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pGluAβ increases accumulation of Aβ in vivo and exacerbates its toxicity

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Abstract

Several species of β-amyloid peptides (Aβ) exist as a result of differential cleavage from amyloid precursor protein (APP) to yield various C-terminal Aβ peptides. Several N-terminal modified Aβ peptides have also been identified in Alzheimer's disease (AD) brains, the most common of which is pyroglutamate-modified Aβ ($Aβ_{pE3-42}$). $Aβ_{pE3-42}$ peptide has an increased propensity to aggregate, appears to accumulate in the brain before the appearance of clinical symptoms of AD, and precedes $Aβ_{1-42}$ deposition. Moreover, in vitro studies have shown that $Aβ_{pE3-42}$ can act as a seed for full length $Aβ_{1-42}$. In this study, we characterized the *Drosophila* model of $Aβ_{pE3-42}$ toxicity by expressing the peptide in specific sets of neurons using the GAL4-UAS system, and measuring different phenotypic outcomes. We found that $Aβ_{pE3-42}$ peptide had an increased propensity to aggregate. Expression of $Aβ_{pE3-42}$ in the neurons of adult flies led to behavioural dysfunction and shortened lifespan. Expression of $Aβ_{pE3-42}$ constitutively in the eyes led to disorganised ommatidia, and activation of the c-Jun N-terminal kinase (JNK) signaling pathway. The eye disruption was almost completely rescued by co-expressing a candidate Aβ degrading enzyme, neprilysin2. Furthermore, we found that neprilysin2 was capable of degrading $Aβ_{pE3-42}$. Also, we tested the seeding hypothesis for $Aβ_{pE3-42}$ in vivo, and measured its effect on $Aβ_{1-42}$ levels. We found that $Aβ_{1-42}$ levels were significantly increased when $Aβ_{1-42}$ and $Aβ_{pE3-42}$ peptides were co-expressed. Furthermore, we found that $Aβ_{pE3-42}$ enhanced $Aβ_{1-42}$ toxicity in vivo. Our findings implicate $Aβ_{pE3-42}$ as an important source of toxicity in AD, and suggest that its specific degradation could be therapeutic.

Keywords: Neurodegeneration, Alzheimer's disease, pyroglutamate Abeta, *Drosophila*

Introduction

Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by amyloid beta (Aβ) deposits and neurofibrillary hyperphosphorylated tau tangles [1]. The amyloid cascade, which has undergone some revision in recent years, is the leading hypothesis for the pathology associated with AD, and states that amyloidogenic Aβ is the trigger of the pathogenic process leading to neuronal cell death [2, 3]. Aβ induces several stressors, which could lead to neuronal cell death [4]. The c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) signaling pathway which influences cell death is activated in response to many forms of stress, such as oxidative stress and endoplasmic reticulum (ER) stress [5, 6]. In particular, Aβ is able to activate the JNK/SAPK pathway, and increased phosphorylation of JNK/SAPK has been observed in post-mortem AD brain tissue in comparison to control cases [5, 7].

Aβ peptides form as a cleavage product from the amyloid precursor protein (APP) [8]. Several species of Aβ peptides exist, as a result of differential cleavage from APP to yield various C-terminal Aβ peptides. Aβ40 and Aβ42 are the most abundant, with Aβ42 being the more toxic form [9]. More recently, Aβ43 has also been identified as a pathogenic species [10, 11]. There are also several N-terminal truncated/modified Aβ peptides that have been identified in AD brains, the most common of which is pyroglutamate-modified Aβ [12–14].

Pyroglutamic (pGlu) acid is generated from N-terminal glutamine during pro-hormone maturation in the secretory pathway; the enzyme glutaminyl cyclase (QC) is directed to the secretory pathway and catalyses the conversion from N-terminal glutamic to pGlu acid [15, 16]. Interestingly, Aβ undergoes this post-translational modification at its amino terminus, also catalyzed by QC [17], which is up-

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regulated in the cortex of patients with AD [18]. Of particular interest is the highly abundant $A\beta_{pE3-42}$, which is generated by cleavage of the first 2 amino acids of the Aβ peptide, followed by pGlu-modification of glutamate in the third amino acid position, which is thought to stabilize it and/or promote its aggregation propensity [14].

Interestingly, water soluble Aβ, which appear before plaques is made up predominantly of $A\beta_{pE3-42}$ [19], and indeed $A\beta_{pE3-42}$ accumulate early on in the brain before the appearance of clinical symptoms and $A\beta_{1-42}$ deposition [20–23]. Moreover, in vitro studies have shown that $A\beta_{pE3-42}$ has an increased propensity to aggregate in comparison to $A\beta_{1-42}$ and can act as a seed/primer for full length $A\beta_{1-42}$ [14, 24, 25]. $A\beta_{pE3-42}$ behaves in a prion-like manner, whereby only small quantities of $A\beta_{pE3-42}$ are able to increase the amount of metastable low-n $A\beta_{1-42}$ oligomers in vitro [25]. These attributes have created a lot of interest in $A\beta_{pE3-42}$ peptides, and several groups have suggested that they are important in initiating the pathological cascade of AD [14, 20].

Interestingly, $A\beta_{pE3-42}$ plaque load has been observed in brain autopsies of familial, sporadic cases and controls although, importantly, oligomeric $A\beta_{pE3-42}$ was only found in the familial and sporadic cases [26]. There is also a likely role for $A\beta_{pE3-42}$ in intra-neuronal AD toxicity. A study that expressed $A\beta_{Q3-42}$ under the Thy-1 promoter in mice (glutamine was used instead of glutamate because it is a better substrate for pyroglutamate conversion, [15]) showed increased levels of intra-neuronal $A\beta_{pE3-42}$, severe neurological impairment and loss of Purkinje cells [27]. Furthermore, $A\beta_{pE3-42}$ expressing mice which display neuronal loss [28], when crossed into a tau KO background were almost completely protected against neuronal loss, establishing a functional connection between pGluAβ and tau [25]. Moreover, transgenic mouse models of AD that develop more severe pathology, as measured by the appearance of early neurological phenotypes and amyloid plaque deposition, tend to have high levels of $A\beta_{pE3-42}$ [14].

Over-expression or reduction of QC has also been shown to exacerbate or rescue behavioural phenotypes and plaque pathology in an AD mouse model [29]. Interestingly, QC KO mice showed a reduction in both $A\beta_{pE3-42}$ and $A\beta_{1-42}$ levels, again supporting the idea that $A\beta_{pE3-42}$ plays a role in seeding $A\beta_{1-42}$ [29]. The data also demonstrate the importance of QC, and suggest that a reduction of QC might be a promising therapeutic strategy.

Many signaling pathways/molecules are conserved between flies and humans, and QC is 1 of them. *Drosophila* has 2 QCs – Drome QC and isoDrome QC, which have different subcellular locations [30]. IsoDrome QC more closely resembles the mammalian homologue [30]. Interestingly, treatment of $A\beta_{Q3-42}$ transgenic flies with a QC inhibitor led to reduced $A\beta_{pE3-42}$ levels [18], highlighting the usefulness of *Drosophila* to investigate the molecular pathogenicity of $A\beta_{pE3-42}$. Several labs have generated fly models that express various Aβ peptides [31–33]. $A\beta_{Q3-42}$ fly models are available, but have not been fully characterized or utilized to test the "seeding hypothesis".

