Presynaptic Aβ40 prevents synapse addition in the adult Drosophila neuromuscular junction

Begoña López-Arias, Enrique Turieñano, Ignacio Monedero, Inmaculada Canal, Laura Torroja*

Department of Biology, Universidad Autónoma de Madrid, Madrid, Spain

* laura.torroja@uam.es

Abstract

Complexity in the processing of the Amyloid Precursor Protein, which generates a mixture of βamyloid peptides, lies beneath the difficulty in understanding the etiology of Alzheimer’s disease. Moreover, whether Aβ peptides have any physiological role in neurons is an unresolved question. By expressing single, defined Aβ peptides in Drosophila, specific effects can be discriminated in vivo. Here, we show that in the adult neuromuscular junction (NMJ), presynaptic expression of Aβ40 hinders the synaptic addition that normally occurs in adults, yielding NMJs with an invariant number of active zones at all ages tested. A similar trend is observed for Aβ42 at young ages, but net synaptic loss occurs at older ages in NMJs expressing this amyloid species. In contrast, Aβ42arc produces net synaptic loss at all ages tested, although age-dependent synaptic variations are maintained. Inhibition of the PI3K synaptogenic pathway may mediate some of these effects, because western analyses show that Aβ peptides block activation of this pathway, and Aβ species-specific synaptoxic effects persists in NMJs overgrown by over-expression of PI3K. Finally, individual Aβ effects are also observed when toxicity is examined by quantifying neurodegeneration and survival. Our results suggest a physiological effect of Aβ40 in synaptic plasticity, and imply different toxic mechanisms for each peptide species.

Introduction

Mounting evidences suggest that Alzheimer’s disease (AD) is primarily a disease of synaptic dysfunction [1], and place the amyloid Aβ peptide as the culprit of AD etiology. Indeed, in vivo and in vitro studies have shown that Aβ interferes with synaptic plasticity: it impairs excitatory transmission, inhibits LTP and enhances LTD [2,3]; it leads to spine retraction and synaptic loss [2,4,5]; and it causes robust behavioral deficits, particularly in learning and memory [2,6,7]. However, there are still important opened questions as to exactly which Aβ peptide forms trigger these synaptic alterations.

The latest prevalent version of the amyloid hypothesis states that AD arises from synaptic toxicity mediated by amyloid peptide (Aβ) soluble oligomers [8,9]. Proteolytic processing of
the APP transmembrane protein by β and γ secretases generates a mixture of Aβ species that
differ by few amino acids in their C-terminal sequence. Currently, it is unclear whether synap-
totoxicity in AD is attributable to single specific Aβ species or rather to a quantitatively and/or
qualitatively defined combination [10], or even if individual species harbor different toxic
activities. In vitro experiments using biochemically defined Aβ species provide insights into
these questions, but their relevance to the in vivo situation remains unresolved. Moreover,
recent data suggest that Aβ may have a physiological role in synaptic plasticity: synaptic activ-
ity increases Aβ production [11], Aβ is necessary for memory formation [12], and low concen-
trations of Aβ enhance LTP and memory formation [12–15]. However, whether this is actually
the case and its implication to AD is still undetermined [16].

Drosophila has proven a useful model to address these questions, because toxicity of specific
amyloid species can be dissected in vivo [17,18]. Expression of amyloid peptides in Drosophila
neurons recapitulates many of the pathological hallmarks of AD, including progressive neuro-
regeneration and behavioral deficits. In general, the severity of the behavioral phenotype cor-
related with peptide aggregation propensity and in vivo toxicity measured by longevity,
neurodegeneration, and mobility assays [19,20–24]. However, Aβ40, which was proven non-
toxic in these assays, induced early learning deficits in young flies [20,22].

The behavioral data suggest that all forms of Aβ, including Aβ40, produce synaptic defects
early in life, yet electrophysiological and structural studies Drosophila have reported synaptic
alterations only in response to Aβ42 or Aβ42arc [19,20,25,26]. However, the effects of Aβ40 on
synaptic structure were assessed primarily in larval or pupal preparations. Because Aβ40 affects
olfactory learning, but have no obvious effects on other behavioral processes such as climbing
ability, it may exert subtle alterations in adult synaptic plasticity. Moreover, the progressive
nature of AD cognitive decline indicates that Aβ synaptotoxicity is age-dependent. Therefore,
we aimed at finding a model that could uncover small changes in age-related synaptic dynam-
ics elicited by particular Aβ species.

The Drosophila adult NMJ should provide a simple, reproducible and quantifiable
system to examine alterations on synaptic dynamics. A few studies in the adult NMJ have
described age-dependent morphological changes [27,28]. These changes are consistent with an
early period of synaptic refinement and maturation, analogous to that described in the fly
antennal lobes [29] and mushroom bodies [30], and with a subsequent phase of age-related
motor decline [31,32]. Thus, we hypothesized that the adult ventral NMJ would provide a
model suitable for studying how particular Aβ peptides affect age-dependent synaptic
remodeling.

In this study, we systematically compared the age-dependent synaptotoxic effect of presyn-
aptic secretable forms of Aβ40, Aβ42, or Aβ42arc (a mutant form with increased aggregation
propensity which causes early onset familial AD; [33]) in the Drosophila adult glutamatergic
NMJ. We show that in wild type NMJs, there is an age-dependent variation in the number of
active zones, which peaks at 15 days after eclosion. Our data shows that Aβ40 blocks these
changes, so that it renders a slightly reduced, but constant number of synapses throughout
adult life. On the contrary, both Aβ42 peptides cause net synaptic loss, albeit age-dependent
variations are observed. These qualitative differences were apparent even in the presence
of PI3K overexpression, which causes a dramatic increase in the number of synaptic con-
tacts [34]. Our data provide new evidences of qualitative differences between the three Aβ pep-
tides in their synaptotoxic activities, and suggest a physiological role for Aβ40 in synaptic
plasticity.
Materials and methods
Fly culture
Fly stocks used were: w^1118, w^1118;D42-GAL4 -drives expression in all motor neurons and in some neuronal populations in the adult brain [35], and w^1118;elav-GAL4 -drives panneural expression - (all from Bloomington Drosophila Stock Center http://flystocks.bio.indiana.edu/); w^1118;UAS-αβ42, w^1118;UAS-αβ42 Arc2E, and w^1118;UAS-αβ40 (generously donated by Dr. Crowther D.C., [21]); w^1118;UAS-PI3K92E [36]. Lines harboring two UAS transgenes (Aβ and PI3K) were generated by standard genetic procedures.

For all experiments, males were analyzed from the progeny of crosses between UAS transgenic males and either w^1118;Gal4 (experimental flies) or w^1118 (control flies) females. The Gal4 control males were collected from a cross between w^1118;Gal4 and w^1118. All crosses were kept at 26˚C and parents transferred to new tubes every 2–3 days to maintain similar crowding conditions. When adults started to eclose, tubes were emptied and males collected after 24h and kept in tubes at 26˚C for aging.

Longevity assay
Males of the specified genotypes were incubated at 26˚C in groups of 20 in plastic vials and transferred every 2 days to fresh food. Death or lost flies were counted every 2 days. A minimum of five experiments per genotype was performed.

Dissection and immunohistochemistry
For neuromuscular junction (NMJ) analysis, ventral abdominal body-wall muscle preparations were dissected in Ca^{2+} free saline from 3, 7, 15, 20 and 30-day old adult males [37]. For assessing Aβ synaptotoxicity, we selected the ventral abdominal NMJ in the third abdominal hemisegment (Fig 1A). Samples were fixed in 4% formaldehyde in PBS, and immunostained with monoclonal antibody nc82 (anti-Bruchpilot 1:20, Developmental Studies Hybridoma Bank) visualized with α-mouse Alexa-488 (1:500, Invitrogen) and with Cy3-conjugated anti-HRP (1:200, Jackson Immuno Research). Anti-HRP signal reveals neuronal membranes, delimiting the motor neuron terminals. Bruchpilot is a CAST (CD3E-associated protein) homolog localized to the presynaptic specialization [38], and thus that was used to reveal active zones in the motor neuron terminal. Each Aβ-expressing genotype was processed simultaneously with its respective age-matched controls, to control for quantitative differences due to culture conditions.

Clearing of brain tissue for neurodegeneration assays
15 and 20 day old adults were decapitated and fly brains dissected in PBS and fixed for 30 minutes in 10% formalin in phosphate buffer (Electron Microscopy Sciences). After 3 washes in PBS (5 minutes each), samples were permeabilized in cold methanol (Panreac) for 5 minutes, treated with clearing solution (1:2 Benzyl Alcohol:Benzyl Benzoate, Sigma Aldrich) for another 5 minutes as described [39], and mounted in the same solution in excavated slides.

