

Original Article

Early A β 42 Exposure Causes Learning Impairment in Later Life

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ABSTRACT: Amyloid cascade hypothesis proposes that amyloid β (A β) accumulation is the initiator and major contributor to the development of Alzheimer's disease (AD). However, this hypothesis has recently been challenged by clinical studies showing that reduction of A β accumulation in the brain does not accompany with cognitive improvement, suggesting that therapeutically targeting A β in the brain may not be sufficient for restoring cognitive function. Since the molecular mechanism underlying the progressive development of cognitive impairment after A β clearance is largely unknown, the reason of why there is no behavioral improvement after A β clearance remains elusive. In the current study, we demonstrated that transient A β expression caused learning deficit in later life, despite the accumulated A β was soon being removed after the expression. Early A β exposure decreased the cellular expression of XBP1 and both the antioxidants, catalase, and dPrx5, which made cells more vulnerable to oxidative stress in later life. Early induction of XBP1, catalase, and dPrx5 prevented the overproduction of ROS, improved the learning performance, and preserved the viability of cells in the later life with the early A β induction. Treating the early A β exposed flies with antioxidants such as vitamin E, melatonin and lipoic acid, after the removal of A β also preserved the learning ability in later life. Taken together, we demonstrated that early and transient A β exposure can have a profound impact on animal behavior in later life and also revealed the cellular and molecular mechanism underlying the development of learning impairment by the early and transient A β exposure.

Key words: A β , learning and memory, XBP1, oxidative stress, aging

It is long believed that amyloid β (A β) peptide accumulation induces memory loss. The A β hypothesis proposes that A β accumulation in the brain initiates a series of signaling cascade, which triggers neuronal dysfunction and cell death. However, this hypothesis has recently been challenged by findings showing that reduced accumulation of A β in the brain does not produce significant improvement on cognitive function in patients with AD [1, 2], raising a question on the role of A β accumulation in the development of this disease, and also suggesting that an unidentified risk factor that can affect memory performance, during or after A β clearance, may be present. While the cause of memory

dysfunction after the clearance of A β remains elusive, the pathologies between early A β accumulation and post-A β clearance are also largely unknown.

Traumatic brain injury (TBI) has been proposed as one of the risk factors for AD [3, 4]. Early TBI experience increases the chance of developing dementia in later life [5]. In addition, it has been shown that TBI increases acute A β accumulation in the region of injury, suggesting a possible cause-effect relationship between the early A β accumulation and the late dementia occurrence [6, 7]. Interestingly, the increased A β accumulation after brain injury is not always elevated, in the PDAPP mice, familial AD mutation V \rightarrow F at amyloid

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precursor protein (APP) position 717, there is increased A β 42 2 hours after brain trauma but the level returns to the basal level 6 hours after injury [8]. Studies from TBI patients has shown that the accumulation of A β 42 reached the peak within one week after injury and then gradually decreased to the levels that are similar to the basal levels of the control group [9]. Furthermore, different results have been reported in a study examining the association between amyloid burden and hippocampal atrophy after TBI. Increased hippocampal atrophy, but with reduced amyloid burden, were observed in the PDAPP mice after injury [10]. Declined CSF A β concentration has also been reported to be correlated with the worsened clinical status in TBI patients [11]. Therefore, even though the molecular mechanism underlying these differential results remains unclear, these findings suggest that transient and short exposure of A β accumulation as an early event may trigger pathological changes in later life.

Accumulating evidence has suggested a role of ER stress in the development of AD [12]. Activation of protein kinase RNA-like ER kinase (PERK) and X-box binding protein 1 (XBP1) has been found in AD patients, as compared to the nondemented people [13]. Increased XBP1 splicing reverses the spatial memory in the AD mice [14], prevents the death of neurons, and extends the life-span of the A β 42 flies [15, 16]. Interestingly, studies showed that XBP1 is transiently increased in the early stage of learning and memory impairment in the APP/presenilin 1 (PS1) and 5x*FAD* mice [17], A β transgenic flies [16], and human AD patients [17]. Despite activation of XBP1 has been considered as a protection for cells and early induction of XBP1 could be a cell protective response against A β toxicity, chronic stresses promote the reduction of XBP1 activity [12].

Oxidative stress is known to play a role in the development of numerous diseases including AD [18, 19]. Increased ROS production or accumulation has been shown to trigger various molecular and cellular abnormalities such as DNA oxidation, lipid oxidation, and mitochondria dysfunction [19, 20]. Early accumulation of oxidative stress is observed in the early stage of AD and mild cognitive impairment (MCI) [21, 22]. Early accumulation of ROS during oligomer formation before plaque and tangle formation could also be found in the triple transgenic mice harboring PS1(M146V), APP(Swe), and tau(P301L) transgenes [23]. Behavioral recovery experiments showed that reducing oxidative stress in the brain, through both genetic and pharmacological approaches, improves the cognitive performance in the AD animal model [24, 25]. However, despite growing amount of evidence has suggested a relationship between the A β accumulation and the oxidative stress induction, the underlying mechanism remains elusive.

It is believed that the molecular mechanism and the process of memory formation are highly conserved among different species [26]. The similarity of pathological phenotypes in between the A β 42 flies and mammals suggests that the A β toxicity may also be preserved in the A β 42 flies [16, 27, 28]. In the current study, we showed that early and transient accumulation of A β affected later learning and memory performance in flies. Transient A β accumulation in the early stage decreased the expression of ER response and antioxidant genes and increased the levels of ROS in later life. Importantly, antioxidant treatments lowered the ROS levels and prevented the development of the learning and memory impairment in flies. Our data, for the first time, not only reveals the pathological role of the early and transient A β accumulation in the development of learning and memory impairment but also partially validates the A β hypothesis. Besides developing novel A β detector or finding biomarkers to indicate the early status of disease, this study offers a new alternative way to investigate the medical intervention for the future disease treatment, a combination of current A β clearance strategy and antioxidant application.

MATERIALS AND METHODS

Fly Stocks

All flies were raised on standard food at 18°C after crossed, then transferred to 30°C until adult. Besides, all flies were stored under normal circadian cycle (12:12 light dark cycle).