In this study, we characterized a *Drosophila* model of $A\beta_{pE3-42}$ toxicity in the fruit-fly. Expression specifically in adult fly neurons led to behavioural dysfunction and shortened lifespan. Expression of the $A\beta_{pE3-42}$ constitutively in the eyes led to disorganised ommatidia, which was ameliorated by neprilysin2. Furthermore, we show for the first time that neprilysin2 was able to degrade pyroglutamate Aβ.

Several recent studies have suggested that $A\beta_{pE3-42}$ can act as a seed for Aβ1-42, and such a role has been demonstrated in vitro. $A\beta_{pE3-42}$ has been shown to increase the amount of metastable low-n $A\beta_{1-42}$ oligomers in vitro [25]. Furthermore, peri-hippocampal injection of $A\beta_{pE3-42}$ into APPswe/NOS2-/- AD mice led to the presence of both $A\beta_{pE3-42}$ and conventional Aβ plaques, which the authors mention was hardly seen in sham injected AD mice or WT mice injected with $A\beta_{pE3-42}$ [25]. However, the direct effect of $A\beta_{pE3-42}$ on $A\beta_{1-42}$ in vivo remains to be assessed, because the genetic background of the AD mouse lines are mutant for several genes that may well be affected by $A\beta_{pE3-42}$. Furthermore, to control for the specificity of the $A\beta_{pE3-42}$ species in causing enhanced plaque formation, an important control of peri-hippocampal injection of $A\beta_{1-42}$ into the AD mice is missing from their studies.

We have utilized the *Drosophila* model to our advantage by expressing multiple transgenes at the same time to test this seeding hypothesis in vivo, and thus determine whether $A\beta_{pE3-42}$ could be a target for therapeutic intervention, and/or a diagnostic marker. We found that total $A\beta_{1-42}$ levels and toxicity are greatly increased when $A\beta_{pE3-42}$ is co-expressed. These data suggest that $A\beta_{pE3-42}$ is able to stabilise $A\beta_{1-42}$ in vivo.

Materials and methods

Fly stocks and maintenance

All fly stocks were maintained either at 25 °C or 28 °C on a 12:12-h light:dark cycle at constant humidity on a standard sugar-yeast (SY) medium ($15gl^{-1}$ agar, 50 gl^{-1} sugar, 100 gl^{-1} autolysed yeast, $100gl^{-1}$ nipagin and 3 ml l^{-1} propionic acid). Adult-onset, neuronal-specific expression of Aβ peptide was achieved by using the elav GeneSwitch (elavGS)-UAS system. ElavGS was derived from the original elavGS 301.2 line [34] and obtained as a generous gift from Dr H. Tricoire (CNRS, France), GMR driver was from Bloomington stock centre. UAS-Aβ1-42 line was obtained from Dr D. Crowther [35]. UAS- $A\beta_{Q3-42}$ line has been previously described [36], briefly the rat pre pro-enkephalin signaling sequence was cloned upstream

$A\beta_{Q3\text{-}42}$ and put into the EcoR1 site of the pUAST vector, the construct then gets processed via prohormone convertases and glutaminyl cyclase to generate $A\beta_{pE3\text{-}42}$ [17, 37] (Additional file 1: Figure S1). GMR, elavGS and UAS-lines used in all experiments were backcrossed six times into the w^{1118} genetic background. Expression by elavGS was induced by treatment with mifepristone (RU486; 200 μM) added to the standard SY medium. In the absence of mifepristone (RU486; -RU), the transgene remains transcriptionally silent. Following treatment with RU486, $A\beta_{Q3\text{-}42}$ peptide is expressed.

Lifespan analyses

For all experiments, flies were raised at a standard density on standard SY medium in 200 mL bottles. Two days after eclosion once-mated females were transferred to experimental vials containing SY medium with or without RU486 (200 μM) at a density of 10 flies per vial (120 flies per genotype were used in Fig. 2, and 150 flies per genotype were used in Fig. 4). Deaths were scored almost every other day and flies were transferred to fresh food. Data are presented as survival curves and statistical analysis was performed using log-rank tests to compare survival of groups.

Negative geotaxis assays

Climbing assays were performed at 25 °C according to previously published methods [31]. Climbing was analysed every 2–3 days post-RU486 treatment. Fifteen adult flies were placed in a vertical column, then their rate of climb to the top of the column was analysed. Flies reaching the top (12 cm) and flies remaining at the bottom of the column after a 30 s period were counted separately, and 3 trials were performed for each experiment. Scores recorded were the mean number of flies at the top (n_{top}), the mean number of flies at the bottom (n_{bottom}) and the total number of flies assessed (n_{tot}). A performance index (PI) defined as $½(n_{tot} + n_{top} - n_{bottom)/} n_{tot})$ was calculated. Data are presented as the mean PI ± SEM obtained in 3 independent experiments for each group, and analyses of variances (ANOVA) were performed using JMP software.

Quantification of Aβ peptide by ELISA

Quantification of Aβ was carried out as previously described [38]. To extract total Aβ, 5 *Drosophila* heads were homogenised in 50 μl GnHCl extraction buffer (5 M Guanidine HCl, 50 mM Hepes pH 7.3, protease inhibitor cocktail (Sigma, P8340) and 5 mM EDTA), centrifuged at 21,000 g for 5 min at 4 °C, and cleared supernatant retained as the total fly Aβ sample. Alternatively, for soluble and insoluble pools of Aβ, 25 fly heads were homogenised in 50 μl tissue homogenisation buffer (250 mM sucrose, 20 mM Tris base, 1 mM EDTA, 1 mM EGTA, protease inhibitor cocktail (Sigma) then mixed further with 50 μl diethyl acetate (DEA) buffer (0.4 % DEA, 100 mM NaCl and protease inhibitor cocktail). Samples were centrifuged at 135,000 g for one hour at 4 °C (Beckman Optima^{TM} Max centrifuge, TLA120.1 rotor), and supernatant retained as the cytosolic, soluble Aβ fraction. Pellets were resuspended in 200 μls ice-cold formic acid (FA; 70 %), and sonicated. Samples were re-centrifuged at 135,000 g for one hour at 4 °C, then 100 μl of supernatant diluted with 1 ml FA neutralisation buffer (1 M Tris base, 0.5 M Na_2HPO_4, 0.05 % NaN_3) and retained as the insoluble, formic acid-extractable Aβ fraction. Aβ content was measured using the hAmyloid β42 ELISA kits, X-42 and N3pE-42 (IBL INTERNATIONAL). N of 3 or 4 individual samples were diluted in sample/standard dilution buffer and ELISA performed according to the manufacturers' instructions. Protein extracts were quantified using the Bradford protein assay (Bio-Rad protein assay reagent; Bio-Rad laboratories (UK) Ltd) and the amount of Aβ in each sample expressed as a ratio of the total protein content (pmol/g total protein).