Image acquisition and quantification
Preparations were visualized using an Olympus IX70 confocal microscope. For body wall preparations, 1μm optical sections were taken. Stacks from hemisegments were used to quantify the number of Bruchpilot-containing active zones, with a macro-function developed in ImageJ that automatically selects the HRP-labeled motor terminal in each section and counts individual Bruchpilot-positive punctae while avoiding repeatedly counting the same synapse...
Fig 1. Age-dependent changes in the synaptic structure of the adult neuromuscular junction. (A) Schematic drawing of the anterior ventral abdomen (segments s2-s4), illustrating the position of the ventral abdominal muscles used for NMJ preparations. The accompanying image displays a representative confocal projection of a preparation labeled with anti-HRP (red) and anti-Brp (green). The inset shows active zones labeled with anti-Brp at a higher magnification. Scale bar = 20 μm. (B-D) Quantification of the number of active zones (synaptic contacts) (B), boutons (C), and branches (D) in control males in three different abdominal segments A3, A4, and A5. The bars represent the number of active zones, boutons, and branches per segment. The data is presented as mean ± SEM. (E) Summary of the number of active zones, boutons, and branches in control males at different time points after eclosion (3, 7, 15, 30, and 45 days after eclosion).
segments (A3, A4, and A5) at five ages (3, 7, 15, 30, and 45 days after eclosion (dae)). Statistical analyses showed that the three parameters showed segmental differences, but only synapses and boutons displayed age-dependent variations, that were similar in all segments. Number of synaptic contacts differed between the three segments, for boutons only segment A3 was statistically different from the other two segments, while branches was different in A4. Number of NMJs analyzed was between 7 and 9 for each segment/age pair, except for 45 days, which was 6 for A3, 5 for A4, and 3 for A5. Each data point represents the average ± SEM.

https://doi.org/10.1371/journal.pone.0177541.g001

in contiguous sections. Number of boutons (ellipsoid enlargements of the HRP-labeled axon terminal) and branches (defined as number of HRP-labeled axonal branch tips per hemisegment) was manually quantified based on the anti-HRP signal using the ImageJ point picker tool.

Autofluorescence from whole brains was recorded with an argon 488 nm laser in 2 μm optical sections. To quantify brain neurodegeneration, a macro function was developed in Fiji-win32 to automatically detect black circular areas (holes of neurodegenerated tissue) in each autofluorescent section of the brain and measure the area of neurodegeneration relative to the total brain area.

**Western blot**

To measure total Aβ content, 10 heads of 15-day-old males of each genotype were homogenized in 20 μl of lysis/monomerization buffer (9M Urea, 1% SDS, 1mM EDTA, 25 mM Tris-HCL pH 7.5), sonicated with 3–4 pulses of 5 seconds, heated at 55 °C during 1 hour, and centrifuged for 2 minutes. The supernatant was collected and proteins were separated by SDS–PAGE in 10–20% Tris–Tricine precast gels (Bio-Rad) and electroblotted onto 0.22 μm nitrocellulose membranes. The membrane was boiled in 1xPBS for 1 minute, and incubated with monoclonal anti-Aβ 6E10 (1:2000; Covance) and anti-αTubulin (1:2000, Sigma) and visualized by enhanced chemiluminescence (ECL, Amersham) in a Bio-Rad GelDoc XR+ system. Quantification was performed with ImageJ, and amount of Aβ signal relative to tubulin was averaged from three experiments.

Levels of phospho-Akt and phospho-GSK3β were used as readouts of PI3K activation. For this, 10 heads of 15-day-old males of each genotype were homogenized in 20 μl of RIPA buffer containing a cocktail of protease inhibitors and phosphatase inhibitors, and centrifuged for 2 minutes. The supernatant was collected and proteins were separated in Any kD TGX precast gels (Bio-Rad), and electroblotted onto 0.22 μm nitrocellulose membranes. Membranes were incubated with rabbit anti phospho(S505)-Akt or rabbit anti phospho(S9)-GSK3β (both 1:1000; Cell Signaling Technology) and visualized by enhanced chemiluminescence (ECL, Amersham) with a Bio-Rad GelDoc documentation system. After stripping, the same membranes were used to measure rabbit anti Drosophila Akt or mouse anti GSK3β signals (both 1:1000; Cell Signaling Technology). Stripped membranes were finally probed with mouse anti Tubulin α (1:2000; Sigma-Aldrich). Quantification was performed with ImageJ, and the ratio p-S505Akt/Akt or p-S9GSK3β/GSK3β was averaged from four experiments.

**Data analysis**

Parameters analyzed in NMJs were as follows: number of synaptic contacts, defined as Bruchpilot-positive active zones (also referred as synapses); number of boutons; number of branches; ratio synapses/bouton; and Aβ-induced synaptic reduction, which represents the percentage of active zones lost in each genotype with respect to the average number of synapses in age-matched controls (\[average \#synaptic contacts in controls \]– "\#synaptic contacts in Aβ")/ "average \#synaptic contacts in controls"). For genotypes expressing only Aβ peptides,
Aβ-induced synaptic reduction was calculated relative to UAS controls, which showed a slightly lower synaptic count than Gal4 controls, and thus provided a conservative measure. For flies expressing Aβ and PI3K, synaptic reduction was calculated with respect to age-matched PI3K-expressing NMJs. To control for possible GAL4 titration effects that could reduce the levels of PI3K when expressed in conjunction with Aβ, nlsGFP instead of Aβ was co-expressed with PI3K (w;UAS-PI3K/+; D42-Gal4 UAS-GFPnls/+), and the number of synaptic contacts in 15 day-old flies was compared to age-matched w;UAS-PI3K/+;D42-Gal4/ flies. Statistical analysis showed no significant difference between both genotypes (number of synaptic contacts: 1035.7 ± 47.3 for w;UAS-PI3K/+; D42-Gal4/ n = 10 NMJs (9 flies); 988.7 ± 44.6 for w;UAS-PI3K/+;D42-Gal4 UAS-GFPnls/+ n = 12 NMJs (7 flies); t = 0.72, p = 0.48).

For NMJ parameters and neurodegeneration, we tested for the normality of all variables and their usual logarithmic transformation. Statistical significance was calculated for the normally distributed variables with a two-way ANOVA test followed by Bonferroni post hoc. Because there is not a widely accepted non-parametric test to analyze the effect of interaction between factors, we also performed ANOVAs on these data, considering that the results obtained with ANOVA and Kruskal-Wallis test were equivalent. The SPSS 15.0.1 software (SPSS Inc., USA) was used throughout. Data are presented as average ± SEM, and statistical functions are reported as F for the ANOVA analysis and H for the Kruskal-Wallis test, where N refers to the number of samples and g to the degrees of freedom. For regression analysis, we used the Real Statistics Resource Pack software (Release 3.5; Copyright (2013–2015) Charles Zaiontz; www.real-statistics.com) on Excel (Microsoft).

Differences in survival were analyzed using the Kaplan-Meier survival plots and log-rank analysis with the online survival analysis package OASIS [40]. Statistical significance was set at a corrected Bonferroni p value of 0.05.

Results

Age-dependent variations in the number of active zones in the adult Drosophila NMJ

Two studies in adult flies suggest that the fly NMJ undergoes morphological changes during the adult life [27,28]. While boutons enlarge throughout the adult life, branches elongate during the first two weeks but become shorter and thinner from this relatively young age onward [27]. The number of active zones was shown to increase during the first 5 days after eclosion [28], although this synaptic trait has not been analyzed at older ages. To characterize age-dependent synaptic structural changes in the Drosophila adult abdominal ventral NMJ (Fig 1A; [37]), we measured the number of branches, boutons, and Bruchpilot-positive active zones (referred to as number of synaptic contacts) in segments A3 to A5 at different ages between 5 and 45 days after eclosion (Fig 1), and analyzed the effect of segment, age, and their interaction on these variables with ANOVA (synapses and boutons) or Kruskal-Wallis (branches) tests.

The three parameters showed segmental differences (synapses: F2,91 = 82.735, p < 0.001; boutons: F2,91 = 34.086, p < 0.001; branches: H2,N = 106 = 17.807, p < 0.001; Fig 1). However, only synapse and bouton number showed significant age-dependent differences (synapses: F4,91 = 36.178, p < 0.001; boutons: F4,91 = 14.833, p < 0.001; branches: H4,N = 106 = 1.343, p = 0.854). Synapses continuously increased from 3 to 15 days after eclosion (dae), decreased from 15 to 30 dae and stabilized until at least 45-days of age (Fig 1B). For boutons, the number remained essentially constant until 15 dae, and decreased by 45 dae (Fig 1C). A similar temporal pattern was observed in all segments, as revealed by the lack of effect of the interaction.
segment age in the statistical analyses (synapses: $F_{8,91} = 1.237, p = 0.287$; boutons: $F_{8,91} = 1.450, p = 0.187$; branches: $F_{8,91} = 1.641, p = 0.124$).