Elav-Gal4 were used as pan-neuronal driver for all experiments. Following fly stocks were come from the Bloomington Drosophila Stock Center (BDSC): Tublin-Gal80^{TS} (BDSC 7017), UAS-dFOXO (BDSC 42221), UAS-Rac1 DN (BDSC 6292), UAS-EGFR DN (BDSC 5364), UAS-JNK DN (BDSC 9311), and UAS-Catalase (BDSC 24621). Elav-gal4, UAS-A β 42 were gifts from Dr. Yi Zhong at Tsinghua University, China. UAS-XBP1d08698 was a gift from Dr. Pedro Fernandez-Funez at the University of Texas Medical Branch, USA. UAS-GADD34 was a gift from Dr. Stefan Marciniak, at the University of Cambridge, UK. UAS-dPrx5 was a gift from Dr. William C. Orr, at the Southern Methodist University, USA.

Pavlovian Olfactory Aversive Conditioning

All experiments were done in a 27°C room with 70% relative humidity as described previously [29, 30]. During training process, about 100 flies were put in training tube containing electric path where two aversive odors, 3-octanol (OCT, Sigma-Aldrich) and 4-methylcyclohexanol

(MCH, Sigma-Aldrich), were given to flies. The odor concentrations were adjusted to avoid bias by daily condition. The flies first exposed to the odor pairing with electric shock (12 pulses of 90 V per 1 minute) for 1 minute. The odor pairing with electric shock was called CS+ odor. Then, through 45 seconds interval of fresh air, flies exposed to the second odor without electric shock. The odor without electric shock was called CS- odor. One training process included one CS+ and one CS- odor exposure.

During test process, for immediate memory measurement, we immediately send the trained flies into a T-maze where flies could choose between CS+ and CS- for 2 minutes. The measurement of immediate memory also referred to as learning. For short term memory (STM) measurement, flies were placed in the same food containing vial they had been kept in before training process until the test. Two hours after training, flies were examined for STM. The distribution of flies in the two T-maze arms is referred to as performance index (PI) [29]. A reciprocal group of flies was trained and tested by using OCT as the CS+ and MCH as the CS+, respectively. The data were finally averaged for one data ($n = 1$) and multiplied by 100. The wonderful learning was performed as PI of 100, indicating all flies avoided the CS+ odor. However, a PI of 0 indicated the equal distribution of two odors, referring to no memory retention. Control groups were age-matched to the experimental groups in each test.

Western Blot Analysis

Five fly heads were homogenized in SDS sample buffer (LC2676, Invitrogen). Lysates were centrifuged. The resulting supernatant was collected, and proteins were resolved on 15% Tris-tricine gels. The protein samples were transferred to nitrocellulose membranes with 0.22 μ m pore size (Pall Corporation). The membranes were blocked with 5% nonfat dry milk and incubated with anti- β -Amyloid (1:2000, #8243 CST), anti-GABARAP (1:2000, #13733 CST), anti-Ubiquitin (1:2000, #3936 CST), anti-Ref2P (1:2000, ab178440 Abcam) and anti-GAPDH (1:50000, GTX100118 GeneTex) primary antibodies in TBST at 4°C overnight. The rabbit horseradish peroxidase-conjugated secondary antibody was used, and the signal was detected by chemiluminescence. The signal intensity was quantified by Image J software.

Survival Assay

Flies were collected under light CO₂ anesthesia and divided into vials containing regular food. Flies were passed onto fresh food vials every 3–4 days and the number of dead flies were recorded. The experiments

were placed in 30°C, with 70% humidity, on a 12-h light/dark cycle and performed at least twice. All results were analyzed from experiments carried out using female flies.

Quantitative RT-PCR

Trizol was used to isolate total RNA from 20 fly heads. Then, RevertAid RT Reverse Transcription Kit (K1691, Thermo Scientific) was used to eliminate DNA traces. Finally, One Step RT-PCR (Applied biosystems) was used to prepare cDNAs. For detection of *Drosophila XBP1s*, the primers dXBP1s550F 5'-ACCAACCTTGGATCTGCCG-3' and dXBP1s697R 5'-CGCCAAGCATGTCTTGTAGA-3' were used to amplify a 140 bp fragment as previously described [15]. For quantification of the expression of *XBP1* in *Drosophila*, the primers XBP1t-F 5'-TGGGAGGAGAAAGTGCAAAG-3' and XBP1t-R 5'-TCCGTTCGTCTGTCAGCTC-3' were used to amplify 125 bp fragment in the 5' exon [15]. For detection of *dPrx5*, the primers dPrx5-F 5'-AGAGAGGAGAAATGCGTGTG-3' and dPrx5-R 5'-CACTGGTTTTGGACAGGGAT-3' were used to amplify a 105 bp fragment. For quantification of the expression of *catalase* in *Drosophila*, the primers Cat-F 5'-CTCTGATTCTGTGGCAAA-3' and Cat-R 5'-AGTAGGAGAACAGACGACCA-3' were used to amplify a 150 bp fragment. As internal control, the primers for *dRpl32*: dRpl32-F 5'-AATCCTCGTTGGCACTCACC-3' and dRpl32-R 5'-TGTTGTGTCCTTCCAGCTTCA-3' were used to amplify a fragment of 135 bp. For qPCR with the SYBR Select Master Mix (#4472913, Applied biosystems) and StepOnePlus™ Real-Time PCR System (Thermo Fisher), we used 10 ng of purified RNA. Cycling conditions were set holding state at 50°C for 2 min, 95°C for 2 min and 40 cycles of 95°C for 15 s, 56°C for 1 min and 72°C for 1 min. The levels of RNA were calculated using the StepOne Software v2.3 (Thermo Fisher), which relied on the comparative Ct method of quantification. All mRNA levels were normalized with *dRpl32*.

Propidium iodide staining

Fly brains were quickly dissected in PBS and fixed in 4% paraformaldehyde in PBS overnight. Then, after vacuumed 3 times for 20 minutes each, the brains were permeabilized with 2% TritonX-100 in PBS overnight. Next, fly brains were incubated with 1:200 propidium iodide in PBS at 4 °C overnight. The brains were washed with PBS 3 times for 20 minutes and subsequently mounted between two glass coverslips in focusclear™, and imaged on a FluoView FV1000 confocal microscope.