Western blotting

For total Aβ extraction, we used a procedure previously described [11]. 20 heads per biological replicate were homogenized in 100 μL of 70 % formic acid. Samples were centrifuged at 16,000 *g* for 20 min at room temperature. The supernatant was collected and evaporated using a SpeedVac. The pellet was resuspended in 100 μL 2× LDS containing reducing agent (Invitrogen) and homogenized by sonication (10 pulses). Samples were then boiled at 100 °C for 10 min and 15 μL of each sample were used for western blotting to determine total Aβ levels. For LDS/SDS oligomer Aβ extraction, 20 heads per biological replicate were homogenized in 100 μL 2× LDS containing reducing agent (Invitrogen). Samples were incubated on ice for 30 min and then boiled at 100 °C for 10 min. 15 μL per sample were used for western blotting to evaluate LDS/SDS-stable Aβ oligomers. Proteins were separated on 16.5 % Tris-Tricine Criterion gels (Biorad) blotted onto nitrocellulose membranes. Membranes were incubated in a blocking solution containing 5 % milk proteins in TBST for 1 h at room temperature, then probed with primary antibody diluted in TBST + 5 % BSA overnight at 4 °C. 82E1 Aβ1-42 Antibody was from Takara, used at 1 in 100 dilution.

For pJNK western blot analyses, total protein was extracted from 5 fly heads in 30 μl 2 × LDS buffer containing reducing agent (Invitrogen). Membranes were incubated in a blocking solution containing 5 % BSA in TBST for 1 h at room temperature, then probed with primary antibody diluted in TBST + 5 % BSA overnight at 4 °C. mouse monoclonal phospho-SAPK/JNK (T183/

Y185) antibody was from cell signaling, used at 1 in 1000 dilution. Rabbit polyclonal actin antibody was from abcam and used at 1 in 1000 dilution.

Quantitative RT-PCR

Total RNA was extracted from 20 to 25 fly heads using TRIzol (GIBCO) according to the manufacturers' instructions. The concentration of total RNA purified for each sample was measured using an *Eppendorf biophotometer*. 1 μg of total RNA was then subjected to DNA digestion using DNAse I (Ambion), immediately followed by reverse transcription using the Superscript II system (Invitrogen) with oligo(dT) primers. Quantitative PCR was performed using the PRISM 7000 sequence-detection system (Applied Biosystems), SYBR Green (Molecular Probes), ROX Reference Dye (Invitrogen), and Hot StarTaq (Qiagen, Valencia, CA) by following manufacturers' instructions. Each sample was analysed at a minimum in triplicate with both target gene ($A\beta_{Q3\text{-}42}$ or NEP2) and control gene (RP49) primers in parallel. The primers for the Aβ transgenes were directed to the 5' end and 3' end of the Aβ coding sequence: forward CGACATGACTCAGGTTAT GAAGTT; reverse GACAACGCCCACCAT Neprilysin2 primers are, forward ACGAGGTCAACTGGATG GAC and reverse GTCGAGCTTGGCGTAGTAGG. RP49 primers were as follows: forward ATGACCATCCGCCC AGCATCAGG; reverse ATCTCGCCGCAGTAAACG.

Eye phenotype

Eye images of 2/3-day-old female flies expressing $A\beta_{pE3\text{-}42}$ under the control of the GMR-Gal4 driver at 28 °C were taken. Nail polish imprint of the external eye was carried out as previously described. For adult eye transverse sections, adult heads were fixed, dehydrated, sectioned (10 microns thick) and stained with Harry's hematoxylin. To investigate the eye phenotype of double transgenic $A\beta_{1\text{-}42;}$ $A\beta_{Q3\text{-}42}$ flies, we kept the flies at 25 °C to minimize $A\beta_{pE3\text{-}42}$ eye phenotype. Images were taken with ZEISS Axioskop2 plus microscope. The eye phenotype was quantified by assigning numbers, from zero to 2 to individual flies chosen at random. Normal looking eyes were given zero, flies with moderate eye phenotype were assigned 1, and flies with strong eye phenotype were given 2, ($N = 5 - 6$ flies per genotype). The scoring was carried out blind by 2 independent researchers.

Statistical analyses

For lifespan analyses, log-rank tests were used to assess for statistical differences. Eye phenotype was presented as means ± SEM, and statistically assessed by Student's *t* test. Other data are presented as means ± SEM obtained in at least 3 independent experiments, and differences between means were assessed by either Student's *t* test or 2-way analysis of variance (ANOVA) using JMP (version 12.0) software (SAS Institute, Cary, NC, USA).

Results

Pyroglutamate Aβ ($A\beta_{pE3\text{-}42}$) expression can be induced in the adult *Drosophila* nervous system

A fly model that expresses $A\beta_{E3\text{-}42}$ has been described [39], however, we are utilising a previously generated $A\beta_{Q3\text{-}42}$ transgenic fly model for this study, since glutamine is a better substrate for pyroglutamate conversion than glutamate [14]. $A\beta_{Q3\text{-}42}$ fly models have been generated but little characterized [18, 36]. To ensure that these flies could express $A\beta_{Q3\text{-}42,}$ we drove expression of the $A\beta_{Q3\text{-}42}$ transgene in adult neurons with the inducible pan-neuronal driver, elav GeneSwitch (elavGS) [31, 34]. We measured RNA levels of $A\beta_{Q3\text{-}42}$ flies in adult neurons, after treating elavGS;UAS-$A\beta_{Q3\text{-}42}$ flies with the activator mifepristone (RU486) for 7 days, starting at 2 days post-eclosion (Fig. 1a). We found a significant increase in $A\beta_{Q3\text{-}42}$ transcripts in RU486-treated elavGS;UAS-$A\beta_{Q3\text{-}42}$ flies in comparison to untreated flies (Fig. 1a). Furthermore, we confirmed that the flies generate pyroglutamate-modified Aβ by measuring $A\beta_{pE3\text{-}42}$ protein levels specifically. $A\beta_{pE3\text{-}42}$ protein levels in adult neurons of elavGS;UAS-$A\beta_{Q3\text{-}42}$ flies treated with RU486 for 21 days, starting at 2 days post-eclosion were significantly increased in comparison to untreated flies (Fig. 1b). These data demonstrate that $A\beta_{pE3\text{-}42}$ can be successfully generated in the flies.

Expression of $A\beta_{pE3\text{-}42}$ causes shortened lifespan, neuronal dysfunction, disorganised eye phenotype, and activates JNK in *Drosophila*

To determine whether expression of $A\beta_{pE3\text{-}42}$ in neurons is toxic, we used the elavGS driver to express $A\beta_{pE3\text{-}42}$ peptide in adult neurons, and measured the effects on behaviour. Impaired geotaxis is a behavioural measure of neuronal dysfunction and can be assessed using a climbing assay [31]. elavGS;UAS-$A\beta_{Q3\text{-}42}$ flies were treated with RU486 starting at 2 days post-eclosion, and their climbing ability was subsequently recorded. Flies expressing $A\beta_{pE3\text{-}42}$ displayed substantially increased rate of decline in negative geotaxis with age in comparison to the −RU or driver (elavGS) alone control flies (Fig 2a and Additional file 2: Figure S2).

We also measured the effects of $A\beta_{pE3\text{-}42}$ on lifespan in comparison to the −RU control flies, by treating elavGS,UAS-$A\beta_{Q3\text{-}42}$ flies with RU486 starting at 2 days post-eclosion and recording their subsequent survival. Expression of $A\beta_{pE3\text{-}42}$ in adult neurons significantly shortened median (54 %) and maximum lifespan (31 %) in comparison to control elavGS,UAS-$A\beta_{Q3\text{-}42}$ -RU flies (Fig. 2b).

