlying mechanisms behind this proteostatic collapse are insufficiently understood. There is evidence for the involvement of genetically-regulated responses to, for example, calorie restriction (Kenyon, et al., 1993) and heat shock (Morimoto, 1993), however several other factors may also play a key part in this process. Among these, the role of oxidative stress and age-related metabolic changes may be of particular importance (Finkel and Holbrook, 2000,Houtkooper, et al., 2011). It is not clear whether proteostatic collapse causes metabolic changes or vice-versa. However, there is strong biochemical evidence showing that long-lived proteins undergo oxidative and other chemical changes that progressively impede their function (Grune, et al., 2001,Terman and Brunk, 2006). In addition, the incidence of metabolic disorders such as diabetes also correlates with ageing and in many patients diabetes and proteostatic disorders such as AD are both present (Biessels, et al., 2006,Munch, et al., 1998).

The importance of metabolic alterations as part of the pathology of age-related neurodegenerative disorders such as Alzheimer's has been supported by an array of studies conducted with mouse models and clinical samples from patients with AD (reviewed in (Barba, et al., 2008,Trushina and Mielke, 2013)). Some plausible biomarkers of AD include n-acetylaspartate that reports neuronal loss and myo-inositol which is a marker of gliosis and inflammation. A recent lipidomic study has indicated that a panel of ten metabolic biomarkers is sufficient to allow confident prediction of which patients with mild cognitive impairment will progress to AD (Mapstone, et al., 2014). Such metabolic markers can be used to detect disease onset and then monitor pathological progression. While the metabolic changes in AD are distinct from normal ageing, it remains unclear whether these changes are the cause or effect of the associated protein aggregation pathology.

Defining the processes underpinning the age-related incidence of AD is of practical importance, as diagnostic assays based on genomics, proteomics and metabolomics are all now possible. By understanding the initial steps that lead to pathology we can identify biomarkers and develop assays that will allow early diagnosis and then treatment of age-related disorders. In this study we have used a well-characterised *Drosophila* model (Crowther, et al., 2005) as a tool to extend our understanding of how proteostatic deficits and metabolomic changes may lead to the age-related toxicity of the Aβ peptide (Rogers, et al., 2012).

Methods

Fly stocks

Drosophila cultures were maintained on standard fly medium containing 1.25% (w/v) agar, 10.5% (w/v) dextrose, 10.5% (w/v) maize, 2.1% (w/v) yeast that was supplemented with a fungicide (methylparaben) and sparse grains of dried yeast on the surface. Fly cultures were maintained in

bottles and vials at 18°C, 25°C or 29°C according to standard *Drosophila* husbandry techniques (Ashburner, et al., 2005). Experiments using the inducible Gene Switch (GS) driver (Osterwalder, et al., 2001) for inducible Aβ expression were performed with flies carrying two transgenes for $A\beta_{42}$. This stock has been characterised previously (Luheshi, et al., 2007). Unless otherwise stated, all other experiments were carried out with single transgenic $A\beta_{40}$, $A\beta_{42}$ and Arctic $A\beta_{42}$ flies which have been described previously (Kruppa, et al., 2013). The Aβ transgene was inserted into the 51D location on the second chromosome using the φC31 integrase system (Bischof, et al., 2007). Temperature titration experiments to measure the protein homeostasis were performed with *Drosophila shibire*^{TS2} constructs which have been described earlier (Grigliatti, et al., 1973).

Longevity assays with constitutive Aβ expression

Longevity assays were conducted as described previously (Crowther, et al., 2005). *Drosophila* stocks carrying various UAS-Aβ isoforms were crossed with the *elav*^{c155}-Gal4 promoter stock to induce pan-neuronal transgene expression in the offspring. Longevity experiments were performed on populations of 90-100 mated females, sorted into groups of 10. The statistical interpretation of longevity data was conservative: we considered the median survival values for each of the 9-10 sub-groups of flies per condition as estimates of the population mean. Accordingly the Wilcoxon-rank test was used to determine whether the median survivals of the population pairs were significantly different from each other. When estimating mortality trajectories a minimum of 150 flies per genotype were assessed. Mortality trajectories were generated by plotting the natural log of the chance of dying over the coming 24 hrs versus the age of the flies. The first and last 5% of all recorded death events were excluded to minimise noise as described previously (Shaw, et al., 1999).

Longevity assays with inducible Aβ expression

The mifepristone (RU 486, Sigma-Aldrich) inducing agent was dissolved at 10 mg/ml in ethanol and further diluted in sterile water to its final concentration. Vials of fly food were treated with 300 µL of the drug solution, or vehicle, and left to air dry for two days at room temperature (25˚C) before use. The food was used within four days of preparation or else discarded. To achieve inducible, pan-neuronal, expression of Aβ with the GS system, *Drosophila* homozygous for the $A\beta_{42}$ transgene on the second and third chromosomes ($A\beta_{42.3}$; $A\beta_{42.4}$, as previously described in (Crowther, et al., 2005)) were first crossed with the *GS-elav-Gal4* driver line and the offspring treated with 200 µg/mL of RU 486. In other respects, longevity assays employing drug-inducible Aβ expression (Osterwalder, et al., 2001), were identical to the protocol for constitutive Aβ expression as described above (Crowther, et al., 2005).

Temperature titration locomotion assays with *Drosophila shibire*^{TS2} mutants

The effect of Aβ expression on the shibire^{TS2}-mediated paralytic phenotype was assessed for flies of various ages. *UAS-Aβ* stocks were crossed with flies carrying the constitutive pan-neuronal *elav-Gal4* driver (chromosome 3). Male offspring carrying both transgenes, over balancer chromosomes, were mated with females homozygous for the *shibire*^{TS2} allele (X chromosome). The walking velocities of male offspring carrying *shibire*^{TS2} +/- Aβ expression were recorded by using the iFly tracking system described previously (Jahn, et al., 2011). The climbing velocity of 30 male flies, in three groups of 10 was assessed in triplicate for each genotype at day 5, 15 and 35 after eclosion. All *Drosophila shibire*^{TS2} mutants were maintained and aged at the permissive temperature of 18°C. Before each measurement, the flies were incubated for 90 sec in pre-heated glass vials in a water bath at required temperature. Differences in climbing performance between genotypes were probed for statistical significance by a two-way analysis of variance (ANOVA) using the GraphPad Prism statistical package (Graph Pad Prism Software, Inc.).

Hydrogen peroxide lethality assays

Mated female flies were sorted into at least 8 groups of 20 and cultured at 25°C throughout the experiment. The control population was maintained on 2% (w/v) agar containing 5% (w/v) sucrose. The food was supplemented with 10% (v/v) hydrogen peroxide for the test population. Survival rates

72 hr post-exposure were tabulated. The statistical significance of mortality differences between genotypes throughout ageing was assessed by a two-way ANOVA.

Brain dissections

Brain dissections were performed as described previously (Costa, et al., 2011).

Quantitative PCR

Quantitative PCR for Aβ mRNA was carried out as described previously (Kruppa, et al., 2013).