Antioxidant Feeding

Lipoic acid (T5625, Sigma-Aldrich) and melatonin (M5250, Sigma-Aldrich) were diluted in ethanol and mixed with the regular food to make the final concentration of 2 mM and 100 μ g/ml respectively. Vitamin E (#258024, Sigma-Aldrich) was made in the final concentration of 20 μ g/ml. All flies were transferred to fresh tubes every 3-4 days to avoid food desiccation and compound degradation.

Starving stress resistance

To trigger starving stress, flies were placed in the vials containing with filter paper moistened with water. The papers were replaced every 12 hours. The survival of flies was recorded every several hours and kept under stress condition until whole flies died. Each experiment was performed at least twice.

Oxidative stress resistance

To trigger oxidative stress, flies were kept on filter paper soaked with 40 mM Methyl Viologen (Paraquet, #856177, Sigma-Aldrich) in 4% sucrose solution in the vials. The papers were replaced every 12 hours. The survival of flies was recorded every several hours and kept under stress condition until whole flies died. Each experiment was performed at least twice.

H2DCF staining

2',7'-dichlorofluorescein (H2DCF, D6883, Sigma-Aldrich) was used to detect ROS following previously described protocols [31]. Briefly, fly heads were dissected in PBS, then placed in 10 μ M H2DCF for 15 minutes at room temperature. Before mounting, samples were washed three times for 5 minutes each in PBS at room temperature on a shaker. Brains were mounted between two glass coverslips in focusclearTM and imaged on a FluoView FV1000 confocal microscope.

Proteasome Activity Assay

Proteasome activity was measured using a Proteasome Activity Fluorometric Assay Kit (K245, Biovision). According to the manufacturer instruction, five fly heads were homogenized in 0.5 % NP-40 in dH₂O and suspended in assay buffer. Then, samples were incubated with an AMC-tagged peptide substrate for 30 minutes at 37° C to produce fluorophore. Finally, the fluorescence was measured by using an ELISA plate reader (SpectraMax iD3 Multi-Mode Microplate Readers,

Molecular Devices) at Ex/Em = 350/440 nm in kinetics mode at 37°C for 30-60 minutes.

Image quantification

We used FV1000 analysis software and Image J software to analyze all the images. Statistical analysis was performed on samples from three or more brains.

For cell loss measurement, the quantified vacuolated areas within cell body region were divided by total area of cell body region around calyx. The final data was presented as the percentage of cell loss. To prevent the bias, we used double blind to analysis our data.

For H2DCF staining, we stacked all frames and used the mean of the summed H2DCF intensities averaged from whole brain for statistical analysis. Three brain intensities within one experiment were finally averaged for one data (n = 1) and we analyzed at least three experiments.

Statistics

All data were analyzed using Graphpad Prism 6.0 software. Comparisons of multiple groups used one-way ANOVA followed by Tukey's multiple comparison test. Comparisons between two groups used two-tailed t-test. Statistical significance was shown with *P* value <0.05. Statistical results were presented as means \pm SEM.

RESULTS

Reduction of A β accumulation temporary reverses A β -induced behavioral damages

We employed conditional expression system, Gal80^{ts} [32], to temporary regulate the A β 42 expression. Gal80^{ts} is the temperature-sensitive version of Gal80. Unless mentioned otherwise, all the experiments were done with using *Elav-Gal4* promoter to induce A β expression in neurons (Fig. 1A). We demonstrated in a previous study that memory deficit can be observed 3 days after A β expression in the adult A β 42 flies [16]. To prove that the accumulated A β (3 days post-induction) can be removed in the brain of fly; we transferred the flies from the restrictive temperature, 30°C, to the permissive temperature, 18°C, to terminate the A β expression process and were subsequently housed under the permissive temperature for 21 days (*i.e.*, 3_{30°C}-21_{18°C} A β 42 flies), we examined the A β level in the brain of 3_{30°C}-21_{18°C} A β 42 flies. As shown in Fig. 1B, the amount A β accumulated in the brain of 3_{30°C}-21_{18°C} A β 42 flies was decreased, as compared to 3_{30°C} A β 42 flies (*i.e.*, only with 3 days induction) (*P* < 0.0001, unpaired Two-tailed T-test). Flies with 45 days post-housing under the permissive

temperature after 3 days of A β induction (*i.e.*, 3_{30°C}-45_{18°C} A β 42 flies) showed very few A β left in the brain (Fig. 1C; $P < 0.0001$, unpaired Two-tailed T-test). Notably, A β flies only housed under the permissive temperature for 48 days had no A β expression, confirming that the UAS-leakage

under the permissive temperature is negligible (Supplementary Fig. 1). Taken together, these results confirmed that induced and accumulated A β can be removed after the induction process being terminated in A β 42 flies.