To assess the effects of $A\beta_{pE3\text{-}42}$ on neurodegeneration, we expressed $A\beta_{pE3\text{-}42}$ constitutively in the fly eye

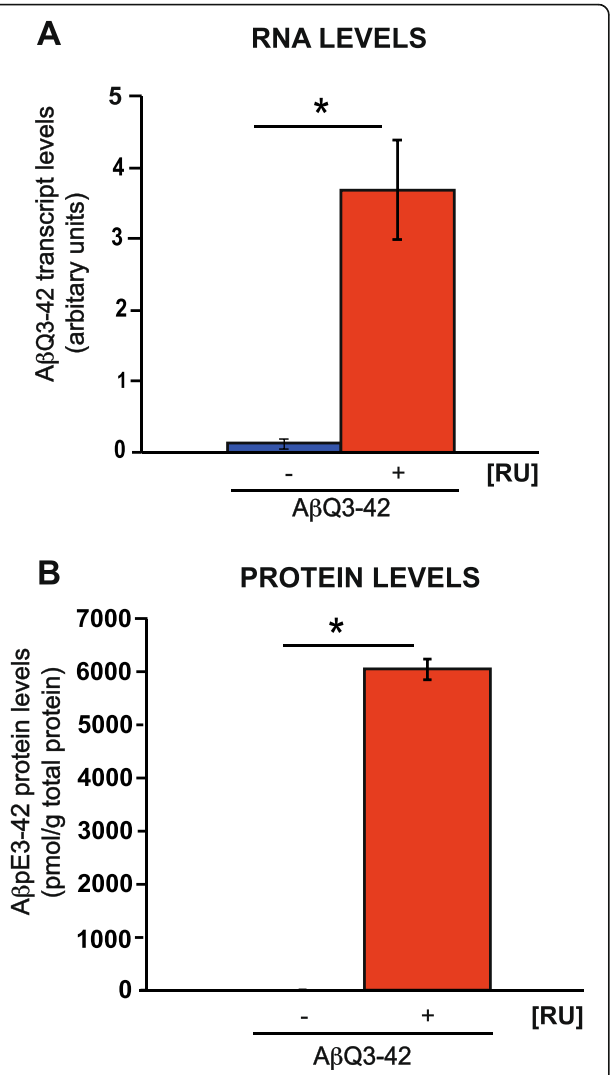

Fig. 1 Pyroglutamate Aβ expression in the *Drosophila* nervous system. **a** RNA levels were quantified at 7 days post-RU486 treatment and (**b**) $Aβ_{pE3-42}$ protein levels were quantified at 21 days post-RU486 treatment. Data are presented as means ± SEM and were analysed by Student *t* test, *$P < 0.001$ comparing Aβ RNA and protein expression in RU486-treated elavGS/UAS-$Aβ_{Q3-42}$ flies to their –RU486 controls

using the GMR-GAL4 driver and observed the effect on the organization of the ommatidia, an assay that has been used extensively to characterise fly models of neurodegenerative diseases [35]. Expression of $Aβ_{pE3-42}$ caused disorganisation of the ommatidia in these flies, presenting with eye roughness and fused ommatidia in comparison to flies expressing EGFP, and w1118 control flies (Fig. 2c).

The JNK/SAPK signaling pathway which influences cell death is activated by Aβ, and has been suggested to contribute to Aβ mediated cell death in fly models expressing $Aβ_{1-42}$ [40, 41]. To determine whether $Aβ_{pE3-42}$ expressing flies are also capable of activating JNK, we measured the levels of phosphorylated JNK as a read-out in the flies, by western blot analyses. We found a significant increase in the level of phosphorylated JNK in flies expressing $Aβ_{pE3-42}$ (GMR-GAL4/+; $Aβ_{Q3-42}$/+) in comparison to control flies expressing LACZ (GMR-GAL4/LACZ), 6 days post-eclosion (Fig. 2d).

Co-expression of neprilysin2 suppresses the toxicity of $Aβ_{pE3-42}$ expressing flies

Neprilysin (NEP) and its close homologue Neprilysin2 (Nep2) are candidate Aβ degrading enzymes, and regulate amyloid protein levels in AD [42–44]. We next determined whether the fly orthologue, *nep2*, which has been shown previously to significantly reduce $Aβ_{1-42}$ levels and toxicity, is able to similarly ameliorate $Aβ_{pE3-42}$ induced toxicity. We made use of the rough eye/disorganised ommatidia phenotype, and found that co-expression of NEP2 using the EP(3)3549 *Drosophila* strain, with the GMR-GAL4 driver almost completely suppressed both the external disorganised ommatidia and internal retinal degeneration of the $Aβ_{pE3-42}$ expressing flies (Fig. 3a). We confirmed that the EP(3)3549 strain had a significant expression of NEP2 levels by RTPCR (Additional file 3: Figure S3A).

Co-expression of neprilysin2 reduces $Aβ_{pE3-42}$ protein levels

To understand the mechanism by which NEP2 ameliorates the $Aβ_{pE3-42}$ eye phenotype, we measured total Aβ load, and $Aβ_{pE3-42}$ protein levels specifically in the flies, 7 days post-eclosion. Interestingly, we found by ELISA analyses that Aβ load and importantly, $Aβ_{pE3-42}$ levels were significantly reduced in flies co-expressing $Aβ_{pE3-42}$ and NEP2 in comparison to flies co-expressing $Aβ_{pE3-42}$ and EGFP as a control (Additional file 3: Figure S3B and Fig. 3b). Furthermore, the reduction we see at the protein level is not due to reduced RNA levels, since this reduction was not observed in the RNA by RTPCR analyses, 7 days post eclosion (Additional file 3: Figure S3C). These data demonstrate a major role of NEP2 in ameliorating $Aβ_{pE3-42}$ induced toxicity, by reducing $Aβ_{pE3-42}$ protein levels.

$Aβ_{pE3-42}$ is more toxic than $Aβ_{1-42}$ in *Drosophila*

Data from mouse models have indicated that the appearance of $Aβ_{pE3-42}$ correlates with increased pathogenicity [45]. To determine whether $Aβ_{pE3-42}$ peptide was more toxic in comparison to $Aβ_{1-42}$, we needed 2 lines with comparable levels of Aβ peptide. We expressed the peptides with the elavGS driver line, by treating $Aβ_{Q3-42}$ and $Aβ_{1-42}$ transgenic flies independently with RU486 starting at 2 days post-eclosion, for 2 days and 21 days, and measured Aβ protein levels in adult neurons, taking advantage of an ELISA kit that recognizes an epitope in the middle of both $Aβ_{pE3-42}$ and $Aβ_{1-42}$ peptides. We