Quantification of Aβ

The procedure has been described elsewhere (Luheshi, et al., 2010). For the quantification of total Aβ levels, heads from 10 flies were collected and homogenized in 20 µL 5 M guanidine hydrochloride (GnHCl; Melford Laboratories, UK) extraction buffer (1x stock: 50 mM Hepes pH 7.3, 5 mM EDTA, 5 M GnHCl) manually for 1 min. The samples were then sonicated in a water bath at room temperature for 480 sec and centrifuged for 7 min at 18,000 g. Five µL of supernatant were diluted down 1:5 in a Meso Scale Discovery (MSD) dilution buffer (50 mM Hepes pH 7.3, 10 mM EDTA, 0.1% (w/v) MSD Blocker A (bovine serum albumin in a PBS-based buffer); MSD, USA). Each 96 well Avidin or Streptavidin plate (MSD, USA) was pre-coated with 3% (w/v) MSD-Blocker A in PBS either for 1 hr at room temperature on a shaker or overnight at 4°C. Between each of the next steps the plate was washed with 0.05% (v/v) PBS-Tween. Samples were incubated for 1 hr at room temperature with the biotinylated 6E10 (Cambridge Biosciences, UK) primary antibody (epitope located within the 3-8 N-terminal amino acids of Aβ), followed by 1.5 hr of incubation with the calibration and test samples in the various wells (the standard curve mixture, water and the *Drosophila* samples). Finally, 1 hr incubation at room temperature with the sulpho-tagged 21F12 secondary antibody (epitope located within the 33-42 C-terminal amino acids of Aβ, Elan Pharmaceuticals, USA) was performed. The plate was read on a MSD plate reader (MSD, USA) according to the manufacturer's instructions. For measuring soluble Aβ 1% (w/v) SDS in PBS buffer was used for the initial extraction instead of 5 M GnHCl. The remaining steps of the protocol were identical except for the centrifugation step: a 7 sec pulse spin was used instead, to ensure the peptide remained in the supernatant. The standard curve for the assay was constructed using the SDS extraction protocol and was used to estimate concentrations for both SDS-soluble and total Aβ assays; this may result in significant underestimation of total Aβ levels due to GnHCl interference in the latter assay. Differences in Aβ concentrations between samples were probed by the Student's *t*-test and the ANOVA Bonferroni test.

^{1}H-Nuclear Magnetic Resonance (NMR) spectroscopy

To induce pan-neuronal expression of Aβ in the progeny, *UAS-Aβ$_{40}$*, *UAS-Aβ$_{42}$*, and *UAS-Aβ$_{42}$ Arctic* stocks were crossed with the *elav^{c155}-Gal4* promoter. To control for metabolic changes in the absence of Aβ expression, *elav^{c155}* flies were crossed with a stock containing the empty 51D insertion site. Subsequently, the progeny of this stock are referred to as "51D control" in the text. The offspring with the desired genotype were aged at 29°C before harvesting. Flies were collected according to a computer-generated random scheme that avoided any time-of-day or position-in-cohort artefacts. Once collected, the flies were frozen in liquid nitrogen and decapitated by vortexing while frozen. Fly heads and bodies were homogenized separately in 350 µL of ice-cold 50% (v/v) acetonitrile (Merck, Germany) in distilled water solution by using an electric homogenizer. The samples were then centrifuged for 20 min at 18000 g (Minispin centrifuge; Eppendorf, Germany) at 4°C and the supernatant was transferred into a new microcentrifuge tube, lyophilized and stored at -80°C. Each sample consisted of either 50 fly heads or bodies. The experiment was performed in triplicate per condition. Prior to NMR analysis, the samples were rehydrated in 650 µL of 50 mM phosphate buffer in D_2O (pH 7.4) supplemented with 50 mg/L 3-trimethylsilyl propionic acid D4 (TSP) as a chemical shift reference, and 50 mg/L sodium azide to avoid bacterial growth. Subsequently, 600 µL of the rehydrated sample were transferred into a 5 mm NMR tube. The NMR measurements were carried out at 25°C on a Bruker Avance-III 600 spectrometer (Bruker Biospin, Germany) which was equipped with a double tuned ^{1}H-^{13}C cryoprobe and operated at a ^{1}H frequency of 600.13 MHz. The ^{1}H NMR

spectra were acquired using a single 90° pulse experiment with a Carr Purcell Meiboom Gill (CPMG) delay added, in order to attenuate broad signals from high molecular weight components. The total CPMG delay was 40 ms and the spin echo delay 200 µs. The water signal was suppressed by pre-saturation of the water peak during the relaxation delay of 4 sec. A total of 98304 data points spanning a spectral width of 24 ppm were collected in 64 and 128 transients for fly bodies and heads, respectively. For assignment purposes two-dimensional ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HSQC-TOCSY and ^{1}H-^{13}C HMBC spectra were acquired. The spectra were processed using iNMR (www.inmr.net). An exponential line broadening of 0.5 Hz was applied to the free induction decay, prior to Fourier transformation. All spectra were referenced to the TSP signal at -0.017 ppm, automatically phased and baseline corrected. The spectra were aligned using Icoshift (Savorani, et al., 2010) and the region around the residual water signal (4.88-4.67 ppm) was removed. Moreover, the high- and low-field ends of the spectrum, where no signals except the reference signal from TSP appeared, were also removed (i.e. leaving data between 9.5 and 0.5 ppm). The integrals were normalized to total intensity to suppress trivial separation based on variations in sample amount and the data was scaled using pareto scaling (Craig, et al., 2006) and centered.

NMR data analysis

Initially, the whole dataset was subjected to principal component analysis (PCA) (Stoyanova and Brown, 2001). Afterwards, orthogonal projection to latent structures discriminant analysis (OPLS-DA) models were created to separate the following three classes of fly populations: (1) control flies, (2) $A\beta_{42}$ expressing flies that showed a minimal decrease in longevity under the tested conditions and (3) Arctic $A\beta_{42}$ flies which showed a significant decrease in median survival in a longevity assessment that was run in parallel. OPLS-DA models are multivariate models that predict group membership based on a multivariate input, in this case the NMR spectra. The model separates variations due to group membership from other (orthogonal) variations (Trygg J, 2003). The models were constructed by using the spectral data of 18 and 21 day old flies and were then implemented to predict the class and age for all other flies. To identify metabolites that responded to non-toxic Aβ expression we constructed OPLS-DA models using data from 14-24 day old 51D controls that do not express Aβ, and compared them to $A\beta_{40}$ and $A\beta_{42}$ flies where Aβ expression was largely non-toxic. Similarly, to probe for metabolites associated with Aβ *toxicity*, we compared profiles of $A\beta_{40}$ and $A\beta_{42}$ flies with those from flies expressing the lethal Arctic $A\beta_{42}$. The loadings and the correlation coefficient (R) between intensities at the individual frequencies and the predictive component were calculated. A cutoff value for R^2 corresponding to $p < 0.01$ with Bonferroni correction for an assumed number of 100 metabolites was calculated from the distribution of R^2 values in 10000 permutated data sets. Xanthurenic acid was confirmed by spiking. The remaining assignments were done based on chemical shifts only, using earlier assignments and spectral databases described elsewhere (Cui, et al., 2008,Malmendal, et al., 2006,Pedersen, et al., 2008), and comparison with *Drosophila* metabolites identified by mass spectrometry (Chintapalli, et al., 2013). OPLS models predict the value of a continuous variable based on a multivariate input (Trygg and Wold, 2002). OPLS models for age were built using 14-40 day old control and $A\beta_{42}$ flies. These models were used to predict the "metabolomic age" of samples for $A\beta_{40}$ and Arctic $A\beta_{42}$ expressing *Drosophila*. All multivariate analysis was performed using the Simca-P software (Umetrics, Sweden). All procedures were done for both fly heads and bodies.