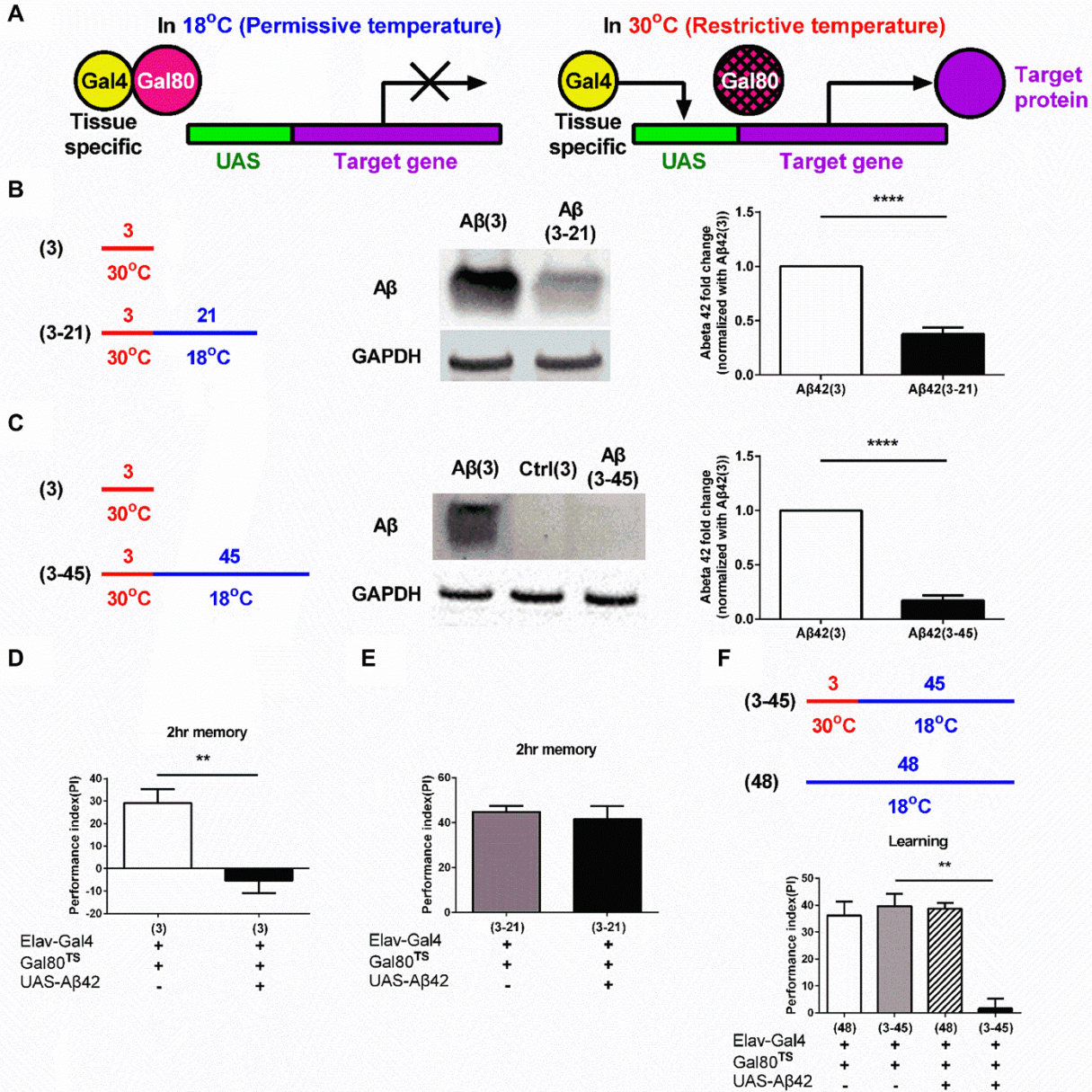


Figure 1. The A β -induced learning and memory deficits are temporally improved by A β clearance. (A) Under the permissive temperature, Gal80^{TS} protein binds to Gal4 protein to repress the transcriptional activity of GAL4; thus, preventing the expression of the target protein. Under the restrictive temperature, the conformation of Gal80^{TS} protein is changed, releasing, and allowing the GAL4 protein to drive the expression of the target protein. (B) The A β level in the brain of 3_{30°C}-21_{18°C} flies was reduced, as compared to 3_{30°C} flies. Results of the respective quantitative analysis is shown on the right, N = 8 respectively. **** $p < 0.0001$. (C) The A β level in the brain of 3_{30°C}-45_{18°C} flies was almost undetectable. The quantitative data is shown on the right, N = 4 respectively. **** $p < 0.0001$. (D) Overexpression of A β for 3 days induced memory deficit, N = 6 respectively. ** $p < 0.01$. (E) The memory deficit in 3_{30°C} flies was reversed in 3_{30°C}-21_{18°C} flies, N = 6 respectively. (F) Learning impairment was reoccurred in 3_{30°C}-45_{18°C} A β flies (Ctrl (3-45) vs. A β 42 (3-45); $p = 0.0041$, N = 6 respectively). ** $p < 0.01$. All Ctrl represents Elav-Gal4+Gal80^{TS}.