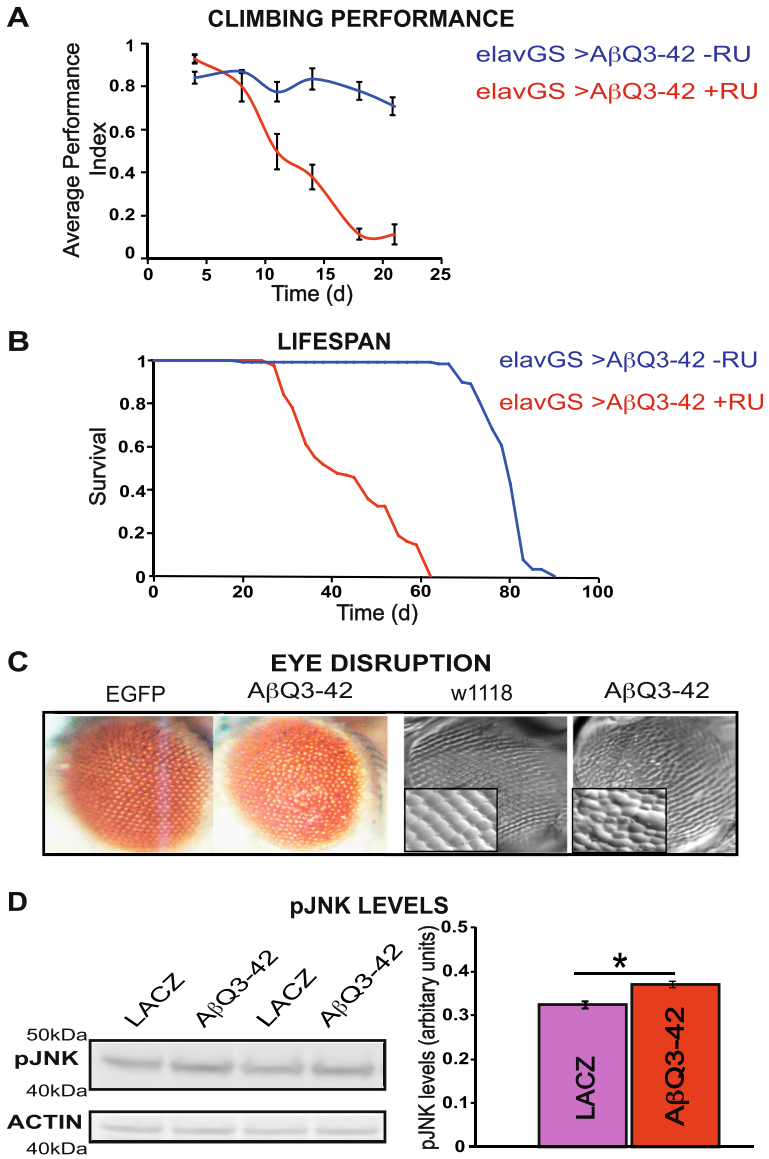

Fig. 2 Expression of $A\beta_{pE3-42}$ causes locomotor dysfunction, shortened lifespan, eye disruption, and JNK activation in *Drosophila*. **a** Climbing ability of elavGS/UAS-$A\beta_{Q3-42}$ flies on + and − RU486 SY medium were assessed at the indicated time-points. Data are presented as the average performance index (PI) ± SEM and were compared using 2-way ANOVA (number of independent tests (n) = 3 **P* < 0.001 (**b**) Expression of $A\beta_{pE3-42}$ in adult neurons shortens lifespan. Survival curves are depicted and data were compared using the log-rank test, **P* < 0.001 comparing elavGS/UAS-$A\beta_{Q3-42}$ + RU flies to -RU flies. **c** Expression of $A\beta_{pE3-42}$ causes a neurodegenerative eye phenotype. First 2 images from left to right are light microscopy images and latter 2 images are nail varnish imprints of eyes (magnification is 25×, and 40× objectives for close up images). From left to right, GMR-GAL4/UAS-EGFP, GMR-GAL4/+;UAS-$A\beta_{Q3-42}$/+, GMR-GAL4/+, GMR-GAL4/+;UAS-$A\beta_{Q3-42}$/+. Note compressed and fused ommatidia in $A\beta_{Q3-42}$ expressing flies in comparison to control flies (GMR-GAL4/UAS-EGFP or GMR-GAL4/+). Flies were grown at 28 °C. **d** Expression of $A\beta_{pE3-42}$ increases the levels of phosphorylated JNK. Data are presented as means ± SEM and were analysed by Student *t* test, **P* < 0.05 comparing GMR-GAL4/+;UAS-$A\beta_{Q3-42}$/+ flies to control GMR-GAL4/UAS-LACZ flies

found similar levels of total Aβ protein in the $A\beta_{pE3-42}$ and $A\beta_{1-42}$ expressing flies (Fig. 4a and b). However, the solubility/aggregation propensity of Aβ differed between the $A\beta_{pE3-42}$ and $A\beta_{1-42}$ expressing flies, with $A\beta_{pE3-42}$ expressing flies having a significantly increased ratio of insoluble to total Aβ in comparison to $A\beta_{1-42}$ expressing flies (Fig. 4c). $A\beta_{pE3-42}$ expressing flies also suffered increased toxicity, because expression of $A\beta_{pE3-42}$ in adult neurons significantly shortened median (27 %) and maximum (23 %) lifespan in comparison both to control w1118;;elavGS and $A\beta_{1-42}$ expressing flies (Fig. 4d), demonstrating a more toxic effect of the $A\beta_{pE3-42}$ peptide.

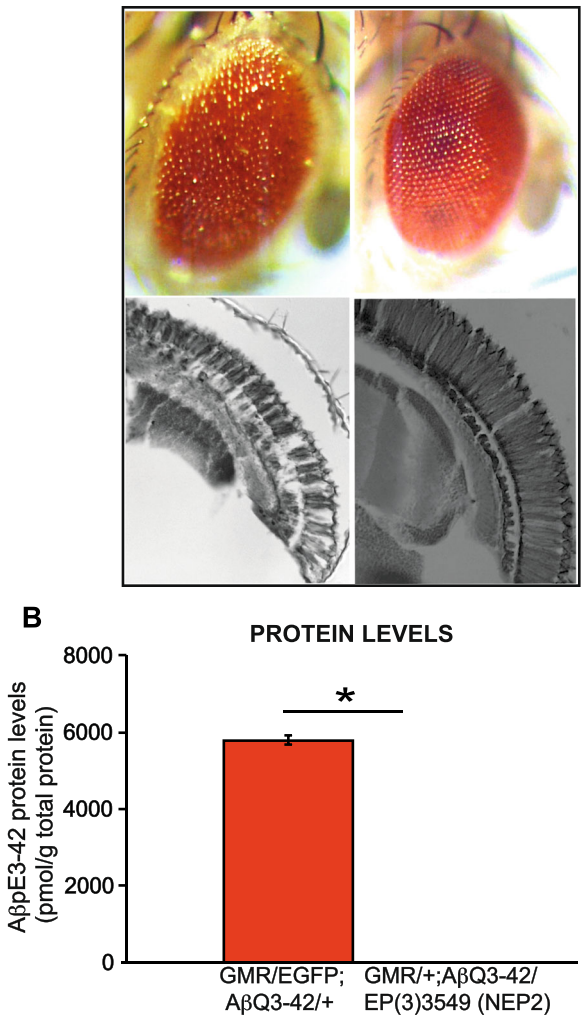

Fig. 3 **a** Co-expression of neprilysin2 suppresses the toxicity of $A\beta_{pE3\text{-}42}$ expressing flies. Top, light microscopy images and bottom, transverse sections. From left to right, GMR-GAL4/UAS-EGFP;UAS-$A\beta_{Q3\text{-}42}$/+ and GMR-GAL4/UAS-EGFP;UAS-$A\beta_{Q3\text{-}42}$/NEP2 (EP(3)3549). Flies were grown at 28 °C. **b** Neprilysin2 significantly reduces $A\beta_{pE3\text{-}42}$ protein levels, *$P < 0.001$. $A\beta_{pE3\text{-}42}$ levels were quantified by ELISA. Data are presented as means ± SEM and were analysed by Student *t* test