Results

Ageing increases the sensitivity of *Drosophila* to the toxic effects of Aβ

The toxicity of Aβ peptides in our *Drosophila* model system is dependent on both the aggregation propensity of each isoform and the intensity of its expression. When cultured at 25 °C, flies carrying $A\beta_{40}$ and $A\beta_{42}$ transgenes inserted at the 51D genomic locus and driven by *elav^{c155}-Gal4* exhibit no reduction in longevity (fig. 1A) and do not show Aβ deposition (fig. S1). By contrast, flies expressing the aggregation prone Arctic (E22G) variant (Nilsberth, et al., 2001) of $A\beta_{42}$ under the same conditions have a shorter lifespan and display Aβ deposits in the brain (figs. 1A and S1). These fly lines have been extensively characterised and the transgenes have been shown to express mRNA at equivalent levels (Crowther, et al., 2005). Moreover, we also confirmed that Aβ mRNA levels did not change significantly throughout life (fig. S2).

When we calculated the mortality trajectories (logarithm of the daily probability of death), we found that the 51D control, $A\beta_{40}$ and $A\beta_{42}$ lines followed the same linear trajectory, reflecting the expected exponential increase in the likelihood of death with age. The equivalent trajectory for Arctic $A\beta_{42}$ flies was also linear but was steeper, indicating that the age-related probability of death in these flies was rising more rapidly than for flies expressing $A\beta_{40}$ and $A\beta_{42}$. Remarkably, when the linear fits were extrapolated back in time, the Arctic $A\beta_{42}$ flies did not diverge from the other lines until a point in mid-adult life around day 15 (fig. 1B).

Thus, young flies (<15 days) appeared more robust and showed less marked increases in the likelihood of death in the presence of Aβ isoforms that are otherwise lethal when present later in life. To investigate this observation directly, we set up a series of flies that carried two $A\beta_{42}$ transgenes under the control of an inducible promoter. Our initial experiments examined the longevity effects of 15-day windows of Aβ induction, specifically days 0-15, 10-25 and 20-35 (fig. 2A). Here, we did not observe any substantial reductions in longevity when Aβ-expression was triggered only before day 15. However, when Aβ expression was induced after this age, we recorded significant reductions in lifespan.

A subsequent experiment compared the survival of four groups of *Drosophila*: i) where Aβ expression was never induced, ii) where Aβ was continuously induced, iii) where Aβ expression was induced from hatching until day 15 and iv) where Aβ was induced only after day 15 (fig. 2B). To ensure that the levels of expressed Aβ were similar for all groups, we undertook a series of experiments in parallel to calibrate the induction of Aβ expression for all conditions (fig. S3). When we examined the median survivals of the groups we observed that, as expected, flies exposed to continuous Aβ induction had a shorter lifespan than those in which Aβ expression was never induced. Interestingly, restricting the expression of Aβ to mid-adult life and later (after day 15) resulted in a median survival that was essentially identical to the median survival of flies where Aβ was expressed continuously. On the other hand, when Aβ was expressed only up to day 15, the lifespan of flies was similar to controls with no Aβ induction at all. Taken together, these survival data support the hypothesis that older flies are more sensitive to the lethal effects of Aβ expression as compared to younger flies.

Using mortality trajectory analysis we were able to estimate this relative robustness of young flies. Specifically, in fig. 2C the filled squares represent the mortality of flies with continuous Aβ expression; by comparison the open squares represent flies that are identical except that they did not experience Aβ expression from days 0-15. These traces are essentially parallel and are separated on average by a mortality displacement of 1.2. Assuming that this relative displacement accrued linearly over days 0-15 this corresponds to an Aβ-induced increase in the gradient of the mortality curve of 0.08 day^{-1} (SEM: $9x10^{-3}$ day^{-1}) (see table S1A). For filled circles the only Aβ expression is from day 0-15 and these are compared to empty circles where Aβ was never expressed. Where paired data points are available, the average mortality displacement between these traces is 0.7, resulting in a calculated daily rate of mortality increase of 0.05 day^{-1} (SEM: $8x10^{-3}$ day^{-1}) over days 0-15. The average of these two estimates of the Aβ-attributable increase in the mortality trajectory gradient between days 0-15 is 0.06 day^{-1}.

By contrast, the daily increase in mortality that is attributable to Aβ expression in mature adults can be quantified directly by comparing the gradients of the following paired traces: filled squares vs. filled circles (difference in gradients 0.10 day^{-1}) and empty squares vs. empty circles (difference in gradients 0.09 day^{-1}; see table S1B). The average of these two estimates of the Aβ-attributable increase in the mortality trajectory gradient days 15+ is 0.10 day^{-1}. This allows us to estimate that the Aβ-attributable increase in the mortality trajectory gradient is approximately 50% greater in older adult flies (days 45-85), as compared to young adults (days 0-15).

Ageing alters Aβ metabolism, favouring its insoluble deposition

Using inducible pan-neuronal Aβ expression, we compared the accumulation of soluble and total peptide in young and aged *Drosophila* (Osterwalder, et al., 2001). One percent w/v SDS extraction yielded soluble peptide and these levels were compared to total Aβ as defined by GnHCl-extracted material. We observed an initial accumulation of soluble peptide in flies expressing Aβ from day 1-15,

which dropped to a base level after the inducing agent was withdrawn (fig. 3, green). Under such circumstances there was little increase in total Aβ levels, indicating that the soluble material was largely cleared. When induction was continued, then soluble and subsequently insoluble peptide progressively accumulated (30-40 days, black). Induction of Aβ exclusively in the older flies (red, day 15+), yielded relatively little soluble material with a higher proportion accumulating as insoluble deposits rather than being cleared.

Aβ toxicity is not accompanied by a generalised protein homeostasis collapse

Previous studies, particularly in the nematode worm *C. elegans*, have indicated that a concerted loss of protein homeostasis accompanies the onset of protein misfolding and amyloid deposition. It was also shown that additional misfolding stressors can destabilise the proteome (Ben-Zvi, et al., 2009,Gidalevitz, et al., 2006). To test whether Aβ expression precipitates proteostatic collapse in *Drosophila* we assessed the locomotor behaviour of a temperature-sensitive dynamin variant (*shibire*^{TS2}) using paralysis as a reporter of proteome stability (van der Bliek and Meyerowitz, 1991). We have developed a novel assay in which *shibire*^{TS2} flies expressing various isoforms of Aβ, or not, undergo a temperature titration to determine how sensitive they are to thermal stress (fig. 4). In all tested *Drosophila* we observed an age-related increase in temperature sensitivity, specifically the temperature at which the flies walked at half their normal velocity, was reduced from 22°C at day 5 to 19°C at day 35. Similarly the temperature at which the flies were all paralysed was reduced from 28°C at day 5 to 25°C at day 35.