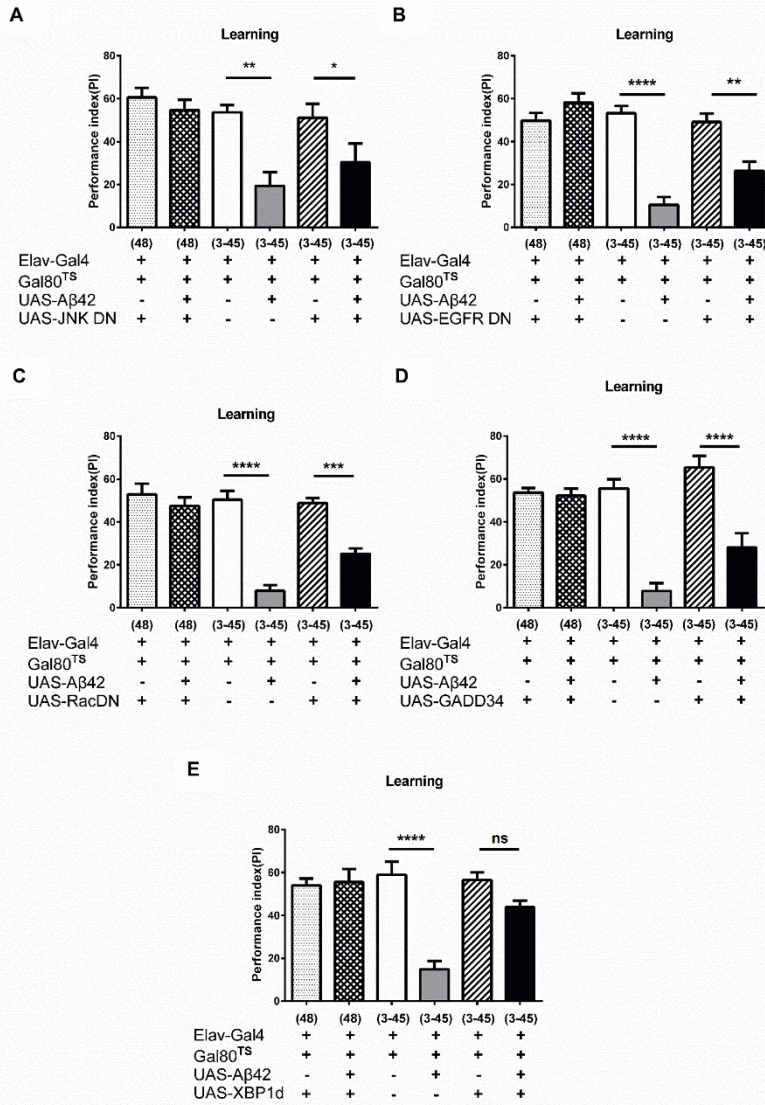


Figure 2. Early XBP1 overexpression prevents the early-transient $A\beta$ accumulation induced learning impairment in later life. (A) Early JNK DN overexpression did not prevent the learning impairment developed in 33°C - 45/18°C $A\beta$ flies (Ctrl (3-45) vs. $A\beta$ 42 (3-45): $p = 0.0043$ respectively; $A\beta$ 42 (3-45) vs. $A\beta$ 42+JNK DN (3-45): $p = 0.7867$, $N = 6$ respectively). ** $p < 0.01$. (B) Learning impairment was partially improved by early EGFR DN overexpression in 33°C - 45/18°C $A\beta$ flies (Ctrl (3-45) vs. $A\beta$ 42 (3-45): $p < 0.0001$ respectively; Ctrl+EGFR DN (3-45) vs. $A\beta$ 42+EGFR DN (3-45): $p = 0.0012$ respectively; $A\beta$ 42 (3-45) vs. $A\beta$ 42+EGFR DN (3-45): $p = 0.0030$, $N = 6$ respectively). ** $p < 0.01$, **** $p < 0.0001$. (C) Learning impairment was partially improved by Rac DN overexpression in 33°C - 45/18°C $A\beta$ flies (Ctrl (3-45) vs. $A\beta$ 42 (3-45): $p < 0.0001$ respectively; Ctrl+Rac DN (3-45) vs. $A\beta$ 42+Rac DN (3-45): $p = 0.0007$ respectively; $A\beta$ 42 (3-45) vs. $A\beta$ 42+Rac DN (3-45): $p = 0.0197$, $N = 6$ respectively). *** $p < 0.001$, **** $p < 0.0001$. (D) Learning impairment was partially improved by GADD34 overexpression in 33°C - 45/18°C $A\beta$ flies (Ctrl (3-45) vs. $A\beta$ 42 (3-45): $p < 0.0001$ respectively; GADD34 (3-45) vs. $A\beta$ 42+GADD34 (3-45): $p < 0.0001$ respectively; $A\beta$ 42 (3-45) vs. $A\beta$ 42+GADD34 (3-45): $p = 0.0349$, $N = 6$ respectively). * $p < 0.05$, **** $p < 0.0001$. (E) Learning impairment was fully improved by XBP1 overexpression in 33°C - 45/18°C $A\beta$ flies (Ctrl (3-45) vs. $A\beta$ 42 (3-45): $p < 0.0001$ respectively; Ctrl+XBP1 (3-45) vs. $A\beta$ 42+XBP1 (3-45): $p = 0.3710$ respectively; $A\beta$ 42 (3-45) vs. $A\beta$ 42+XBP1 (3-45): $p = 0.0010$, $N = 6$ respectively). ** $p < 0.01$, **** $p < 0.0001$. ns represents non-significant. All Ctrl represents Elav-Gal4+Gal80^{TS}.

The memory performance of 33°C-21/18°C $A\beta$ 42 flies was examined. As shown in Fig. 1D ($P = 0.0020$, unpaired Two-tailed T-test) and 1e ($P = 0.6168$, unpaired Two-tailed T-test), $A\beta$ expression-induced memory damage in 33°C $A\beta$ 42 flies was recovered in 33°C-21/18°C $A\beta$ flies. We also examined the memory performance of 33°C-3/18°C $A\beta$ 42 flies. $A\beta$ 42 flies kept in the 18°C after 3 days in the 30°C displayed damaged memory performance (Supplementary Fig. 2; $P = 0.0001$, unpaired Two-tailed T-test). The results confirmed that the recovery effect of memory damage in 33°C-21/18°C $A\beta$ flies was due to $A\beta$ removal. However, the recovery of behavioral performance was only temporary in the flies. Despite 33°C-30/18°C $A\beta$ 42 flies (*i.e.* flies being housed under the permissive temperature for 30 days after the termination

of $A\beta$ induction) did not show any learning difficulties (Supplementary Fig. 3; $P = 0.6066$, $F(3, 12) = 0.6350$, One-way ANOVA), 33°C-45/18°C $A\beta$ 42 flies (*i.e.* flies being housed under the permissive temperature for 45 days after the termination of $A\beta$ induction) exhibited noticeable learning deficit (Fig. 1F; $P = 0.4877$, $F(3, 15) = 0.9324$, One-way ANOVA). However, the pain sensation and olfactory function of 33°C-45/18°C $A\beta$ 42 flies were still retained (Supplementary Fig. 4; Left: $P = 0.1622$, unpaired Two-tailed T-test; Right: $P = 0.6257$, unpaired Two-tailed T-test). These results suggest that although the accumulated $A\beta$ in the brain of fly can be cleared after the induction of $A\beta$ being terminated, memory recovery is only temporary and behavioral impairment can be reoccurred in later life.