pGluAβ increases accumulation of Aβ in vivo

$A\beta_{pE3\text{-}42}$ has been shown to increase the amount of metastable low-n $A\beta_{1\text{-}42}$ oligomers in vitro [25]. To study in vivo, the seeding effect of $A\beta_{pE3\text{-}42}$, we co-expressed $A\beta_{pE3\text{-}42}$ and $A\beta_{1\text{-}42}$ peptides directly and compared the effects to those seen in flies expressing $A\beta_{1\text{-}42}$. We expressed $A\beta_{1\text{-}42}$ or $A\beta_{pE3\text{-}42}$ constitutively with the GMR-GAL4 driver line, and measured at 2–3 days post-eclosion total protein levels of flies expressing either peptide. We found a substantial increase in insoluble Aβ levels and a significant decrease in soluble Aβ detected in $A\beta_{pE3\text{-}42}$ expressing flies in comparison to $A\beta_{1\text{-}42}$ expressing flies with ELISA (Fig. 5a). There was also a significant increase in total Aβ levels in $A\beta_{1\text{-}42}$; $A\beta_{pE3\text{-}42}$ expressing flies in comparison to $A\beta_{1\text{-}42}$;$A\beta_{1\text{-}42}$ expressing flies (Fig. 5a and Additional file 4: Figure S4). Furthermore, we found a shift in Aβ solubility in $A\beta_{1\text{-}42}$; $A\beta_{pE3\text{-}42}$ expressing flies, with a significant decrease in soluble Aβ and a substantial increase in insoluble Aβ in comparison to $A\beta_{1\text{-}42}$;$A\beta_{1\text{-}42}$ expressing flies (Fig. 5a).

We next investigated the effect of co-expressing $A\beta_{1\text{-}42}$; $A\beta_{pE3\text{-}42}$ specifically on $A\beta_{1\text{-}42}$ stability, and whether this contributed to the increased total Aβ levels. We measured $A\beta_{1\text{-}42}$ levels by western blot, by selecting an antibody that detects $A\beta_{1\text{-}42}$, but not $A\beta_{pE3\text{-}42}$ (Fig. 5b and Additional file 5: Figure S5). Flies co-expressing $A\beta_{1\text{-}42}$; $A\beta_{pE3\text{-}42}$ had increased levels of $A\beta_{1\text{-}42}$ levels in comparison to flies expressing a single copy of $A\beta_{1\text{-}42}$, which do not accumulate enough protein levels for quantification by western blot analyses (Fig. 5a and Additional file 5: Figure S5). However, $A\beta_{1\text{-}42}$ was specifically detected in flies containing 2 copies of the $A\beta_{1\text{-}42}$ transgene ($A\beta_{1\text{-}42}$;$A\beta_{1\text{-}42}$), confirming protein expression of this line with the antibody (Fig. 5b). Interestingly, the flies co-expressing $A\beta_{1\text{-}42}$;$A\beta_{1\text{-}42}$ significantly expressed lower levels of $A\beta_{1\text{-}42}$ in comparison to flies co-expressing $A\beta_{1\text{-}42}$; $A\beta_{pE3\text{-}42}$ (Fig. 5b).

pGluAβ enhances Aβ toxicity

Since $A\beta_{pE3\text{-}42}$ increased the stability of $A\beta_{1\text{-}42}$, we assessed whether it also increased the toxicity of $A\beta_{1\text{-}42}$, using the rough eye/disorganised ommatidia phenotype. Flies co-expressing $A\beta_{1\text{-}42}$; $A\beta_{pE3\text{-}42}$, but not those expressing $A\beta_{1\text{-}42}$;$A\beta_{1\text{-}42}$ presented with disorganised ommatidia, 2 days post eclosion, and this phenotype was stronger than in flies expressing $A\beta_{Q3\text{-}42}$ alone (Fig. 5c and Additional file 6: Figure S6) suggesting that $A\beta_{pE3\text{-}42}$ enhances the toxicity of $A\beta_{1\text{-}42}$.

Collectively, these data suggest that $A\beta_{pE3\text{-}42}$ is able to increase the stability of the $A\beta_{1\text{-}42}$ peptide and exacerbate its toxicity in vivo.

Discussion

$A\beta_{pE3\text{-}42}$ is increasingly thought to play a pivotal role in the pathogenesis of Alzheimer's disease [14]. Although previous studies in vitro have suggested that $A\beta_{pE3\text{-}42}$ acts as a seed for Aβ stability, and some correlative work has been done in vivo [25], this seeding behaviour and its consequences have not been examined in vivo. Our study demonstrates that $A\beta_{pE3\text{-}42}$ increases the levels of $A\beta_{1\text{-}42}$, presumably by increasing its stability, and that it enhances toxicity of the $A\beta_{1\text{-}42}$ peptide, as observed in flies co-expressing $A\beta_{pE3\text{-}42}$ and $A\beta_{1\text{-}42}$ in comparison to flies expressing 2 copies of $A\beta_{1\text{-}42}$.