Our data shows that in the ventral abdominal NMJ, net synaptic addition occurs during the first two weeks after eclosion, while net synaptic elimination prevails afterwards in the aging fly. Thus, this adult NMJ arises as a simple suitable model for analyzing age-dependent alterations in synaptic dynamics due to Aβ expression. To differentiate physiological age-dependent changes from Aβ-induced synaptic alterations, the two temporal phases of synaptic remodeling will be referred to as "age-dependent synaptic addition" and "age-dependent synaptic elimination".

**Presynaptic Aβ40 prevents synapse addition in the adult NMJ, while Aβ42 and Aβ42arc yield net synaptic elimination**

To examine how Aβ influences synapses during ageing, we performed a systematic quantitative analysis comparing the effect of presynaptic Aβ40, Aβ42 and Aβ42arc on the morphology of the adult NMJ at different ages between 3 and 30 dae. Measures were taken at segment A3 and the effects of peptide (genotype), age, and their interaction, were statistically analyzed (Figs 2 and 3, Table 1).

Our results provide the first demonstration for a synaptic structural defect induced by Aβ40 on Drosophila synapses (Fig 2A; Table 1). All three peptides caused a significant synaptic reduction from 7 dae on. At 3 dae, Aβ40 and Aβ42arc also decreased synaptic contacts, while the number of active zones in Aβ42-expressing NMJs was intermediate between controls and NMJs with Aβ40, although it was not significantly different from either one. Consistent with its higher toxicity, Aβ42arc showed the strongest synaptic reduction (Fig 2A and 2B; Table 1) at all ages tested (at 30 days after eclosion, most Aβ42arc flies had died, and therefore this age was not analyzed). In contrast, Aβ42 induced a larger synaptic reduction than Aβ40 at 20 and 30 dae, but a similar effect at younger ages (Fig 2A and 2B). These peculiarities in Aβ-induced synaptotoxicity could not be solely attributed to quantitative differences in peptide levels, because western analysis showed similar levels of total Aβ40 and Aβ42arc at 15 dae (Fig 2F), yet a stronger synaptic reduction induced by the mutant peptide at this age. Overall, these data suggests that all amyloid species show an early tendency to decrease synapses, which is consistent with reported learning defects induced by the three Aβ peptides at young ages [22,23].

An intriguing observation was that the number of active zones in Aβ40-expressing NMJs remained constant throughout the ages tested. Indeed, statistical analyses showed an effect of the interaction between peptide type (genotype) and age on synapses (Table 1), denoting that Aβ expression alters the age-dependent changes of synapse number that occurs in physiological conditions. To further explore this question, we approximated the rate of synaptic change by the slope of the linear regression for the dataset covering the phase of age-dependent synapse addition (from 3 to 15 dae), and of age-dependent synapse elimination (from 15 to 30 dae; 15–20 dae for Aβ42arc)(Table 2).

In control NMJs, the age-dependent synaptic addition occurring from 3 to 15 dae rendered a slope with a positive value, while the age-dependent synaptic elimination that occurs between 15 and 30 dae yielded a negative slope (Table 2). The synaptic temporal pattern of Aβ40-expressing NMJs produced a slope which was not different from 0 for both age segments, and which was statistically different from both control regression lines (Table 2). Moreover, *post hoc* analyses identified Aβ40 as the only genotype in which the number of active zones did not differ between ages, while all other genotypes showed age dependent variations (Fig 2A). These data demonstrate that the effect of Aβ40 is qualitatively different from the
other Aβ peptides. But more importantly, the results suggest that Aβ40 restraints the addition of new synapses that normally occurs during the first days of adult life. For Aβ42-expressing NMJs, regression analysis revealed a modification in the temporal biphasic pattern of active sites. At young ages, from 3 to 15 dae, the rate of synaptic change was not different from 0 or from Aβ40, but differed from controls (Table 2). This suggests that in early adulthood, Aβ42 acts similarly to Aβ40, hindering synapse addition. In contrast, from 15 to 30 dae, age-dependent synaptic elimination occurred in Aβ42-expressing NMJs at a rate not different from controls (Table 2), suggesting a transformation in the synaptic action of this peptide with aging. Alternatively, the lower peptide content detected in Aβ42-expressing fly heads could account for its reduced early synaptotoxicity, while its age-dependent

Fig 2. Age-dependent effects of three Aβ peptides on the synaptic structure of the adult neuromuscular junction. Parameters were quantified in the adult A3 ventral NMJ at 3, 7, 15, 20, and 30 days after eclosion (dae), in males expressing Aβ40, Aβ42, or Aβ42arc in motoneurons (w; D42-Gal4/UAS-Aβ* or in control males (w; D42-Gal4/+ and w; UAS-Aβ*/*). For simplicity, only the Gal4 control, common to all experimental genotypes, is shown. Parameters measured were (A) number of synaptic contacts, (B) percentage of synaptic reduction measured as: (average number of synaptic contacts in control – number of synaptic contacts in Aβ*)/average number of synaptic contacts in control. (C) number of boutons, (D) number of branches, and (E) ratio of synaptic contacts per bouton. Each data point represents the average of 10 hemisegments of the same genotype/age ±SEM. Asterisks denote significant differences (** p<0.001) with their respective age-matched controls (Gal4 and UAS) and the other Aβ-expressing genotypes. Circled discontinuous lines surround data points that are not statistically different between each other but are statistically different with the other data points outside the circle. The symbol # indicates that post hoc analyses detected statistical differences (p<0.01) within each particular genotype between data from that age and data from 3 dae. Notice that the number of synapses in Aβ40-expressing NMJs (A) remains constant throughout all ages tested, while it shows age-dependent variations for the other genotypes. Number of flies analyzed, N, is larger than 20 for all ages in the control genotype except for 30 dae (N = 16). For all Aβ*-expressing genotypes N = 8, except for: w; D42-Gal4/UAS-Aβ40 20 dae (N = 9); w; D42-Gal4/UAS-Aβ42 at 7 and 30 dae (N = 9) and at 15 dae (N = 7); w; D42-Gal4/UAS-Aβarc at 7 and 15 dae (N = 9) and at 3 and 30 dae (N = 7). (F) Detection of total Aβ peptide (4 KDa) by western blot in head homogenates from 15 day-old flies expressing Aβ40, Aβ42, Aβ42arc (Aβarc), or from Gal4 control flies (D42). The right lane contains purified Aβ42 mixed with extract from control fly heads. αTubulin (50 KDa) was used to control for total protein content. Amount of Aβ was normalized with respect to αTubulin. Each data point in the graph represents the average from 3 experiments ±SEM. ** p<0.01, * p<0.05.

https://doi.org/10.1371/journal.pone.0177541.g002
Fig 3. Morphology of 15-day-old fly NMJs expressing amyloid peptides, with or without elevated PI3K levels. Representative images showing active zones (green; anti-Bruchpilot) and neuronal membrane (red; anti-HRP) of A3 abdominal ventral NMJs from 15-day-old control males (w, A and E), and males expressing Aβ40 (B and F), Aβ42 (C and G), or Aβ42arc (D and H), with normal (A-D) or elevated (E-H) levels of PI3K. Genotypes analyzed were (A) w; D42-Gal4/+, (B-D) w; D42-Gal4/UAS-Aβ*, (E) w; D42-Gal4/UAS-PI3K, and (F-H) w; D42-Gal4/UAS-PI3K, UAS-Aβ*. The complete NMJ is shown in images A-H (scale bar 20 μm), and a higher magnification of part of the NMJ is shown in images A'-H' (scale bar 10 μm).
Table 2. Linear regressions examining the rate of synaptic change in control and Aβ-expressing NMJs during two age intervals.

| Age interval | Genotype | Regression Analysis | Slope comparisons |
|--------------|----------|---------------------|------------------|
|              |          | b (slope) | t    | df  | p          | R²   | t    | df  | p          |
| 3–15 dae     | Control  | 2.825     | 6.778| 98  | <0.001*   | 0.319|       |      |            |
|              | Aβ40     | 0.202     | 0.245| 28  | 0.806     | 0.002| 2.921| 126 | 0.004*     |
|              | Aβ42     | -0.521    | -0.825| 28  | 0.417     | 0.024| 3.925| 126 | <0.001*    |
|              | Aβ42arc  | 2.693     | 3.716| 28  | <0.001*   | 0.330| 0.151| 126 | 0.880      |
| 15–30 dae    | Control  | -5.135    | -13.806| 98  | <0.001*   | 0.660|       |      |            |
|              | Aβ40     | -0.111    | -0.233| 28  | 0.817     | 0.002| 7.450| 126 | <0.001*    |
|              | Aβ42     | -5.49     | -10.712| 28  | <0.001*   | 0.804| 0.520| 126 | 0.604      |
|              | Aβ42arc  | (15–20 dae)| -9.5 | -5.972| 18  | <0.001*   | 0.665| 1.345| 96  | 0.182      |

Linear regression analyses were performed for each genotype with data of the number of active zones pooled from 3 to 15 or from 15 to 30 (15 to 20 for Aβ42arc) days after eclosion (dae). We additionally performed six analyses comparing the slopes of the regression lines for each Aβ-expressing genotype with the slope obtained for the corresponding control genotype (“slope comparisons”).