These data indicate that the *shibire*^{TS2} variant of dynamin becomes thermally destabilized as the flies age. However this effect was not modified by Aβ peptide expression, including the Arctic isoform that is toxic under these conditions (fig. S4). The failure of Aβ expression to modulate the age-related thermal destabilization of dynamin is consistent with the hypothesis that accelerated proteostatic collapse is not the primary mechanism of Aβ toxicity in our model system.

Ageing and Aβ expression predispose *Drosophila* to oxidative damage

Age-related changes in metabolism, in particular the generation of reactive oxygen species, are an alternative factor that may mediate the increased sensitivity of older flies to Aβ toxicity (Finkel and Holbrook, 2000,Rival, et al., 2009). To test this possibility we gave an oxidative stressor, in the form of 10% (v/v) H_2O_2 in the food, to both young and older flies. At day two, control flies and those expressing $A\beta_{40}$ were remarkably resistant to this insult with few dying after 72 hrs of exposure. By contrast the young $A\beta_{42}$ expressing flies were more sensitive with approximately half dying following the same treatment (fig. 5, left bars). Lethality was even higher for $A\beta_{42}$ Arctic flies. In this regard the day two $A\beta_{42}$ flies resemble older, day 20 flies, which uniformly exhibit increased sensitivity to H_2O_2 regardless of Aβ expression (fig. 5, right bars). Thus, day 2 flies are proteostatically robust but sensitive to oxidative stress when they express $A\beta_{42}$, putting the biochemical changes pathologically upstream of proteome dysfunction.

A systematic analysis of the metabolic consequences of Aβ expression in the *Drosophila* brain

To assess the age-related changes in the metabolome in control flies and those expressing Aβ, we have compared the water-soluble metabolite levels in tissue extracts by ^{1}H-NMR spectroscopy. Flies were aged and at specified times frozen and separated into heads and bodies so that the local and systemic effects of Aβ expression could be assessed. Our initial approach was to analyse the NMR spectra using PCA. When we plotted principal component 2 (PC2) versus PC3 it became apparent that there was an age-related trajectory that all the samples were following (fig. 6). In almost all cases, the greatest differences were between days 1 and 8 (open circles) and these were broadly conserved between genotypes. Moreover, the expression of any Aβ isoform produced a displacement of the trajectories as compared to the scores of 51D control samples. This indicates the presence of profound metabolic changes around the time of hatching in addition to those that can be attributed to Aβ expression. PC1 reported metabolic changes that did not correlate with genotype or age and was not analysed further. Furthermore, a two way MANOVA on all PCs revealed significant ($p < 1\times10^{-15}$) differences in the

age-linked metabolomic profiles for 51D controls vs. $A\beta_{40}/A\beta_{42}$ (controls vs. non-toxic Aβ expression) and for $A\beta_{40}/A\beta_{42}$ vs. Arctic $A\beta_{42}$ (non-toxic Aβ– vs. toxic Aβ-expression), for both bodies and heads.

Aβ expression induces a distinct metabolite response

After day 14 the fly metabolome becomes less temporally dynamic and so we created an OPLS-DA model implementing data from mature flies. The goal was to discriminate metabolite profiles between a) 51D control flies (fig. 7, black) and those expressing, b) $A\beta_{42}$ which is aggregation prone but remains non-toxic under the experimental conditions (blue) and c) Arctic $A\beta_{42}$ which is both aggregation-prone and toxic (red). Accordingly, the model was trained using data from day 18 and 21 flies only; furthermore data from flies expressing $A\beta_{40}$, which has a low aggregation propensity and is non-toxic (green), were not included. When the model was applied to samples that were not part of the training dataset three groupings became apparent. Firstly, we observed that the expression of any Aβ isoform resulted in a shift away from controls along the x-axis, this may be interpreted as an adaptive response to aggregation prone peptide expression as this signal is compatible with normal longevity. Among the Aβ-expressing group, there was an additional separation that was associated with reduced longevity as seen in Arctic Aβ flies (fig. 7, panels A and B). Notably, flies expressing $A\beta_{40}$ were located in the same region as those expressing $A\beta_{42}$. Incidentally the oldest (30 - 40 days) control and $A\beta_{42}$ flies became more similar to the $A\beta_{42}$ Arctic flies, but only in the systemic metabolome (fig. 7B).

We also created an OPLS model, linking age to metabolic profiles, based on data from day 14 - 40 control and $A\beta_{42}$ flies. This model was used to estimate the "metabolic age" of flies expressing $A\beta_{40}$ and $A\beta_{42}$ Arctic, which were not included in the model. The predicted ages for these flies corresponded closely to calendar age except for systemic samples from $A\beta_{42}$ Arctic flies that consistently appeared to be 5 days older than expected (fig. S5A).

Metabolic changes are associated with Aβ expression and Aβ toxicity

Next we identified which key metabolites accounted for the metabolomic signals that are apparent in fig. 7. Firstly we compared controls with $A\beta_{40}$ and $A\beta_{42}$ flies to determine the Aβ-linked metabolic changes in the absence of a significant longevity phenotype. These flies have successfully adapted to protein aggregation stress and so metabolic changes may represent adaptive cellular responses. In the presence of $A\beta_{40}/A\beta_{42}$ there was a fourfold increase in maltose (fig. 8 panels A and B, chemical shifts 5.40, 4.64 ppm *inter alia*, fig.9 panels C and I) and a decrease in tyrosine (7.18, 6.88 ppm, fig. 8 panels A and B) in both head and body samples. In the head extracts there was a marked increase in xanthurenic acid (7.53, 7.37, 7.16, 6.92 ppm fig. 8 B, fig. 9K) upon $A\beta_{40}/A\beta_{42}$ expression.

Secondly we compared $A\beta_{40}$ and $A\beta_{42}$ with Arctic $A\beta_{42}$ flies to identify how the metabolome specifically reported decompensated protein aggregation. Flies expressing the toxic Arctic $A\beta_{42}$ isoform showed a distinct series of metabolic changes when compared to those expressing $A\beta_{40}/A\beta_{42}$. In particular the levels of gluconic acid (4.10, 4.01, 3.75 ppm fig.8 panels C and D, fig.9 panels A and G) and histidine (7.77, 7.05 ppm fig. 8 panels C and D, fig.9 panels B and H) were increased in older (14-24 day) Arctic $A\beta_{42}$ expressing flies. While maltose levels were still much higher than in control flies, they were lower than in $A\beta_{40}$ and $A\beta_{42}$ expressing *Drosophila* (fig.8, panels C and D, fig.9 panels C and I). Specific changes in fly heads were higher lactate (1.31 ppm fig. 8D, fig. 9J) and lower alanine (3.77, 1.47 ppm fig. 8D). In fly bodies acetate (1.90 ppm) and fatty acid CH_2 groups (1.26 ppm) were found to be increased (fig. 8C), while adenosine nucleotides (AXP) (8.52, 8.26, 6.13 ppm) and phosphocholine levels (4.17, 3.58, 3.21 ppm) were reduced (figs. 8C and 9D). In both heads and bodies signals from unidentified aromatic compounds (7.93, 6.93 ppm, and 8.03, 7.80, 7.20, 6.90 ppm for heads and bodies, respectively) appeared in NMR spectra from the Arctic $A\beta_{42}$ expressing flies (fig. 9 panels F and L). These signals could not be unambiguously assigned, but when the chemical shifts were matched against the Human Metabolome Database (Wishart, et al., 2013), 8 of the 10 best matches were hydroxylated, carboxylated, or chlorinated compounds. As an example the ^1H next to a hydroxyl substituted carbon typically appears around 6.9 ppm. An additional signal, which likely corresponds to a methyl or methylene attached to an aromatic ring, appeared at 3.25 ppm