Early induction of XBP1 prevents later life learning deficit

To identify molecules or pathways that are responsible for the development of learning deficit in later life, we examined the importance of various molecules that had been shown to be participated in the A β -induced learning and memory damage in the past. Increased inflammatory response in the brain has been suggested as one of the major factors affecting neuronal functions and behavioral performance in AD patients [33]. Here, although 8 days overexpression of the dominant-negative K53R c-Jun N-terminal kinase (JNK DN) recovered A β -induced learning deficit in A β flies (*i.e.* 830°C A β flies, 3 days overexpression of JNK DN did not recover the A β -induced learning deficit in A β flies (*i.e.* 330°C-4518°C A β 42 flies) (Supplementary Fig. 5; $P=0.0004$, $F(3, 23) = 9.016$, One-way ANOVA and Fig. 2A; $P=0.0001$, $F(5, 30) = 72.82$, One-way ANOVA). Previous studies showed that downregulation of EGFR and its downstream signaling pathway reverses learning and memory deficit in A β flies [27, 28]. However, in the current study, early overexpression of the dominant-negative EGFR (EGFR DN) and Rac1 dominant-negative mutant, N17, Rac DN did not show any prominent recovery effect in 330°C-4518°C A β 42 flies (Fig. 2B; $P < 0.0001$, $F(5, 30) = 22.80$, One-way ANOVA and 2C; $P < 0.0001$, $F(5, 30) = 25.93$, One-way ANOVA). ER stress has been suggested to play an important role in mediating A β -induced pathology [16]. Antagonizing the PERK pathway by overexpression GADD34 has been shown to recover A β -induced learning deficit [16]. Here, although the ability of learning was improved after early expression of GADD34 in 330°C-4518°C A β 42 flies, the performance recovery level was not as good as that of the control flies (Fig. 2D; $P < 0.0001$, $F(5, 24) = 23.57$, One-way ANOVA). XBP1 has been shown to play an important role in maintaining the neuronal cell viability of A β flies [15, 16]. Here, we found the learning performance was recovered in 330°C-4518°C A β 42 (Fig. 2E; $P < 0.0001$, $F(5, 30) = 28.05$, One-way ANOVA). Altogether, these behavioral results suggest that the A β 42-induced memory impairment is reversible, and the molecular mechanism involved in 330°C-4518°C A β 42-induced learning impairment is different from the learning damage induced by the continuous A β accumulation.

Increased oxidative stress in 330°C-4518°C A β flies induces learning impairment

To determine the XBP1 expression level in 330°C-4518°C A β flies, quantitative PCR was used. The results showed that expression of both the total and the splicing form of XBP1 was reduced in 330°C-4518°C A β flies, as compared

to the age-matched control flies (Fig. 3A; Left: XBP1s: $P < 0.0001$, unpaired Two-tailed T-test; XBP1t: $P < 0.0001$, unpaired Two-tailed T-test; Right: $P = 0.9524$, unpaired Two-tailed T-test). Although the reduction of PERK and ATF6 was also found in 330°C-4518°C A β flies (Supplementary Fig. 6; PEK: $P < 0.0001$, unpaired Two-tailed T-test; CRC: $P < 0.0001$, unpaired Two-tailed T-test; ATF6: $P < 0.0001$, unpaired Two-tailed T-test); however, considering the behavioral results as shown in Fig. 2D and E, we believed that reduction of XBP1 is one of the major causes of the induction of learning impairment in 330°C-4518°C A β flies. XBP1 regulates numerous cellular functions such as proteasome activity, autophagy and oxidative stress [18, 34, 35]. Western blot analysis was used to examine the protein ubiquitination and autophagy level in the brain of 330°C-4518°C A β flies. As shown in Fig. 3B ($P = 0.7148$, unpaired Two-tailed T-test), the overall protein ubiquitination level remained similar in the brain of 330°C-4518°C A β flies, as compared to the control flies. We also used proteasome activity assay to confirm that the proteasome activity of 330°C-4518°C A β flies remained similar to the control flies (Fig. 3C; $P = 0.5958$, unpaired Two-tailed T-test). Despite increased of Ref(2)p, a homolog of mammalian p62/SQSTM1, was found in the brain of 330°C-4518°C A β flies, the expression ratio of GABARAP II/I remained similar compared to the control flies (Fig. 3C; $P = 0.0336$, unpaired Two-tailed T-test and 3d; $P = 0.1141$, unpaired Two-tailed T-test). In addition, feeding rapamycin, which is a widely used autophagy inducer, for 4 weeks did not affect the learning performance of 330°C-4518°C A β flies (Fig. 3E; $P < 0.0001$, $F(3, 20) = 54.22$, One-way ANOVA). Co-induction of Drosophila FOXO (dFOXO) with A β for 3 days did not affect the learning performance of 330°C-4518°C A β flies (Supplementary Fig. 7; $P < 0.0001$, $F(5, 30) = 28.05$, One-way ANOVA). These results suggest that autophagy is not involved in the development of learning deficit of 330°C-4518°C A β flies. To examine the role of ROS in 330°C-4518°C A β flies, flies were fed with three different types of antioxidants, vitamin E, melatonin (M) or lipoic acid (L), for 4 weeks prior to the behavioral analysis. Results showed that antioxidants treatment prevented the development of learning deficit in 330°C-4518°C A β flies (Fig. 3F; Left: $P < 0.0001$, $F(3, 20) = 15.08$; Right: $P < 0.0001$, $F(5, 30) = 33.87$, One-way ANOVA). 2',7'-dichlorodihydrofluorescein (H2DCF, an indicator for ROS) staining further confirmed that 330°C-4518°C A β flies have higher intracellular ROS accumulation than to the control flies (Fig. 3G; Right Top: $P = 0.0827$, $F(3, 8) = 3.218$; Right bottom: $P = 0.0146$, $F(3, 8) = 6.632$, One-way ANOVA). These results suggest that increased ROS level plays an important role in the development of learning deficit in 330°C-4518°C A β flies.