* indicates that the slope of the regression line is significantly different from 0.

# indicates that significant differences were found between the slopes.

https://doi.org/10.1371/journal.pone.0177541.1002
differences between all Aβ-expressing genotypes, including Aβ42arc, and age-matched controls at most ages tested (Fig 2E), suggesting a specific impact of the peptide on synaptic contacts, independent of its influence on bouton and branch number.

Aβ peptides induce synaptic reduction in adult NMJs enlarged by PI3K over expression

Presynaptic activation of the PI3K pathway promotes synaptogenesis both in Drosophila and mammals [34,41], while reducing the activity of the pathway, or increasing GSK3, a target inhibited by the pathway, has the opposite effect [34]. This pathway is altered in patients with Alzheimer’s disease, which show increased GSK3 activity, a condition that is thought to directly contribute to AD synaptic dysfunction [42,43]. Thus, we wondered whether PI3K activation might counteract Aβ induced synaptotoxicity. To test this, we overexpressed the PI3K catalytic subunit, Dp110, in motor neurons together with single Aβ species, and analyzed their combined effect on the adult NMJ. Synaptic morphological parameters were measured at 15 and 20 dae, two ages at which each Aβ peptide shows clear differences with respect to controls and to the other amyloid species (Figs 3 and 4, Table 3).

Consistent with previous findings on the Drosophila larval NMJ and central adult synapses [34], overexpression of PI3K lead to a robust expansion of the adult NMJ, which showed increased number of synaptic contacts, boutons and branches at both ages relative to controls with normal PI3K levels (Figs 3 and 4). The ratio of synapses per bouton also increased (Fig 4D), supporting independent mechanisms to regulate bouton and active zone formation. Interestingly, the number of synaptic contacts decreased from 15 to 20 dae (Fig 4A), therefore following the pattern of age-dependent variations observed in controls.

Simultaneous expression of PI3K and any of the three Aβ peptides also produced an increase in the number of active zones, boutons and branches when compared to Aβ expressing NMJs with normal PI3K levels, demonstrating that overactivation of the PI3K pathway can induce synaptic overgrowth even in the presence of Aβ peptides (compare Figs 2 and 4; Table 3). However, our data demonstrate that Aβ peptides can still exert their synaptotoxic effects on synapses with elevated PI3K levels, because synaptic parameters were significantly reduced in NMJs co-expressing PI3K and any of the three amyloid peptides when compared to NMJs expressing PI3K in the absence of Aβ (Fig 4).

In general, specific Aβ effects on active zones in NMJs overexpressing PI3K followed a similar tendency to that observed with normal PI3K levels, providing further support for the specific effects uncovered in this study (compare Figs 2 and 4). For example, Aβ42arc induced the largest synaptic reduction also in the presence of elevated PI3K (Aβ42arc: 37.3±9.2% at 15 dae, 39.2±6.1% at 20 dae; Aβ40: 31.8±5.0% at 15 dae, 19.0±4.6% at 20 dae; Aβ42: 22.9±7.3% at 15 dae, 17.8±9.4% at 20 dae). However, some differences were detected when assessing how the level of PI3K activity modifies amyloid outcome. First, post hoc analyses showed that amyloid peptides induced significantly larger synaptic reduction in the presence of elevated PI3K levels (p<0.0001 for all three comparisons D42;UAS-Aβ vs. D42;UAS-Aβ, UAS-PI3K). Second, as with normal PI3K levels, Aβ42arc exerted the strongest effect on branch and boutons in conditions of elevated PI3K (Fig 4B and 4C), but deleterious effects of Aβ40 and Aβ42 on these structures also became significant when PI3K signaling was augmented (p<0.0001 for all three comparisons D42;UAS-Aβ vs. D42;UAS-Aβ, UAS-PI3K).

Noticeably, Aβ40 also altered the age-dependent synaptic pattern when PI3K was overexpressed, so that the number of active zones remained constant between 15 and 20 dae (Fig 4A). This confirms our initial observations with normal PI3K levels, and provides strong support for a specific role of Aβ40 in opposing synapse addition during synaptic refinement.
Fig 4. Specific effects of the three amyloid peptides on synaptic structure persists in expanded adult neuromuscular junctions over expressing PI3K. (A-D) Parameters were quantified in the adult A3 ventral NMJ at 15 and 20 days after eclosion, in males overexpressing in motor neurons: PI3K (w; D42-Gal4/UAS-PI3K), PI3K and either Aβ40, Aβ42, or Aβ42arc (w; D42-Gal4/UAS-PI3K UAS-Aβ*), and in control males (w; D42-Gal4/+ and w; UAS-PI3K UAS-Aβ*/+). For simplicity, only the Gal4 control, common to all experimental genotypes, is shown (light blue line). Parameters measured were (A) number of synaptic contacts, (B) number of boutons, (C) number of branches, and (D) ratio of synaptic contacts per bouton. Each data point represents the average of 8–10 hemisegments of the same genotype/age ±SEM. Asterisks denote significant differences (**p<0.01) with their respective age-matched controls (Gal4 and UAS), and the other Aβ-expressing. The symbol # associated to the genotype label indicates that post hoc analyses detected statistical differences (p<0.01) between the two ages within that particular genotype. Notice that the number of synapses in Aβ40-expressing NMJs (A) remains constant from 15 to 20 dae, while it shows age-dependent...
Aβ synaptotoxicity in the adult fly NMJ

Our data show that the synaptotoxic effects of each Aβ peptide persist in NMJs overexpressing PI3K. Aβ peptides have been reported to inhibit the PI3K pathway both in vivo and in cell culture [25,44,45]. Hence, the observed Aβ-induced synaptic reduction in flies with elevated PI3K may be mediated by inhibition of the PI3K synaptogenic pathway. We therefore compared the activity of the PI3K pathway in 15 day-old flies co-expressing PI3K and each Aβ peptide. For this purpose, we quantified the degree of phosphorylation of Akt, the main effector kinase of the pathway, and the level of inhibitory phosphorylation of GSK3β, a target of the pathway especially relevant to AD. Over expression of PI3K alone induced a marked increase of the ratio p-S505-Akt/Akt when compared with flies with normal PI3K levels (Fig 4E). All three peptides showed a tendency to reduce this ratio, albeit this reduction was statistically significant only for the Aβ42arc peptide (Fig 4E). A similar trend was observed for GSK3β inhibitory phosphorylation, but differences were not statistically significant for any of the genotypes (Fig 4F). These data suggest that in NMJs with elevated PI3K levels, amyloid peptides can persist in NMJs overexpressing PI3K.