The concentrations of a number of key metabolites were age- and/or Aβ-dependent. The compounds that most faithfully reported the presence of Aβ, irrespective of lethality, include maltose, in the head and body and xanthurenic acid levels in the fly head (fig. 9 panels C, I and K) spectra. Both of these metabolites were increased in flies expressing $A\beta_{40}$, $A\beta_{42}$ and Arctic $A\beta_{42}$, as compared to controls. Metabolomic changes specifically induced by the expression of the toxic Arctic $A\beta_{42}$ included age-related increases in gluconic acid, histidine and the appearance of unidentified aromatic compounds in both head and body samples (fig. 9 panels A, B, E, F, G, H and L). Levels of lactate were also higher in head samples (fig. 9 panel J), and levels of AXP and phosphocholine lower in body samples (fig. 9 panel D), in Arctic $A\beta_{42}$ as compared to $A\beta_{40}$/$A\beta_{42}$ flies.

Discussion

AD is one of the most pressing age-related disorders facing society. Ageing may be considered as the time-dependent accumulation of deficits in protective functions that result in a multisystem syndrome defined by increased fragility and propensity to die (Kirkwood and Austad, 2000,Partridge and Gems, 2002). In many organisms this produces a log-linear increase in mortality rates with time (Curtsinger, et al., 1992,Partridge, et al., 2005). In some respects the gradient of the mortality trajectory may be understood as the rate of physiological ageing with chronological time. *Drosophila* ageing can be slowed by reducing the ambient temperature and delayed by restricting calorie intake (Mair, et al., 2003,Partridge, et al., 2005). One important contributor to increased age-related fragility is a progressive degradation in the structural integrity of proteins. Such deficits in proteostatic mechanisms have been described primarily in *C. elegans* but a similar process is thought to underpin the strongly age-related incidence of protein misfolding disorders such as Alzheimer's, Parkinson's and other, systemic, amyloid diseases (Chiti and Dobson, 2006,Taylor and Dillin, 2011).

In this study we have used our Aβ-expressing *Drosophila* as a model of AD to probe how protein aggregation may modulate mortality. We asked the question whether a generalised increase in protein aggregation propensity is a cause or a consequence of the ageing process. Our initial experiments indicated that expression of the lethal Arctic $A\beta_{42}$ increased the gradient of the mortality trajectory. To our surprise we observed that extrapolation of the plots to early life indicated that Arctic flies diverged from the control flies at a point around day 15 of adult life (fig. 1B). This implies that young *Drosophila* before this time point are robust with respect to protein misfolding stress and that they becomes susceptible to the lethal effects of protein aggregation during mid-adult life. We tested this hypothesis directly using an inducible version of the fly model in which Aβ expression only occurred upon oral dosing with RU486. In the first set of experiments (fig. 2A), when Aβ was expressed in 15 day intervals, we found that the earliest, day 1-15, exposure window had only minimal consequences for the flies; exposure to Aβ later in life resulted in a reduction in the median survival. In the second set of experiments (fig. 2B) we compared four cohorts of flies: those in which Aβ was expressed early (day 1-15), late (after day 15), continuously or never. In this study the lifespan of flies expressing Aβ only after day 15 was essentially identical to those where Aβ expression was continuous. This in turn indicates that there was little or no contribution of early Aβ to the accumulated deficits later in life. In order to quantify the early and late contributions of Aβ expression to mortality, we plotted and compared the four mortality trajectories (fig. 2C). Remarkably the gradient of the mortality trajectories was determined by whether Aβ was induced post day 15, whereas early Aβ expression resulted in a displacement along the y-axis. Assuming that this displacement accumulated steadily over the first 15 days of life, we can estimate that older flies were 50% more susceptible to the lethal effects of the peptide, as compared to the young population.

The differential effects of early and late Aβ exposure might be due to the way in which the organism handles the peptide at different stages of its life. To investigate this possibility we collected flies from the four cohorts at various ages and made both total- and SDS-soluble- Aβ extracts. We observed that early expression of Aβ led to the accumulation of soluble material but this was quickly cleared, provided that expression was stopped at day 15 (figs. 3A and S7A); otherwise continued expression resulted in the conversion of this soluble peptide into insoluble isoforms (figs. 3B and S7B). It is remarkable that soluble Aβ is lower in flies following induction at day 15 as compared to continuous

expression. That these two groups of flies have identical longevity suggests that toxicity is generated by post-day 15 insoluble peptide that is elevated similarly in both. This observation stands in contrast to the current consensus in the literature where small, SDS-soluble, oligomers (dimers) are thought to be the primary toxic Aβ species (Shankar, et al., 2008). Overall, our findings support the study by Rogers et al. (Rogers, et al., 2012) who observed acute Aβ expression in older (>20 day) flies results in higher levels of Aβ and stronger reductions in longevity as compared to Aβ expression in younger flies.

These data give us a picture of an organism that is robust in youth but, having passed an age threshold, becomes sensitive to Aβ toxicity; thereafter the peptide seems to accelerate the ageing process. Before this threshold age, the peptide appears to be held in a less-toxic, soluble and labile conformation and its subsequent conversion into insoluble material is associated with increasing mortality. There is evidence, particularly in *C elegans*, that the stress placed upon cells by such protein misfolding and deposition may unmask temperature sensitive phenotypes. Specifically age and polyQ expression sensitise the worm to the paralysis caused by a temperature sensitive dynamin mutation (Ben-Zvi, et al., 2009,Gidalevitz, et al., 2006). Similar age-related protein destabilisation is also seen in *Drosophila* and so we investigated its role in the increased Aβ-related sensitivity seen in older flies (Demontis, et al., 2013). Using a novel temperature titration assay in *shibire*^{TS2} flies that, like the worm carry a temperature sensitive dynamin mutation (Chen, et al., 1992,van der Bliek and Meyerowitz, 1991), we found that locomotion slowed progressively with increasing ambient temperature (fig. 4). As predicted by the proteostatic hypothesis we found that older *shibire* flies became paralysed at progressively lower temperatures. However, under conditions where Arctic $A\beta_{42}$ accumulated in brain sections, with lethal consequences (figs. S4 and S6), there was no effect on the paralysis titration profiles (compare fig. 4, panels A with C). Assuming that the increased sensitivity of older *shibire* flies to heat-induced paralysis is reporting proteostatic decompensation, we conclude that Aβ accumulation and deposition is not an effective proteome-destabilizing factor.