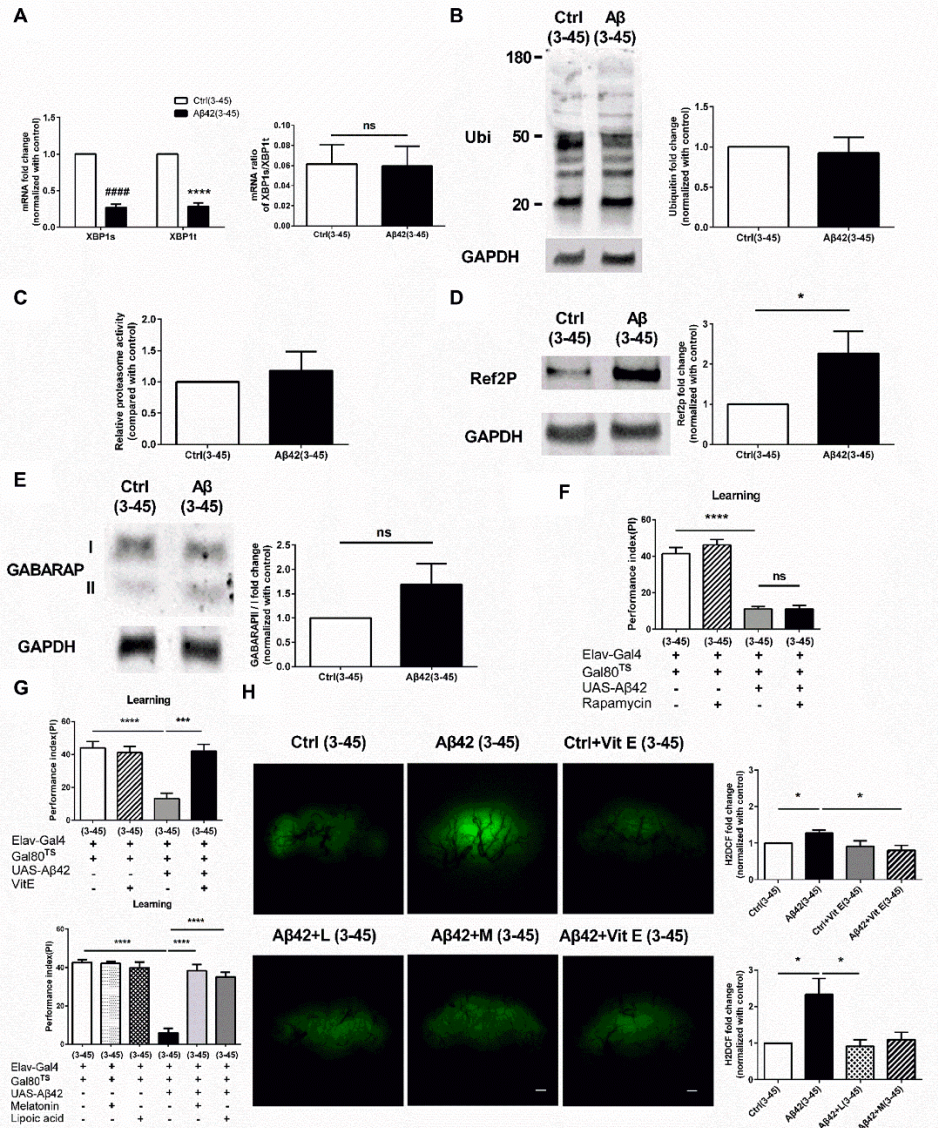


Figure 3. Dysregulation of the oxidative stress response causes learning impairment in 30°C -45°C $A\beta$ flies. (A) Both XBP1t and XBP1s mRNA levels were reduced in the brain of 30°C -45°C $A\beta$ flies, but the ratio of XBP1s/XBP1t remained similar, N = 3 respectively. **** p < 0.0001 in XBP1t, #### p < 0.0001 in XBP1s. (B) The level of protein ubiquitination was not changed in the brain of 30°C -45°C $A\beta$ flies. The quantitative data is shown on the right, N = 7 respectively. (C) The proteasome activity in brain of 30°C -45°C $A\beta$ flies, N = 3 respectively. (D) The amount of Ref2P was increased in the brain of 30°C -45°C $A\beta$ flies. The quantitative data is shown on the right, N = 12 respectively. * p < 0.05. (E) The GABARAP II/I ratio was not changed in the brain of 30°C -45°C $A\beta$ flies. The quantitative data is shown on the right, N = 15 respectively. (F) Rapamycin treatment for 4 weeks could not improve the learning impairment in 30°C -45°C $A\beta$ flies. (Ctrl (3-45) vs. $A\beta$ 42 (3-45): p < 0.0001 respectively; $A\beta$ 42 (3-45) vs. $A\beta$ 42+Rapamycin (3-45): p > 0.9999, N = 6 respectively). **** p < 0.0001. (G) Vitamin E, melatonin (M) or lipoic acid (L) treatment for 4 weeks improved the learning impairment in 30°C -45°C $A\beta$ flies. (Left: Ctrl (3-45) vs. $A\beta$ 42 (3-45): p < 0.0001; $A\beta$ 42 (3-45) vs. $A\beta$ 42+Vit. E (3-45): p = 0.0002, respectively, n = 6; Right: Ctrl (3-45) vs. $A\beta$ 42 (3-45): p < 0.0001; $A\beta$ 42 (3-45) vs. $A\beta$ 42+L (3-45): p < 0.0001; $A\beta$ 42 (3-45) vs. $A\beta$ 42+M (3-45): p < 0.0001, N = 6 respectively). *** p < 0.001, **** p < 0.0001. (H) Vitamin E, melatonin (M) or lipoic acid (L) treatment for 4 weeks decreased the levels of ROS in 30°C -45°C $A\beta$ flies. The quantitative data is shown on the right (Right Top: Ctrl (3-45) vs. $A\beta$ 42 (3-45): p = 0.0270; $A\beta$ 42 (3-45) vs. $A\beta$ 42+Vit. E (3-45): p = 0.0438; Right bottom: Ctrl (3-45) vs. $A\beta$ 42 (3-45): p = 0.0389; $A\beta$ 42 (3-45) vs. $A\beta$ 42+L (3-45): p = 0.0201; $A\beta$ 42 (3-45) vs. $A\beta$ 42+M (3-45): p = 0.0391, N = 3 respectively). * p < 0.05. ns represents non-significant. All Ctrl represents Elav-Gal4+Gal80^{TS}. White bar indicates 50 μ m.