### Table 3. Summary of statistical results for the effect of different Aβ species with normal or elevated PI3K levels, on parameters of the adult neuromuscular junction, at 15 and 20 days after eclosion.

| GENOTYPE          | AGE            | GENOTYPE*AGE |
|-------------------|----------------|--------------|
| SYNAPTIC CONTACTS|                |              |
| F_{14,326} = 457.206 | p<0.001   | F_{1,326} = 138.497 | p<0.001   |
|                   | H(14, N = 356) = 268.191 | p<0.001   | F_{14,326} = 8.309 | p>0.001   |
| BOUTONS           |                |              |
| F_{14,326} = 292.856 | p<0.001   | F_{1,326} = 7.031  | p = 0.008  |
|                   | H(14, N = 356) = 140.761 | p<0.001   | F_{1,326} = 4.609  | p<0.001   |
| SYNAPSES/BOUTON   |                |              |
| F_{14,326} = 47.843  | p<0.001   | F_{1,326} = 110.890 | p<0.001   |
|                   | H(14, N = 356) = 178.545 | p<0.001   | F_{1,326} = 4.603  | p<0.001   |
| BRANCHES          |                |              |
| F_{14,326} = 411.999  | p<0.001   | F_{1,326} = 1.233   | p = 0.268  |
|                   | H(14, N = 356) = 146.587 | p<0.001   | F_{1,326} = 1.014  | p = 0.439  |
| SYNAPTIC REDUCTION|                |              |
| F_{14,326} = 47.782   | p<0.001   | F_{1,326} = 6.649   | p = 0.010  |
|                   | H(14, N = 356) = 9.270    | p>0.001    |

Data on synaptic parameters measured in the adult ventral abdominal NMJs at segment A3, from fifteen genotypes (w;D42-Gal4/+; UAS-Aβ*/+, w; UAS-PI3K/+; w;UAS-Aβ* UAS-PI3K/+, w;D42-Gal4/UAS-PI3K, w;D42/UAS-Aβ*, and w;D42-Gal4/UAS-Aβ* UAS-PI3K, being Aβ* either Aβ40, Aβ42, or Aβ42arc) and 2 ages (15 and 20 days after eclosion (dae)) were used for the analysis. Data with normal distribution were analyzed with a two-way ANOVA (F) that tested the effect of two variables (age and genotype) and their interaction (genotype*age). For data with non-normal distribution, the effect of either genotype or age was analyzed with the non-parametric one-way Kruskal-Wallis test (H). In these cases, to analyze the effect of age*genotype interaction, a two-way ANOVA was also performed that tested the effect of genotype, age, and their interaction. Therefore, for non-normally distributed data, both ANOVA (F) and Kruskal-Wallis (H) tests results are included. 8–10 NMJs were measured for each genotype and age (for details on number of flies analyzed, see Figs 2 and 4). The two subscripted numbers after F functions indicate the degrees of freedom for the between-group and within-group variance. For H function, the first subscript indicates the degrees of freedom.

https://doi.org/10.1371/journal.pone.0177541.t003

https://doi.org/10.1371/journal.pone.0177541.g004
inhibit the PI3K pathway, opposing its synaptogenic activity. To test if a similar mechanism might explain the synaptotoxic effect of Aβ in NMJs with normal PI3K levels, we quantified inhibitory phosphorylation of GSK3β in flies expressing each of the three Aβ species, and found a significant reduction only for NMJs expressing Aβ42arc (Fig 4F). Together, our data suggest that in Drosophila, amyloid peptides can also block PI3K pathway activation, and that this inhibition contributes to synaptic loss.

**PI3K over expression can partially rescue early neurodegeneration induced by Aβ42, but not by Aβ42Arc**

Our previous results show that Aβ40 and Aβ42-derived peptides display qualitatively different synaptotoxic effects, which may be partially mediated by inhibition of the PI3K pathway. Data gathered from various studies in Drosophila suggest that Aβ-induced synaptic deficit, cell loss, locomotor decline, and reduced life span may be elicited by different mechanisms [19,22,23,46,47], which could have different requirements for PI3K signaling. To gain insights into this question, we analyzed how PI3K over expression influences Aβ effects on neurodegeneration and life span.

Neuroprotective roles have been well established for PI3K [48]. Because possible neuroprotective effects would be easier to detect at early phases of the neurodegenerative process, we quantified neurodegeneration at relatively early ages (15 and 20 dae), using a pan-neural driver and an automated image analysis tool that measured brain tissue loss in whole brains. Indeed, this technique allowed detection of significant neurodegeneration as early as 15 dae with Aβ42-derived peptides (Fig 5), while prior experiments using very similar experimental conditions revealed first signs of neurodegeneration at 30 dae [22]. At these young ages, and despite the difference in peptide content (Fig 2F), the extension of the neurodegenerated area was similar in Aβ42- and Aβ42arc-expressing brains. Even though the two-way ANOVA detected significant age dependent differences, post hoc analyses did not reveal differences between 15 and 20 dae within each genotype. Flies expressing Aβ40 or PI3K, or both, were not significantly different from controls (Fig 5I), and thus did not evidence brain tissue loss.

Importantly, overexpression of PI3K was able to reduce Aβ42-induced neurodegeneration to almost wild type levels at both ages (Fig 5I). On the contrary, toxicity of Aβ42arc was not influenced by PI3K levels at any of the two ages tested (Fig 5I). These results are consistent with the level of PI3K pathway activity measured by Akt phosphorylation (Fig 4E), which show higher activity levels in Aβ42 than in Aβ42arc expressing flies, and suggest that activation of this pathway can protect against Aβ-induced cytotoxicity.

We next tested if PI3K positively affected life span in males expressing each of the Aβ peptides. Although most controls showed undistinguishable cumulative survival curves (Fig 6), there were significant differences in some control lines which complicated interpretation of the results. UAS-PI3K control flies displayed an abnormally high survival (p<0.001 vs. all other genotypes), while D42-Gal4 control males had slightly reduced survival when compared to the other control genotypes (p<0.001 vs. all other control genotypes). Life span of flies overexpressing PI3K alone was undistinguishable from the Gal4 control (χ² = 1.03, p = 1), but lower than the other control genotypes (p<0.001).

Consistent with previous reports [21,22,26,46], expression of Aβ40 did not reduce life span, not even in the presence of overexpressed PI3K (Experiment Aβ40 vs. Experiment Aβ40 +PI3K: χ² = 0.22, p = 1) (Fig 6A). Furthermore, longevity of Aβ40 flies, with or without elevated PI3K levels, was higher than Gal4 controls (Control Gal4 vs. Experiment Aβ40: χ² = 11.36, p = 0.011; Control Gal4 vs. Experiment Aβ40 +PI3K: χ² = 10.38, p = 0.018) and flies expressing PI3K alone (Experiment PI3K vs. Experiment Aβ40: χ² = 16.65, p<0.001; Experiment PI3K vs.
Experiment Aβ40+PI3K: $\chi^2 = 14.95$, $p = 0.002$), which points to a possible neuroprotective effect of this peptide. In contrast, Aβ42 significantly reduced survival rate ($p < 0.001$ vs. all other genotypes) (Fig 6B), as has been previously shown [21–23,26,46]. Simultaneous expression of Aβ42 and PI3K reduced longevity even further ($p < 0.001$ vs. all other genotypes) (Fig 6B), indicating a synergistic deleterious effect between the two. In line with earlier studies [22,23,25,26,47], Aβ42arc showed the highest toxicity in this assay, which was not affected by the level of PI3K expression (Experiment Aβ42arc vs. Experiment Aβ42arc+PI3K $\chi^2 = 6.25$, $p = 0.1738$; $p < 0.001$ for both vs. all other genotypes) (Fig 6C). Thus, overexpression of PI3K
does not seem to reduce Aβ toxicity in longevity assays. In summary, our data reveal that the implication of the PI3K pathway in the toxicity of Aβ differs for different Aβ species and in different contexts (synapse formation, cytotoxicity, and life span).

Discussion

Synapse dysfunction and loss are key to dementia in Alzheimer’s disease (AD). It is currently established that amyloid-β peptides are important contributors to these synaptic alterations, but the precise identity of the Aβ species involved is not well defined [10,49]. Likewise, it is unclear how aging, the most influential non-genetic risk factor in AD, influences Aβ synaptotoxicity. These are relevant questions to designing suitable therapeutic strategies. We postulated that the Drosophila adult glutamaergic neuromuscular junction (NMJ) would provide an ideal system in which to address these issues, because its stereotypic morphology would allow uncovering subtle albeit relevant changes. Moreover, our work has revealed a temporal biphasic process of synaptic remodeling at the adult NMJ. An early phase of net synapse addition takes place during the first two weeks of adult life, a critical time period in which several areas of the young fly brain have been shown to undergo experience-dependent structural plasticity [29,50,51]. Thenceforth, net synaptic elimination occurs, consistent with the onset of behavioural and synaptic senescence [31,32,50,52]. Thus, this model allows assessing Aβ influence on synaptic dynamics during synaptic maturation and aging. In this model we show that: (1) Aβ40 seems to reduce the number of synaptic contacts by preventing synapse addition; (2) Aβ42 synaptotoxicity gradually increases with age; and (3) Aβ42arc produces net synaptic loss from early adulthood, but does not impede synapse addition. In summary, we demonstrate specific age-dependent synaptic effects for each Aβ peptide, and establish the fly adult NMJ as a suitable model to investigate the mechanisms underlying these peculiarities.

Early amyloid synaptotoxicity or a physiological role for Aβ?