While Aβ appears not to exert its ageing effects by accelerating a proteostatic collapse, our previous work has indicated that metabolic consequences, not least oxidative stress, may be responsible (Rival, et al., 2009). To test whether we could detect an early metabolic effect of Aβ expression, we challenged flies with hydrogen peroxide in their food. In particular we tested how flies, expressing $A\beta_{40}$ or $A\beta_{42}$, responded to oxidative stress before and after the day 15 threshold. We observed an age-related increase in hydrogen peroxide sensitivity in control and $A\beta_{40}$ expressing flies. Remarkably, we also found that day 2 flies, expressing the $A\beta_{42}$ isoform, were already as sensitive to oxidative stress as day 20 flies. These data are compatible with there being a biochemical signal, rather than a proteostatic defect, underpinning the transition from the robustness of youth to the fragility of older age. Surprisingly, we also noticed that 20 day old control flies were more sensitive to the peroxide challenge than $A\beta_{40}$ expressing flies. While the precise mechanisms for this need further investigation, we speculate that the presence of non-toxic $A\beta_{40}$ can act as a mild stressor and lead to an increase in ROS defence without triggering Aβ toxicity

To systematically investigate the metabolic changes associated with Aβ expression, we made extracts of water-soluble biochemicals from fly tissues at various ages and subjected them to analysis by NMR spectroscopy. The flies were separated into heads, to allow us to measure the local consequences of Aβ production, and bodies, where systemic metabolic changes could be assessed. Initially, the NMR spectra were subjected to PCA to provide an unbiased summary of the Aβ- and age-dependent metabolic changes (fig. 6). We first noted an age-related trajectory that was largely similar for all genotypes. The metabolome of all *Drosophila* variants changed most rapidly during the first 8 days of life, while there was a smaller rate of change in the older flies. However the expression of any Aβ isoform, whether it be $A\beta_{40}$, $A\beta_{42}$ or Arctic $A\beta_{42}$, resulted in a displacement in the age-related trajectories away from the control population. This displacement was already evident from the earliest time points, indicating that the expression of Aβ results in early metabolic alterations.

Our next OPLS-DA model helped us visualise the differences between three groups of flies: a) healthy controls, b) asymptomatic flies expressing the aggregation-prone $A\beta_{42}$ and c) flies expressing the lethal Arctic $A\beta_{42}$ (fig. 7). We observed that the systemic metabolome of the oldest control and $A\beta_{42}$

expressing flies changed so that it became more similar to, albeit younger, $A\beta_{42}$ Arctic flies, indicating a related systematic response between aging and Aβ toxicity. Likewise $A\beta_{42}$ Arctic flies appeared older than other genotypes in an OPLS model of age in the systemic metabolome (fig. S5).

We then identified a set of metabolites that could be used to differentiate control flies from those expressing any isoform of Aβ (fig. 8 panels A and B), and a distinct set (fig. 8 panels C and D) that could differentiate flies expressing Arctic $A\beta_{42}$, from flies with a normal life-expectancy (controls, $A\beta_{40}$ and $A\beta_{42}$). These two orthogonal sets of metabolites provided two-way differentiation in a nervous environment from heads, and for the systemic metabolites from the bodies.

Remarkably, all Aβ-expressing flies exhibited a shift in carbohydrate metabolism, with maltose, a dietary glucose disaccharide, increasing three- to four-fold in both head and body extracts (fig. 9 panels C, I). Similar increases were observed in *Drosophila* in response to both acute stress and artificial selection for stress resistance (Malmendal, et al., 2013,Overgaard, et al., 2007); interestingly maltose has been suggested to have a chaperone-like function for proteins and membranes (Kaplan and Guy, 2004,Pereira and Hunenberger, 2006). Levels of xanthurenic acid were raised in flies expressing all three Aβ isoforms (fig. 9K) indicating potential involvement of the kynurenine pathway that generates vitamin B3 from tryptophan, using vitamin B6 as a cofactor. Low levels of vitamin B6 result in raised proximal intermediates that are converted, by oxidation, to xanthurenic acid. It is notable that such intermediates as kynurenine and 3-OH-kynurenine are raised, as a ratio to tryptophan, in patients with AD (Gulaj, et al., 2010,Schwarz, et al., 2013). Furthermore, several studies have also shown that vitamin B therapy is protective in AD (Douaud, et al., 2013).

The metabolites that separate Arctic $A\beta_{42}$ flies from the other isoforms can be divided into those that are deviating from day one and those that mainly change after day 14 and deviate further with age. The unidentified, potentially hydroxylated, carboxylated or chlorinated aromatic compounds appeared in both heads and bodies of Arctic $A\beta_{42}$ flies, already from day one (fig. 9 panels E, F, L). If these are end products of oxidative stress it provides an interesting perspective on the H_2O_2 challenge experiments, since it indicates that the Aβ-induced chemical stress is taking place already from day one, and with rather constant intensity. Decreased AXP levels in the bodies (fig. 8C) follow a similar pattern, and indicate that there is a reduction in ATP production, and a resulting loss in total adenosine phosphate, through deamidation of AMP to inosine monophosphate, already from day one.

In contrast, gluconic acid was increased from around day 8 in bodies and day 14 in heads (fig. 9 panels A, G). Accumulation of this metabolite was induced by severe oxidative stress and apoptosis in breast cancer cells (Morvan, 2013) and a 100 fold increase was induced by oxidative stress in *Arabidopsis* (Chaouch, et al., 2012,Han, et al., 2013) . An increase in gluconic acid may either reflect oxidation of glucose or enhanced production of 6-phosphogluconate through the oxidative pentose phosphate pathway (OPPP) . When yeast is submitted to oxidative stress, part of the central carbohydrate metabolism is rerouted away from respiratory pathways into the OPPP to provide reducing equivalents to support antioxidant metabolism (Godon, et al., 1998). The results are consistent with the view that the tricarboxylic acid cycle is impacted significantly by Aβ, resulting in oxidative stress (Baxter, et al., 2007,Noctor, et al., 2015).

Histidine is increased from day one in heads and from day eight in bodies, and the increase is accelerated between day 14 and 26 (fig. 9 panels B, H). This amino acid is a precursor for histamine and carnosine biosynthesis, and a powerful antioxidant and anti-inflammatory factor (Peterson, et al., 1998). Low levels of histidine have been associated with inflammation and oxidative stress in humans (Niu, et al., 2012,Watanabe, et al., 2008). Moreover, histidine is increased in urine from patients with Parkinson's disease (Luan, et al., 2015).

Aβ-expressing flies had lower systemic levels of phosphocholine (fig. 9D), reflecting similar reductions, in most brain regions, in a murine model of AD (Salek, et al., 2010). By contrast, phosphocholine levels were higher in blood from patients with the earliest stages of AD (Mapstone, et al., 2014).

While still much higher than in control flies, maltose decreases to levels below $A\beta_{40}$ and $A\beta_{42}$ in middle-aged $A\beta_{42}$ Arctic flies (fig. 9 panels C, I). Abnormalities in sugar metabolism combined with raised lactate levels in fly heads are indicative of defects in aerobic respiration and this is further supported by the reductions in AXP levels in the body extracts. Similarly lactate is increased in brains of mice that model AD (Salek, et al., 2010).

The changes in histidine (fig. 9 panels B, H) and gluconic acid (fig. 9 panels A, G) levels are remarkable, because they occur just at the time when the flies were undergoing the transition from robust youth to fragile older age. In this respect they are potential biomarkers of the metabolic fragility that accompanies the lethal effects of Aβ accumulation. The aromatic oxidation products (fig. 9 panels E, F, L) and the changes in AXP, on the other hand, appear already from day one in agreement with the increase susceptibility to H_2O_2 challenge already at an early age.