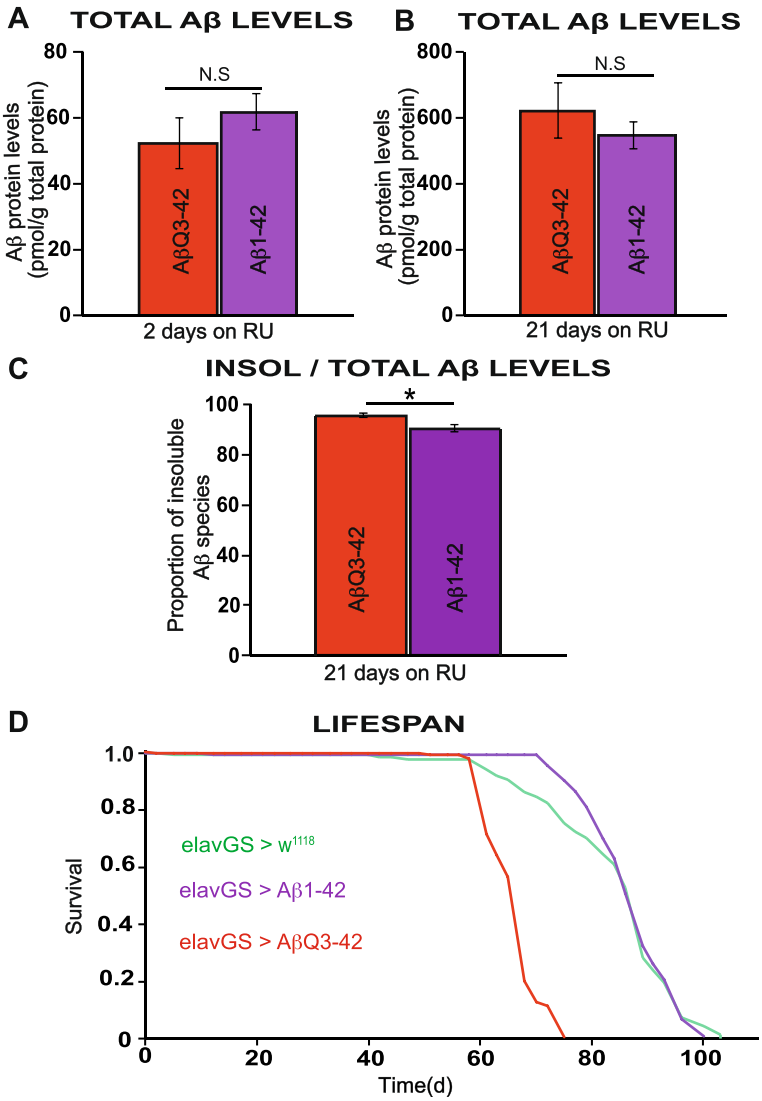

Fig. 4 $A\beta_{pE3-42}$ is more toxic than $A\beta_{1-42}$ in *Drosophila*. Following treatment with RU486, $A\beta_{1-42}$ and $A\beta_{pE3-42}$ peptides were expressed at similar levels, quantified at 2 days and 21 days post-RU486 treatment (**a**) and (**b**) respectively. **c** A significant increase in the amount of insoluble to total Aβ protein levels in $A\beta_{pE3-42}$ expressing flies in comparison to $A\beta_{1-42}$ expressing flies was observed when quantified at 21 days post-RU486 treatment. Data are presented as means ± SEM and were analysed by Student's *t* test, $P < 0.01$. **d** Expression of $A\beta_{pE3-42}$ specifically in adult neurons shortens lifespan significantly relative to both $A\beta_{1-42}$ and w1118 control. Survival curves are depicted and data were compared using the log-rank test. *$P < 0.001$ comparing elavGS/UAS-$A\beta_{Q3-42}$ + RU flies to elavGS/+ and UAS-$A\beta_{1-42}$ /+;elavGS/+ +RU flies

First, we characterized the model. Expression of the $A\beta_{pE3-42}$ peptide specifically in adult fly neurons led to behavioural dysfunction and shortened lifespan, and constitutive expression in the eyes led to disorganised ommatida. Furthermore, we found that $A\beta_{pE3-42}$ was able to activate the JNK signaling pathway, suggesting a role for this cell death activating pathway in $A\beta_{pE3-42}$ mediated toxicity.

Interestingly, we found that we could ameliorate the $A\beta_{pE3-42}$ toxicity by over expressing Neprilsyin2. Increasing the expression of several candidate in vivo Aβ degrading enzymes, such as NEP or Insulin degrading enzyme (IDE) have been shown to reduce the cerebral amyloid plaque burden observed in APP over-expressing mice [46]. However, direct interactions between $A\beta_{pE3-42}$ and Neprilysin have not been investigated. We found that over-expression of *Drosophila* NEP2 was able to reduce $A\beta_{pE3-42}$ levels and improve considerably the disorganised ommatidia, demonstrating for the first time that, although $A\beta_{pE3-42}$ may aggregate more than $A\beta_{1-42}$, NEP2 is capable of degrading pyroglutamate-modified Aβ.

Also, we found that these flies had an increase in the ratio of insoluble to total Aβ levels in comparison to flies expressing $A\beta_{1-42}$, confirming the propensity of $A\beta_{pE3-42}$

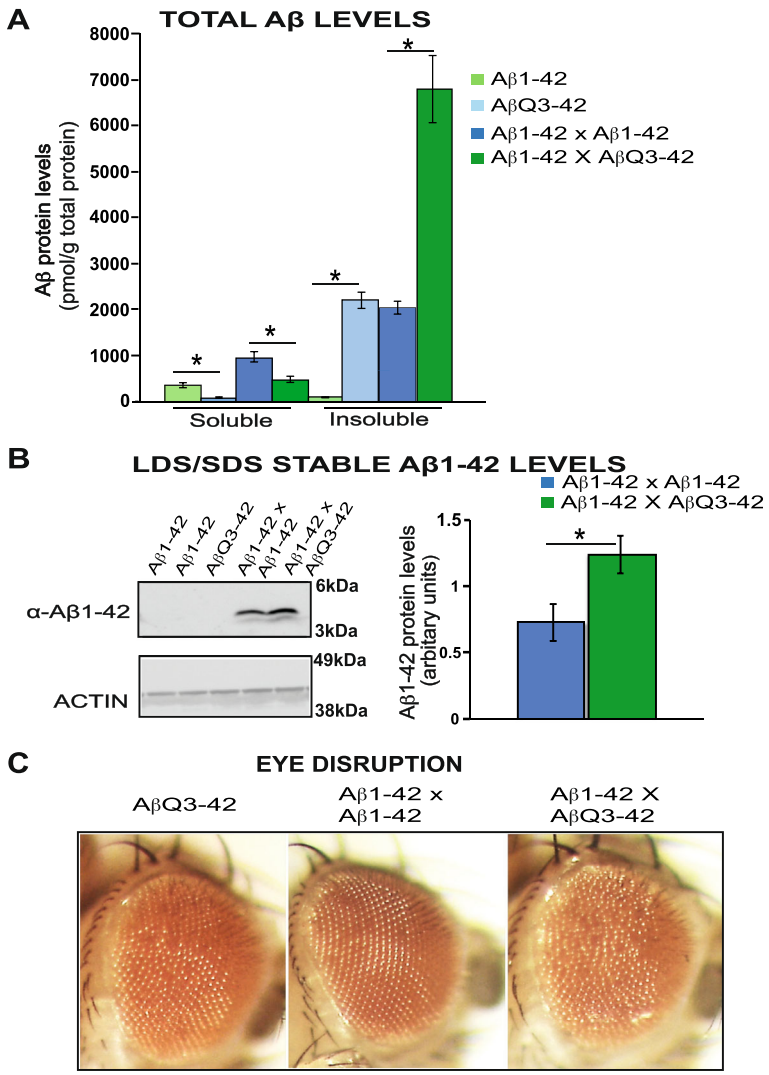

Fig. 5 pGluAβ increases accumulation of Aβ in vivo, and exacerbates toxicity (**a**). Flies expressing $Aβ_{1-42}$ had significantly more soluble Aβ, P <0.05 but less insoluble Aβ, P <0.0001 in comparison to flies expressing $Aβ_{pE3-42}$. Flies co-expressing $Aβ_{1-42}$ and $Aβ_{pE3-42}$ had significantly more insoluble Aβ, P <0.01 but less soluble Aβ P <0.05 Aβ in comparison to flies co-expressing $Aβ_{1-42}$ and $Aβ_{1-42}$. Data are presented as means ± SEM and were analysed by Student's *t*-test. **b** There was a significant increase in $Aβ_{1-42}$ accumulation in flies co-expressing $Aβ_{1-42}$;$Aβ_{pE3-42}$ in comparison to flies expressing either a single copy of $Aβ_{1-42}$ (expressing one single copy of $Aβ_{1-42}$ does not accumulate enough protein levels for quantification by western blot analyses), or flies expressing 2 copies of $Aβ_{1-42}$, $P < 0.05$. Data are presented as means ± SEM and were analysed by Student's *t* test. GMR-GAL4/+;UAS-$Aβ_{1-42}$/+, GMR-GAL4/+;UAS-$Aβ_{Q3-42}$/+, GMR-GAL4/UAS-$Aβ_{1-42}$;UAS-$Aβ_{1-42}$/+ and GMR-GAL4/+;UAS-$Aβ_{1-42}$/UAS-$Aβ_{Q3-42}$ flies were used for (**a**) and (**b**). **c**. Co-expression of $Aβ_{1-42}$ and $Aβ_{pE3-42}$ led to a disorganized eye phenotype that was absent in flies co-expressing $Aβ_{1-42}$ and $Aβ_{1-42}$, and worse than in $Aβ_{pE3-42}$ expressing flies. From left to right, GMR-GAL4/+;UAS-$Aβ_{Q3-42}$/+, GMR-GAL4/UAS-$Aβ_{1-42}$;UAS-$Aβ_{1-42}$/+ and GMR-GAL4/+;UAS-$Aβ_{1-42}$/UAS-$Aβ_{Q3-42}$ flies. Flies were grown at 25 °C

to aggregate as previously described [14]. Furthermore, we found that $Aβ_{pE3-42}$ was more toxic than $Aβ_{1-42}$ peptide.