Studies in Drosophila had shown early memory defects induced by expression of both Aβ40 and Aβ42-derived peptides [19,20,22,23], but a structural and/or functional synaptic correlate had only been found for Aβ42-derived peptides [19,20,25,26]. Here, we show for the first time that Aβ40 reduces the number of synaptic contacts, and it seems to do so by preventing the
addition of new synapses that normally occurs in the adult NMJ. At young ages (3–15 days), Aβ42 effect was remarkably similar to Aβ40, which suggests that early in life, Aβ42 may act similarly to Aβ40 by opposing synapse addition. However, Aβ42-expressing brains had the lowest amount of total peptide, which might explain these relatively mild early effects. In contrast, the mutant amyloid peptide Aβ42arc elicited net synaptic loss also at young ages, yet it showed a pattern of age-dependent variation in synapse number similar to controls, indicating that it does not hinder synapse addition. These are significant qualitative differences that point to a physiological role for wild type amyloid species in the process of synaptic plasticity.

In general, exposure to Aβ is associated with weakening of synapses, which is consistent with a postulated Aβ function in promoting activity-dependent synaptic elimination during mammalian postnatal development [16]. Our data suggest that rather than eliciting synapse removal, Aβ40, and possibly Aβ42, prevents the formation and/or maturation of new synapses in Drosophila. Interestingly, early defects in ocular dominance plasticity in APPswe transgenic mice, which generate elevated levels of wild type amyloid species, are associated with reduced strengthening and expansion of non-deprived eye cortical representations, while deprived eye weakening remains intact [53]. These data argue in favor of a similar effect for Aβ at synapses from juvenile mice and flies. In the Drosophila larval NMJ, the transition from immature to mature synapse involves changes in the relative contribution of specific glutamate receptors in postsynaptic receptor fields opposed to presynaptic active zones [54]. In mammals, synaptic elimination and maturation also involves changes in expression and trafficking of AMPA and NMDA glutamate receptors [55], a process that can be altered by Aβ [5,56–58]. The model described in this work represents an invaluable tool for genetically assessing the contribution of Glutamate receptors, and other molecules, in the amyloid-dependent alterations of synaptic refinement.

Although we cannot provide a mechanistic basis for the specific synaptotoxic activities of each Aβ species, the data on NMJs overexpressing PI3K suggest the direct implication of this pathway. First, despite its strong synaptogenic activity, PI3K over expression was not able to block Aβ-induced synaptic reduction. Second, western quantification showed that the activity of the pathway is reduced by Aβ expression, which is consistent with data from mammalian systems that suggest that amyloid peptides block Akt activation downstream of the PI3K enzyme [44,45], and increase GSK3 activity [43]. Moreover, the degree of PI3K pathway inhibition correlated with the extent of synaptic reduction, both being maximum for Aβ42arc. Altogether, evidences point to a direct relationship between Aβ and the PI3K/Akt/GSK3 pathway as central to Aβ-induced synaptic dysfunction, but further studies would be necessary to unambiguously prove it. The importance of this matter is underscored by the relevance of GSK3 in AD, which has been used as a therapeutic target [42,43].

Aging and amyloid synaptotoxicity

The risk of AD increases with age, but the link between aging and Aβ toxicity is not fully understood. We have shown that after a stage of synaptic growth, synaptic elimination commences in the fly NMJ sometime between 15 and 20 days after eclosion. This age also represents a point of transition from growth to retraction for the abdominal longitudinal NMJ [27] and the mushroom bodies [30], and thus defines an age-dependent change in synaptic dynamics. Our data suggest that this age also delimits a change in the synaptic action of Aβ42, which would oppose synapse addition in early adulthood, but cause synaptic removal in aged flies. In contrast, Aβ42arc induces net synaptic loss at a similar rate at all ages tested, while Aβ40 maintains an unvarying number of active sites at least until 30 days. These data suggest that the impact of age on Aβ synaptotoxicity differs for each amyloid species. Understanding the
mechanisms underlying age-dependent synaptic changes in wild type adult NMJs will be necessary for explaining the observed effects.

Several in vitro and in vivo studies have demonstrated different aggregation kinetics for the three Aβ species, which result in higher effective concentrations of specific aggregation forms with diverse toxic activities. Specifically, a recent in vivo study expressing tandem dimeric Aβ peptides in Drosophila has shown that the aggregation kinetics of Aβ42 favors a relatively high population of toxic oligomeric species, while this population is undetectable for Aβ40, which seems to more rapidly transit from monomeric to insoluble inert forms [59]. The E22G mutation in Aβ42arc has been shown to accelerate both Aβ oligomerization and fibrillogensis [60,61]. Thus, it is tempting to speculate that Aβ monomeric forms inhibit synapse addition, while oligomeric forms promote synapse removal. Assessing the synaptic effects of the various tandem dimeric Aβ peptides [59] on the Drosophila adult NMJ would provide invaluable data to test this hypothesis.

Amyloid-dependent synaptotoxicity, cytotoxicity, and life span shortening

Multiple studies in Drosophila, and in other models, suggest that the mechanisms underlying Aβ synaptotoxicity, cytotoxicity, and reduction of life span are different [19,22,23,46,47]. Even more, in vivo data demonstrate that manipulations that alter aggregation propensity can induce qualitative, rather than quantitative, shifts in the pathology induced [24]. Our data provide further support to this notion. First, we show that Aβ40 disturbs synapses, but not cell survival or life span. Second, neurodegeneration levels were similar in 20 day-old flies expressing either Aβ42 or Aβ42arc, yet both genotypes displayed dramatically different life expectancy. Third, over expression of PI3K reduced early Aβ42 cytotoxicity, measured by degree of neurodegeneration, but it enhanced the deleterious effects of this peptide on life span.

The differential effect of PI3K hyperactivation on Aβ-related phenotypic outcomes is intriguing. PI3K is an essential signaling pathway with multiple developmental and physiological functions; these include widespread functions such as regulation of cell proliferation and metabolism, or control of cellular remodeling and migration, but also cell-type specific roles such as synapse plasticity in neurons [62]. Moreover, different levels of pathway activation seems to trigger distinct responses [63,64]. In view of this complexity, we can only speculate on how PI3K alters Aβ-induced phenotypes. Our data show that PI3K overexpression had no influence on Aβ42arc-related phenotypes, a finding that might well be explained by the significant reduction in the activity of the PI3K pathway displayed by Aβ42arc-expressing flies. In contrast, the observed reduced neurodegeneration in flies co-expressing Aβ42 and PI3K could reflect residual hyper-activation of the pro-survival PI3K pathway, as suggested by western quantification. This hypothesis is consistent with studies in mice which suggest that low levels of Akt activity are sufficient to support neuronal survival responses [64]. However, PI3K overexpression had a negative effect on life span in flies expressing Aβ42. Lifespan is an extremely complex trait which may be particularly sensitive to unbalanced conditions, and age-dependent accumulation of toxic amyloid aggregates might further disturb PI3K and related signaling networks, advancing deterioration. These data underscore the complexity of Aβ toxicity and the necessity to test it at different levels when assessing the consequences of therapeutic approaches.

In summary, using an in vivo model we demonstrate that (1) different amyloid species disturb synapses by differentially influencing synapse addition or synapse elimination, (2) age-dependent changes in their synaptotoxicity are species-specific, and (3) the toxic actions of each Aβ peptide differ in different contexts. Furthermore, our work demonstrates the value of
the *Drosophila* adult NMJ as an ideal *in vivo* model for understanding specific effects of amyloid peptides on synaptic plasticity.

**Acknowledgments**

We thank DC. Crowther, the Developmental Studies Hybridoma Bank, and the Bloomington Stock Center for antibodies and fly lines, A.B. Fernández, M. Ruiz, and J. Benito for reagents, and A. Ferrús and A. Acebes for comments on the manuscript.

**Author Contributions**

*Conceptualization:* IC LT.

*Formal analysis:* BLA IM LT ET.

*Funding acquisition:* IC LT.

*Investigation:* BLA LT.

*Project administration:* LT.

*Software:* IM.

*Supervision:* LT.

*Visualization:* BLA ET LT.

*Writing – original draft:* BLA ET LT.

*Writing – review & editing:* LT.

**References**

1. Selkoe DJ. Alzheimer's disease is a synaptic failure. Science. 2002; 298(5594):789–91. [https://doi.org/10.1126/science.1074069](https://doi.org/10.1126/science.1074069) PMID: 12399581

2. Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, et al. Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. Nat Med. 2008; 14(8):837–42. [https://doi.org/10.1038/nm1782](https://doi.org/10.1038/nm1782) PMID: 18568035

3. Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, et al. Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature.2002; 416(6880):535–9. [https://doi.org/10.1038/416535a](https://doi.org/10.1038/416535a) PMID: 11932745

4. Lacor PN, Buniel MC, Furlow PW, Clemente AS, Velasco PT, Wood M, et al. Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. J Neurosci Off J Soc Neurosci. 2007; 27(4):796–807.