The metabolite changes observed here (fig. 10) only show a limited overlap with those detected in mouse (Salek, et al., 2010) and man (Trushina and Mielke, 2013). There are multiple explanations: We use whole body parts rather than organs or body fluids, we separate effects of Aβ-expression and Aβ-toxicity, and we do of course use flies, and as it seems, the dominating effects here are those of decreased oxidative phosphorylation and increased oxidative stress.

In conclusion, while Aβ aggregation accelerates mortality in older flies, it does not seem to do this by exacerbating proteostatic collapse. Rather, the flies exhibit earlier oxidative stress, in response to Aβ expression that closely mirrors increases in mortality. The effects of this stress become obvious at older ages, inducing changes both in lethality and the metabolomic profile.

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Abbreviations

Aβ, amyloid beta; AD, Alzheimer's disease; ANOVA, analysis of variance, AXP, adenosine nucleotides; CPMG, Carr Purcell Meiboom Gill; GS, gene switch; GnHCl, guanidine hydrochloride; MSD, meso scale discovery; NMR, nuclear magnetic resonance; OPLS, orthogonal projection to latent structures; OPLS-DA, orthogonal projection to latent structures discriminant analysis; OPPP, oxidative pentose phosphate pathway; polyQ, poly-glutamine; PC, principal component; PCA, principal component analysis; TSP, 3-trimethylsilyl propionic acid D4.

Competing interests

The authors declare no competing interests.

Author contributions

SO, AV and AM performed the experiments. SO, AV, DCC and AM analysed the metabolomic data. AM and DCC conceived and designed the experiments. SO, AM and DCC drafted the manuscript.

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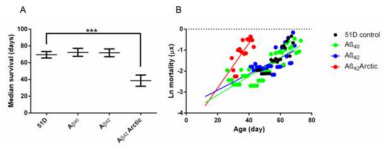