Another interesting finding from our analyses is the increase in Aβ levels observed in response to $Aβ_{pE3-42}$. We found a general increase in total Aβ levels in flies co-expressing $Aβ_{pE3-42}$ and $Aβ_{1-42}$, and there was also an increase in the ratio of insoluble Aβ to total Aβ levels in these flies. To determine what role $Aβ_{1-42}$ might have in this, we measured $Aβ_{1-42}$ levels specifically by western blot analyses. Interestingly, we found that total $Aβ_{1-42}$ levels were increased when $Aβ_{pE3-42}$ was co-expressed. Nussbaum et al. showed that Aβ peptides oligomerise by different pathways, and that the low-n oligomers of $Aβ_{pE3-42}$ are structurally distinct from $Aβ_{1-42}$, and far more cytotoxic, in the order of $Aβ_{pE3-42}$ /$Aβ_{1-42}$ > $Aβ_{pE3-42}$ > $Aβ_{1-42}$ [25]. We found that co-expressing 1 copy each of $Aβ_{1-42}$ and $Aβ_{pE3-42}$ increased the accumulation of $Aβ_{1-42}$, and was more toxic than expressing 2 copies of $Aβ_{1-42}$,

indicating that $A\beta_{pE3-42}$ is able to stabilise $A\beta_{1-42}$ in a different manner to over expressing the $A\beta_{1-42}$ peptide, perhaps by affecting its structure.

Conclusions

We have tested and validated the $A\beta_{pE3-42}$ seeding hypothesis and shown that indeed $A\beta_{pE3-42}$ increases the levels of Aβ, and that $A\beta_{pE3-42}$ enhances pathology in this AD model. These results raise $A\beta_{pE3-42}$ as both a potential biomarker and new therapeutic target in AD. Furthermore, because *Drosophila* does not inherently express Aβ, the observation that expression of $A\beta_{pE3-42}$ is able to cause toxicity independent of its effect on $A\beta_{1-42}$ suggests that it is capable of initiating toxicity in other ways. It would be interesting to uncover downstream pathways that are modulated specifically by $A\beta_{pE3-42}$, and the fly provides a powerful context for pursuing this question with genetic screens.

Additional files

Additional file 1: Figure S1. Generation of $A\beta_{pE3-42}$. The proenkephalin signaling peptide upstream of $A\beta_{Q3-42}$ is cleaved by prohormone convertases, $A\beta_{Q3-42}$ is then released, and glutaminyl cyclase catalyses its conversion to $A\beta_{pE3-42}$. (PDF 271 kb)

Additional file 2: Figure S2. Expression of $A\beta_{pE3-42}$ causes locomotor dysfunction. Climbing ability of elavGS/UAS-$A\beta_{Q3-42}$and elavGS flies on + RU486 SY medium was assessed at the indicated time-points (see Materials & Methods). Expression of $A\beta_{pE3-42}$ in adult neurons reduced climbing ability of the flies in comparison to control elavGS driver flies. Data are presented as the average performance index (PI) ± SEM and were compared using 2-way ANOVA (number of independent tests (n) = 3 $P < 0.01$. (PDF 306 kb)

Additional file 3: Figure S3. (A). Confirming the expression of Neprilysin 2 in the EP(3)3549 fly strain. There was a significant increase in *nep2* transcript levels in the flies expressing the EP(3)3549 EP element, in comparison to the control fly lines expressing LACZ. Data are presented as means ± SEM and were analysed by student's *t*-test, $P < 0.001$. (B) Neprilysin2 significantly reduces Aβ protein levels, $^*P < 0.001$. $A\beta_{X-42}$ levels were quantified by ELISA. Data are presented as means ± SEM and were analysed by Student *t* test. (C) Neprilysin2 does not reduce $A\beta_{Q3-42}$ RNA levels. There was a significant increase in $A\beta_{Q3-42}$ RNA levels in flies co-expressing $A\beta_{Q3-42}$ and NEP2 in comparison to flies expressing $A\beta_{Q3-42}$ and EGFP, by quantitative RTPCR, $P < 0.01$, student's *t*-test. GMR-GAL4 was used to drive expression of $A\beta_{Q3-42}$ transgenic flies. (PDF 369 kb)

Additional file 4: Figure S4. pGluAβ increases accumulation of Aβ in vivo. Flies co-expressing $A\beta_{1-42}$ and $A\beta_{pE3-42}$ peptide, had significantly higher Aβ levels than flies co-expressing $A\beta_{1-42}$ and $A\beta_{1-42}$. Data are presented as means ± SEM and were analysed by student's *t*-test, $P < 0.01$. GMR-GAL4 was used to drive expression of $A\beta_{1-42}$ and $A\beta_{Q3-42}$ transgenic flies. (PDF 417 kb)

Additional file 5: Figure S5. pGluAβ increases accumulation of Aβ1-42 in vivo. (A). Flies co-expressing $A\beta_{1-42}$ and $A\beta_{pE3-42}$ peptide, had substantially more Aβ1-42 levels than flies expressing $A\beta_{1-42}$ alone. Aβ was not detected in flies expressing a single copy of $A\beta_{1-42}$ because it does not accumulate enough protein. However, $A\beta_{1-42}$ was detected in flies expressing 2 copies of $A\beta_{1-42}$ (B), confirming protein expression in these flies with the antibody. Furthermore, to validate specificity, $A\beta_{1-42}$ was not detected in flies expressing either a single copy or double copy of $A\beta_{pE3-42}$. GMR-GAL4 was used to drive expression of $A\beta_{1-42}$ and $A\beta_{Q3-42}$ transgenic flies. (PDF 10727 kb)

Additional file 6: Figure S6. Blind scoring of disorganised eye phenotype. The data demonstrate a significant difference in the degree of severity of eye roughness in flies co-expressing $A\beta_{1-42}$ and $A\beta_{pE3-42}$ in comparison to flies expressing either 2 copies of $A\beta_{1-42}$ (A), or $A\beta_{pE3-42}$ only (B), $^*P < 0.001$ for both. Flies were grown at 25 °C. Data are presented as means ± SEM and were analysed by Student's *t* test. GMR-GAL4 was used to drive expression of $A\beta_{1-42}$ and $A\beta_{Q3-42}$ transgenic flies. (PDF 303 kb)

Abbreviations

AD: Alzheimer's disease; APP: amyloid precursor protein; Aβ: Amyloid beta; elavGS: elav GeneSwitch; ER: Endoplasmic reticulum; IDE: Insulin degrading enzyme; JNK/SAPK: c-Jun N-terminal kinase/stress-activated protein kinase; Nep2: Neprilysin2; pGlu: Pyroglutamic; QC: glutaminyl cyclase; RU486, RU: mifepristone

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Availability of supporting data

Supporting data have been included in the publication as supplementary figures.

Authors' contributions

OS-A conceived experiments, performed experiments, analysed the data and wrote the manuscript. MK and IS performed experiments, and analyzed the data. LT generated UAS-AβQ3-42 transgenic flies. LP contributed to supervision of the project and writing of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interest.

Consent for publication

All authors have given consent for publication.

Ethical approval and consent to participate

Not applicable.

Authors' information

Not applicable.

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