5. Shankar GM, Bloodgood BL, Townsend M, Walsh DM, Selkoe DJ, Sabatini BL. Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. J Neurosci Off J Soc Neurosci. 2007; 27(11):2866–75.

6. Lesnè S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, et al. A specific amyloid-beta protein assembly in the brain impairs memory. Nature. 2006; 440(7082):352–7. [https://doi.org/10.1038/nature04533](https://doi.org/10.1038/nature04533) PMID: 16541076

7. McDonald MP, Dahl EE, Overmier JB, Mantyh P, Cleary J. Effects of an exogenous beta-amyloid peptide on retention for spatial learning. Behav Neural Biol. 1994; 62(1):60–7. PMID: 7945146

8. Ma T, Klann E. Amyloid β: linking synaptic plasticity failure to memory disruption in Alzheimer's disease. J Neurochem. 2012; 120:140–6. [https://doi.org/10.1111/j.1471-4159.2011.07560.x](https://doi.org/10.1111/j.1471-4159.2011.07560.x) PMID: 22122128

9. Wilcox K, Lacor P, Pitt J, Klein W. Aβ Oligomer-Induced Synapse Degeneration in Alzheimer’s Disease. Cell Mol Neurobiol. 2011; 31(6):939–48. [https://doi.org/10.1007/s10571-011-9691-4](https://doi.org/10.1007/s10571-011-9691-4) PMID: 21538118

10. Benilova I, Karran E, De Strooper B. The toxic Aβ oligomer and Alzheimer's disease: an emperor in need of clothes. Nat Neurosci. 2012; 15(3):349–57. [https://doi.org/10.1038/nn.3028](https://doi.org/10.1038/nn.3028) PMID: 22286176
11. Kamenetz F, Tomita T, Hsieh H, Seabrook G, Borchelt D, Iwatsubo T, et al. APP processing and synaptic function. Neuron. 2003; 37(6):925–37. PMID: 12670422

12. Garcia-Osta A, Alberini CM. Amyloid beta mediates memory formation. Learn Mem. 2009; 16(4): 267–72. https://doi.org/10.1101/lm.1310209 PMID: 19318468

13. Abramov E, Dolev I, Fogel H, Cicciotosto GD, Ruff E, Slutsky I. Amyloid-[beta] as a positive endogenous regulator of release probability at hippocampal synapses. Nat Neurosci. 2009; 12(12):1567–76. https://doi.org/10.1038/nn.2433 PMID: 19935655

14. Puzzo D, Privitera L, Leznik E, Fa M, Staniszewski A, Palmeri A, et al. Picomolar Amyloid-[beta] Positively Modulates Synaptic Plasticity and Memory in Hippocampus. J Neurosci. 2008; 28(53):14537–45. https://doi.org/10.1523/JNEUROSCI.2692-08.2008 PMID: 19118188

15. Wasling P, Daborg J, Riebe I, Andersson M, Portelius E, Blennow K, et al. Synaptic Retrogenesis and Amyloid-β in Alzheimer's Disease. J Alzheimers Dis. 2009; 16(1):1–14. https://doi.org/10.3233/JAD-2009-0918 PMID: 19158416

16. Iijima-Ando K, Iijima K. Transgenic Drosophila models of Alzheimer's disease and tauopathies. Brain Struct Funct. 2010; 214(2–3):245–62. https://doi.org/10.1007/s00429-009-0234-4 PMID: 19967412

17. Moloney A, Sattelle DB, Lomas DA, Crowther DC. Alzheimer's disease: insights from Drosophila models. Trends Biochem Sci. 2010; 35(4):228–35. https://doi.org/10.1016/j.tibs.2010.02.005 PMID: 2036556

18. Iijima K, Liu H-P, Chiang H-C, Hearn SA, Konsolaki M, Zhong Y. Dissecting the pathological effects of human Aβ42 in Drosophila: A potential model for Alzheimer's disease. Proc Natl Acad Sci U S A. 2004; 101(17):6623–8. https://doi.org/10.1073/pnas.0400895101 PMID: 15069204

19. Iijima K, Liu H-P, Chiang H-C, Hearn SA, Konsoaki M, Zhong Y. Dissecting the pathological effects of human Aβ40 and Aβ42 in Drosophila: A potential model for Alzheimer's disease. Proc Natl Acad Sci U S A. 2004; 101(17):6623–8. https://doi.org/10.1073/pnas.0400895101 PMID: 15069204

20. Fang L, Duan J, Ran D, Fan Z, Yan Y, Huang N, et al. Amyloid-β depresses excitatory cholinergic synaptic transmission in Drosophila. Neurosci Bull. 2012; 28(5):585–94. https://doi.org/10.1007/s12264-012-1267-x PMID: 23054636

21. Crowther DC, Kinghorn KJ, Miranda E, Page R, Curry JA, Duthie FAI, et al. Intraneuronal Aβ, nonamyloid aggregates and neurodegeneration in a Drosophila model of Alzheimer's disease. Neurosci. 2005; 132(1):123–35. https://doi.org/10.1016/j.neuroscience.2004.12.025 PMID: 15780472

22. Iijima K, Liu H-P, Chiang H-C, Hearn S, Konsoaki M, Zhong Y. Dissecting the pathological effects of human Aβ40 and Aβ42 in Drosophila: A potential model for Alzheimer's disease. Proc Natl Acad Sci U S A. 2004; 101(17):6623–8. https://doi.org/10.1073/pnas.0400895101 PMID: 15069204

23. Iijima K, Chiang H-C, Hakker I, Gatt A, Shenton C, et al. Aβ42 Mutants with Different Aggregation Profiles Induce Distinct Pathologies in Drosophila. PLoS ONE. 2008; 3(2):e1703. https://doi.org/10.1371/journal.pone.0001703 PMID: 18301778

24. Luheisli LM, Tartaglia GG, Brorssojn A-C, Pawar AP, Watson IE, Chiti F, et al. Systematic In Vivo Analysis of the Intrinsic Determinants of Amyloid β Pathogenicity. PLoS Biol. 3 2007; 5(11):e290. https://doi.org/10.1371/journal.pbio.0050290 PMID: 17973577

25. Sofola O, Kerr F, Rogers I, Killick R, Augustin H, Gandy C, et al. Inhibition of GSK-3 Ameliorates Aβ Pathology in an Adult-Onset Drosophila Model of Alzheimer's Disease. PLoS Genet. 2010; 6(9):e1001087. https://doi.org/10.1371/journal.pgen.1001087 PMID: 20824130

26. Zhao X-L, Wang W-A, Tan J-X, Huang J-K, Zhang X, Zhang B-Z, et al. Expression of β-Amyloid Induced Age-Dependent Presynaptic and Axonal Changes in Drosophila. J Neurosci. 2010; 30(4):1512–22. https://doi.org/10.1523/JNEUROSCI.3699-09.2010 PMID: 20107079

27. Beramendi A, Peron S, Casanova G, Reggiani C, Cantero R. Neuromuscular junction in abdominal muscles of Drosophila melanogaster during adulthood and aging. J Comp Neurol. 2007; 501(4): 498–508. https://doi.org/10.1002/cne.21253 PMID: 17278125

28. Rivlin PK, Clair RMS, Vilinsky I, Deitcher DL. Morphology and molecular organization of the adult neuromuscular junction of Drosophila. J Comp Neurol. 2004; 468(4):596–613. https://doi.org/10.1002/cne.10777 PMID: 14689489

29. Devau J-M, Acebes A, Ramaswami M, Ferrús A. Structural and functional changes in the olfactory pathway of adult Drosophila take place at a critical age. J Neurobiol. 2003; 56(1):13–23. https://doi.org/10.1002/neu.10215 PMID: 12767029

30. Technau GM. Fiber number in the mushroom bodies of adult Drosophila melanogaster depends on age, sex and experience. J Neurogenet. 1984; 1(2):113–26. PMID: 6085635