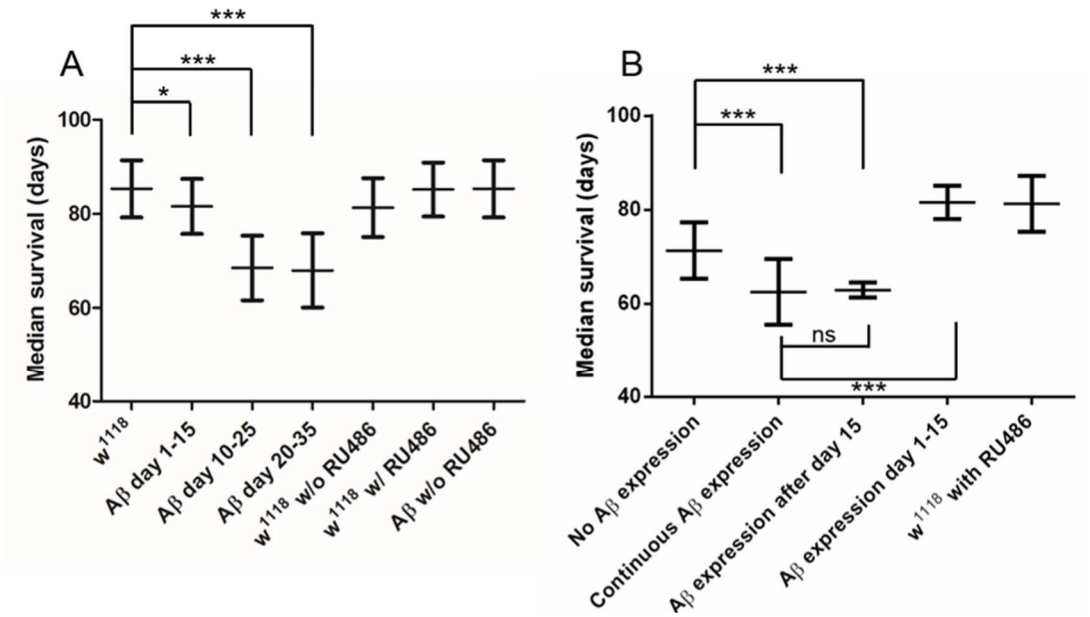

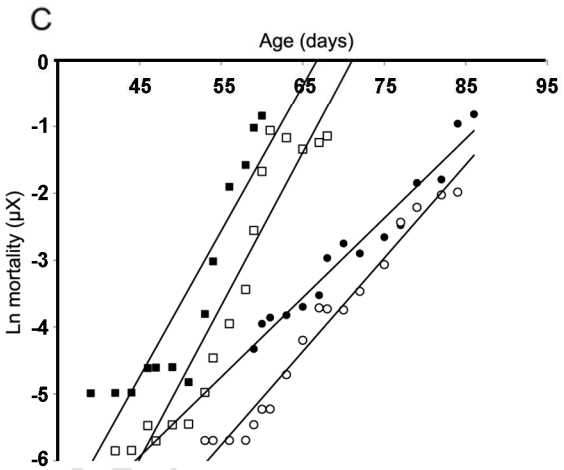

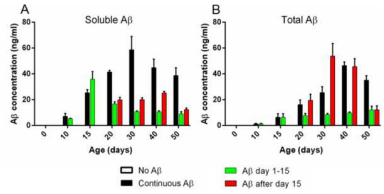

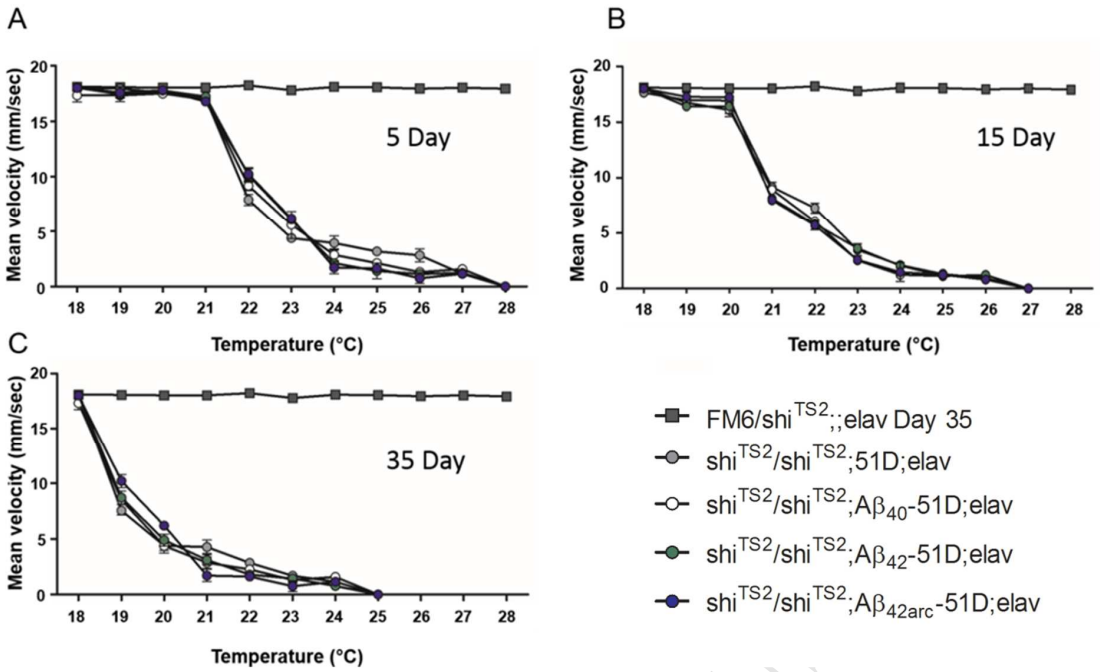

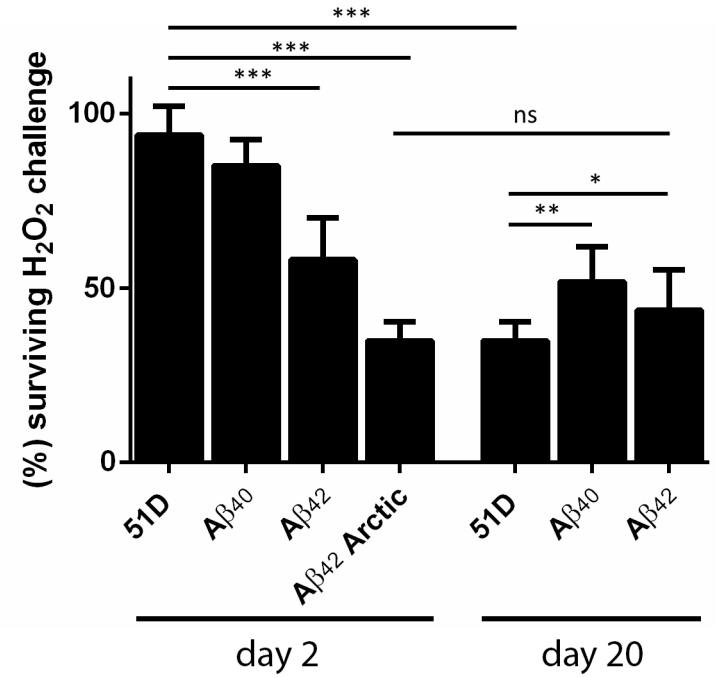

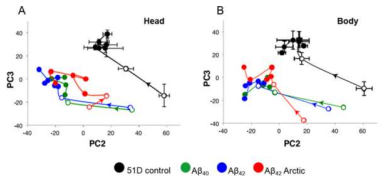

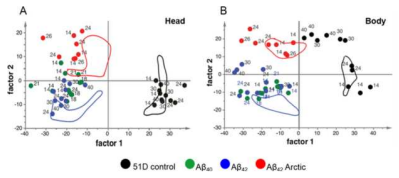

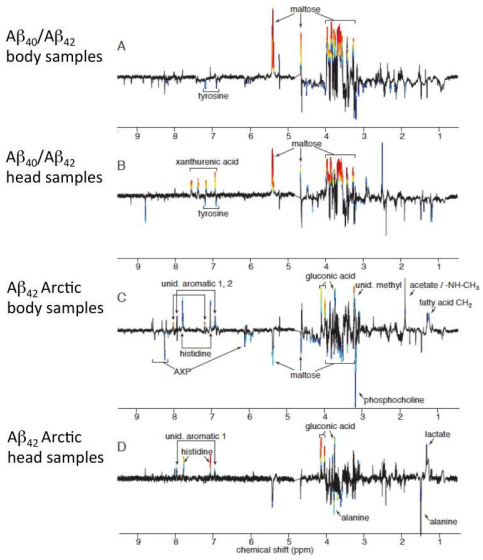

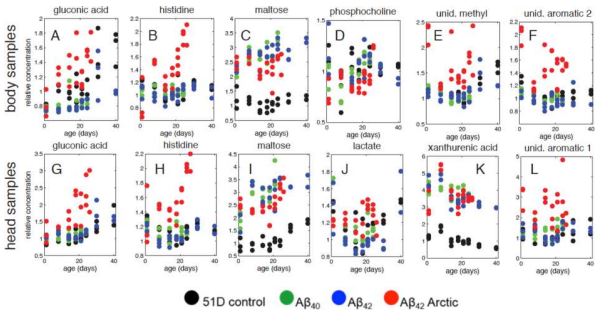

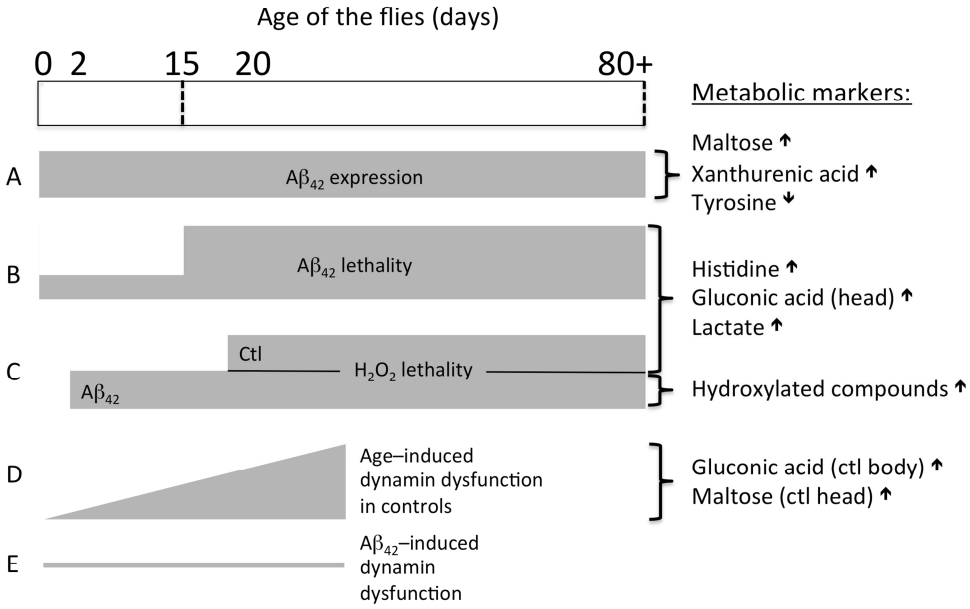

Highlights

- Ageing increases the sensitivity of *Drosophila* to Aβ toxicity. In this work we have shown that young flies are less affected by presence of Aβ isoforms. Instead Aβ becomes lethal when present later in life
- Ageing alters Aβ metabolism, favouring its insoluble deposition. In line with the observed age-related Aβ toxicity, we illustrate that young *Drosophila* are able to clear SDS soluble and insoluble Aβ material which begins to accumulate after passing a critical age threshold

- Aβ toxicity is not accompanied by a generalised protein homeostasis collapse. While our *shibire*^{TS} model reported a decrease in proteome stability throughout ageing , this effect was not exacerbated by the presence of different Aβ isoforms

- Ageing and Aβ expression predispose *Drosophila* to oxidative damage. Our data indicate that young Aβ expressing flies are already as sensitive to oxidative stress as older control flies without Aβ expression.

- Distinct metabolic changes are associated with Aβ expression and Aβ toxicity. Instead of proteostatic changes we observed early alterations in the *Drosophila* metabolome which could be resolved into age- and Aβ-induced effects