31. Grotewiel MS, Martin I, Bhandari P, Cook-Wiens E. Functional senescence in Drosophila melanogaster. Ageing Res Rev. 2005; 4(3):372–97. https://doi.org/10.1016/j.arr.2005.04.001 PMID: 16024299
32. Iliadi KG, Boulianne GL. Age-related behavioral changes in Drosophila. Ann N Y Acad Sci. 2010; 1197(1):9–18.
33. Nilsberth C, Westlund-Danielsson A, Eckman CB, Condron MM, Axelman K, Forsell C, et al. The «Arc» APP mutation (E693G) causes Alzheimer’s disease by enhanced Aβ protofibril formation. Nat Neurosci. 2001; 4(9):87–93. https://doi.org/10.1038/nn0901-887 PMID: 11528419
34. Martin-Peña A, Acebes A, Rodríguez J-R, Sorribes A, de Polavieja GG, Fernández-Fúnez P, et al. Age-Independent Synaptogenesis by Phosphoinositide 3 Kinase. J Neurosci. 2006; 26(40):10199–208. https://doi.org/10.1523/JNEUROSCI.1223-06.2006 PMID: 17021175
35. Legan SK, Rebrin I, Mockett RJ, Radyuk SN, Klichko VI, Sohal RS, et al. Overexpression of Glucose-6-phosphate Dehydrogenase Extends the Life Span of Drosophila melanogaster. J Biol Chem. 2008; 283(47):32492–9. https://doi.org/10.1074/jbc.M805832200 PMID: 18809674
36. Leevers SJ, Weinkove D, MacDougall LK, Hafen E, Waterfield MD. The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth. EMBO J. 1996; 15(23):6584–94. PMID: 8978685
37. Hebbar S, Hall RE, Demska SA, Subramanian A, Fernandes JJ. The adult abdominal neuromuscular junction of Drosophila: A model for synaptic plasticity. J Neurobiol. 2006; 66(10):1140–55. https://doi.org/10.1002/neu.20279 PMID: 16838368
38. Waghi DA, Rasse TM, Asan E, Hofbauer A, Schwenk I, Dürrbeck H, et al. Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in Drosophila. Neuron. 2006; 49(6):833–44. https://doi.org/10.1016/j.neuron.2006.02.008 PMID: 16543132
39. McGurk L, Morrison H, Keegan LP, Sharpe J, O’Connell MA. Three-dimensional imaging of Drosophila melanogaster. PLoS One. 2007; 2(9):e834. https://doi.org/10.1371/journal.pone.0000834 PMID: 17786206
40. Yang J-S, Nam H-J, Seo M, Han SK, Choi Y, Nam HG, et al. OASIS: Online Application for the Survival Analysis of Lifespan Assays Performed in Aging Research. PLoS ONE. 2011; 6(8):e23525. https://doi.org/10.1371/journal.pone.0023525 PMID: 21858155
41. Cuesto G, Enríquez-Barreto L, Caramés C, Cantarero M, Gasull X, Sandi C, et al. Phosphoinositide-3-Kinase Activation Controls Synaptogenesis and Spinogenesis in Hippocampal Neurons. J Neurosci. 2011; 31(8):2721–33. https://doi.org/10.1523/JNEUROSCI.4477-10.2011 PMID: 21414895
42. Kremer A, Louis JV, Jaworski T, Van Leuven F, GSK3 and Alzheimer’s Disease: Facts and Fiction. Neuron. 2006; 49(6):833–44. https://doi.org/10.1016/j.neuron.2006.02.008 PMID: 16543132
43. Llorens-Martín M, Jurado J, Hernández F, Avila J. GSK-3β, a pivotal kinase in Alzheimer disease. Front Mol Neurosci. 2014; 7:46. https://doi.org/10.3389/fnmol.2014.00046 PMID: 24904272
44. Lee H-K, Kumar P, Fu Q, Rosen KM, Querfurth HW. The insulin/Akt signaling pathway is targeted by the PI3K-Akt signaling pathway. Curr Opin Neurobiol. junio de 2001; 11(3):297–305.
45. Kerr F, Augustin H, Piper MDW, Gandy C, Allen MJ, Lovestone S, et al. Dietary restriction delays aging, but not neuronal dysfunction, in Drosophila models of Alzheimer’s disease. Neurobiol Aging. 2011; 32(11):1977–89. https://doi.org/10.1016/j.neurobiolaging.2010.07.015 PMID: 19969390
46. Brunet A, Datta SR, Greenberg ME. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr Opin Neurobiol. junio de 2001; 11(3):297–305.
47. Shankar GM, Walsh DM. Alzheimer’s disease: synaptic dysfunction and Abeta. Mol Neurodegener. 2009; 4:48. https://doi.org/10.1186/1750-1326-4-48 PMID: 19930651
48. Donlea JM, Ramanan N, Silverman N, Shaw PJ. Genetic Rescue of Functional Senescence in Synaptic and Behavioral Plasticity. Sleep. 2014; 37(9):1427–37. https://doi.org/10.5665/sleep.3988 PMID: 25142573
49. Heisenberg M, Heusipp M, Wanke C. Structural plasticity in the Drosophila brain. J Neurosci Off J Soc Neurosci. 1995; 15(3 Pt 1):1951–60.
50. Haddadi M, Jahromi SR, Sagar BKC, Patil RK, Shivanandappa T, Ramesh SR. Brain aging, memory impairment and oxidative stress: a study in Drosophila melanogaster. Behav Brain Res. 2014; 259:60–9. https://doi.org/10.1016/j.bbr.2013.10.036 PMID: 24183945
53. William CM, Andermann ML, Goldey GJ, Roumis DK, Reid RC, Shatz CJ, et al. Synaptic Plasticity Defect Following Visual Deprivation in Alzheimer’s Disease Model Transgenic Mice. J Neurosci. 2012; 32(23):8004–11. https://doi.org/10.1523/JNEUROSCI.5369-11.2012 PMID: 22674275

54. Thomas U, Sigrist SJ. Glutamate receptors in synaptic assembly and plasticity: case studies on fly NMJs. Adv Exp Med Biol. 2012; 970:3–28. https://doi.org/10.1007/978-3-7091-0932-8_1 PMID: 22351049

55. McKinney RA. Excitatory amino acid involvement in dendritic spine formation, maintenance and remodelling. J Physiol. 2010; 588(Pt 1):107–16. https://doi.org/10.1113/jphysiol.2009.178905 PMID: 19933758

56. Almeida CG, Tampellini D, Takahashi RH, Greengard P, Lin MT, Snyder EM, et al. Beta-amyloid accumulation in APP mutant neurons reduces PSD-95 and GluR1 in synapses. Neurobiol Dis. 2005; 20(2):187–98. https://doi.org/10.1016/j.nbd.2005.02.008 PMID: 16242627

57. Hsieh H, Boehm J, Sato C, Iwatsubo T, Tomita T, Sisodia S, et al. AMPAR removal underlies Abeta-induced synaptic depression and dendritic spine loss. Neuron. 2006; 52(5):831–43. https://doi.org/10.1016/j.neuron.2006.10.035 PMID: 17145504

58. Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, et al. Regulation of NMDA receptor trafficking by amyloid-beta. Nat Neurosci. 2005; 8(8):1051–8. https://doi.org/10.1038/nn1503 PMID: 16025111

59. Speretta E, Jahn TR, Tartaglia GG, Favrin G, Barros TP, Imarisio S, et al. Expression in Drosophila of Tandem Aβ Peptides Provides Insights Into the Link Between Aggregation and Neurotoxicity. J Biol Chem 2012; 287(24):20748–54. https://doi.org/10.1074/jbc.M112.350124 PMID: 2246132

60. Brorsson A-C, Bolognesi B, Tartaglia GG, Shammas SL, Favrin G, Watson I, et al. Intrinsic Determinants of Neurotoxic Aggregate Formation by the Amyloid β Peptide. Biophys J. 2 2010; 98(8):1677–84. https://doi.org/10.1016/j.bpj.2009.12.4320 PMID: 20409489

61. Norlin N, Hellberg M, Filippov A, Sousa AA, Grönbäck M, Leapman RD, et al. Aggregation and fibril morphology of the Arctic mutation of Alzheimer’s Aβ peptide by CD, TEM, STEM and in situ AFM. J Struct Biol. 2012; 180(1):174–89. https://doi.org/10.1016/j.jsb.2012.06.010 PMID: 22750418

62. Knafo S, Esteban JA. Common pathways for growth and for plasticity. Curr Opin Neurobiol. 2012; 22(3):405–11. https://doi.org/10.1016/j.conb.2012.02.008 PMID: 22398399

63. Iwanami A, Cloughesy TF, Mischel PS. Striking the balance between PTEN and PDK1: It all depends on the cell context. Genes Dev 2009; 23:1699–704 https://doi.org/10.1101/gad.1832909 PMID: 19651981

64. Zhou X, Cordon-Barris L, Zuravlev T, Bayascas JR. Fine-tuning the intensity of the PKB/Akt signal enables diverse physiological responses. Cell Cycle. 2014; 13(20):3164–8. https://doi.org/10.4161/15384101.2014.962954 PMID: 25485494