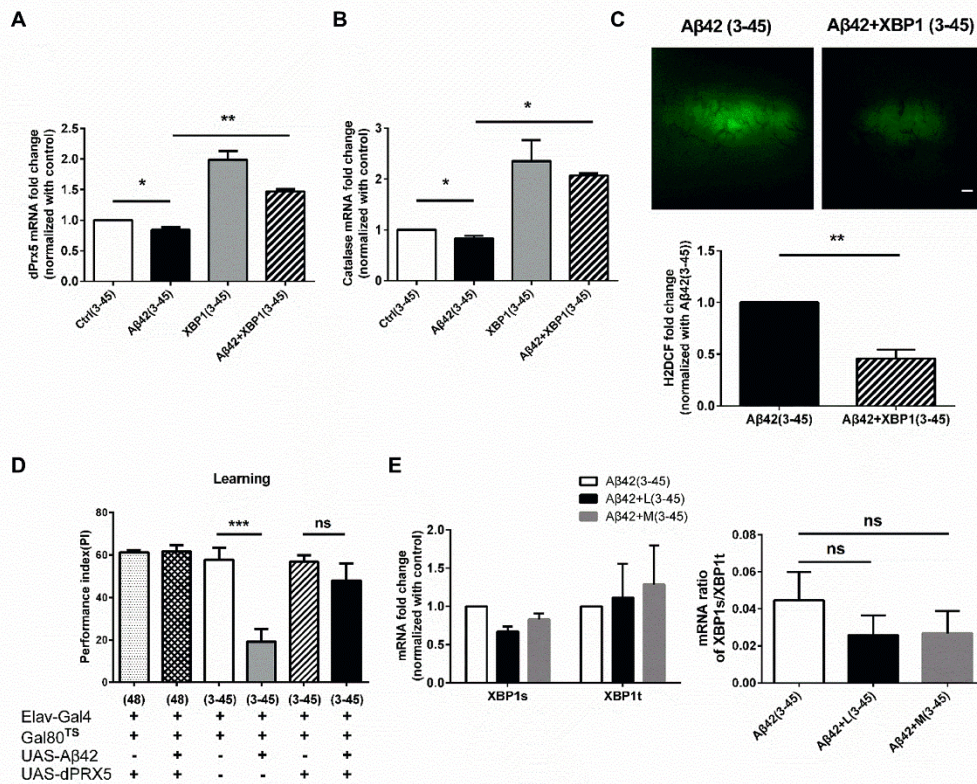


Figure 4. Early XBP1 expression increases the expression of catalase and dPrx5, reduces oxidative stress, and improves learning performance in 30°C -45°C $A\beta$ flies. (A) The reduction of *dPrx5* mRNA in 30°C -45°C $A\beta$ flies was prevented by early XBP1 overexpression (Ctrl (3-45) vs. A β 42 (3-45): $p = 0.0483$; A β 42 (3-45) vs. A β 42+XBP1 (3-45): $p = 0.0017$, $N = 3$ respectively). * $p < 0.05$, ** $p < 0.01$. (B) The reduction of *catalase* mRNA in 30°C -45°C $A\beta$ flies was prevented by early XBP1 overexpression (Ctrl (3-45) vs. A β 42 (3-45): $p = 0.0476$; A β 42 (3-45) vs. A β 42+XBP1 (3-45): $p = 0.0135$, $N = 3$ respectively). * $p < 0.05$. (C) Early XBP1 overexpression for 3 days prevented the oxidative stress elevation in 30°C -45°C $A\beta$ flies. The quantitative data is shown at the bottom, $N = 3$ respectively. ** $p < 0.01$. ns represents non-significant. (D) Learning impairment in 30°C -45°C $A\beta$ flies was fully improved by early dPrx5 overexpression. (Ctrl (3-45) vs. A β 42 (3-45): $p = 0.0002$; A β 42 (3-45) vs. A β 42+dPrx5 (3-45): $p = 0.0057$; Ctrl (3-45) vs. A β 42+dPrx5 (3-45): $p = 0.7417$, $N = 6$ respectively). ** $p < 0.01$, *** $p < 0.001$. (E) The XBP1s and XBP1t mRNA expression level and the ratio of XBP1s/XBP1t did not change in the brain of 30°C -45°C $A\beta$ flies with melatonin (M) or lipoic acid (L) co-treatment (Left: XBP1t: A β 42 (3-45) vs. A β 42+L (3-45): $p = 0.9889$; A β 42 (3-45) vs. A β 42+M (3-45): $p = 0.8599$; XBP1s: A β 42 (3-45) vs. A β 42+L (3-45): $p = 0.8040$; A β 42 (3-45) vs. A β 42+M (3-45): $p = 0.9639$; Right: A β 42 (3-45) vs. A β 42+L (3-45): $p = 0.6928$; A β 42 (3-45) vs. A β 42+M (3-45): $p = 0.7274$, $N = 3$ respectively). All Ctrl represents Elav-Gal4+Gal80^{TS}. White bar indicates 50 μ m.

To dissect the mechanisms underlying ROS upregulation in 30°C-45°C $A\beta$ flies, we examined the gene expression of various antioxidants. Results of the qPCR analysis showed that expression of dPrx5 and catalase was decreased in 30°C-45°C $A\beta$ flies (Fig. 4A; $P < 0.0001$, $F(3, 8) = 45.81$, One-way ANOVA and 4b; $P < 0.0001$, $F(3, 8) = 45.81$, One-way ANOVA). In addition, co-induction of XBP1 and $A\beta$ for 3 days increased the expression of dPrx5 and catalase in 30°C-45°C $A\beta$ flies, suggesting that early induction of XBP1 might prevent antioxidant reduction in later life. This hypothesis was supported by results showing that 3 days co-expression of XBP1 with $A\beta$ reduced the ROS accumulation in 30°C-45°C $A\beta$ flies (Fig. 4C; $P =$

0.0033, unpaired Two-tailed T-test). Accumulating evidence suggested that dPrx5 is one of the antioxidants in flies with abundant expression in the brain [36]. Behavioral study confirmed that 3 days co-expression of dPrx5 with $A\beta$ prevented the occurrence of learning damage in 30°C-45°C $A\beta$ flies (Fig. 4D; $P < 0.0001$, $F(5, 24) = 10.38$, One-way ANOVA). Further study showed that antioxidants, melatonin or lipoic acid, treatment did not increase the XBP1 expression in 30°C-45°C $A\beta$ flies, suggesting that decreased XBP1 expression cannot be reversed by antioxidant treatment (Fig. 4E; Left: XBP1t: $P = 0.8404$, $F(2, 8) = 0.1756$; XBP1s: $P = 0.8404$, $F(2, 6) = 0.1756$; Right: $P = 0.5292$, $F(2, 9) = 0.6836$, One-way ANOVA).

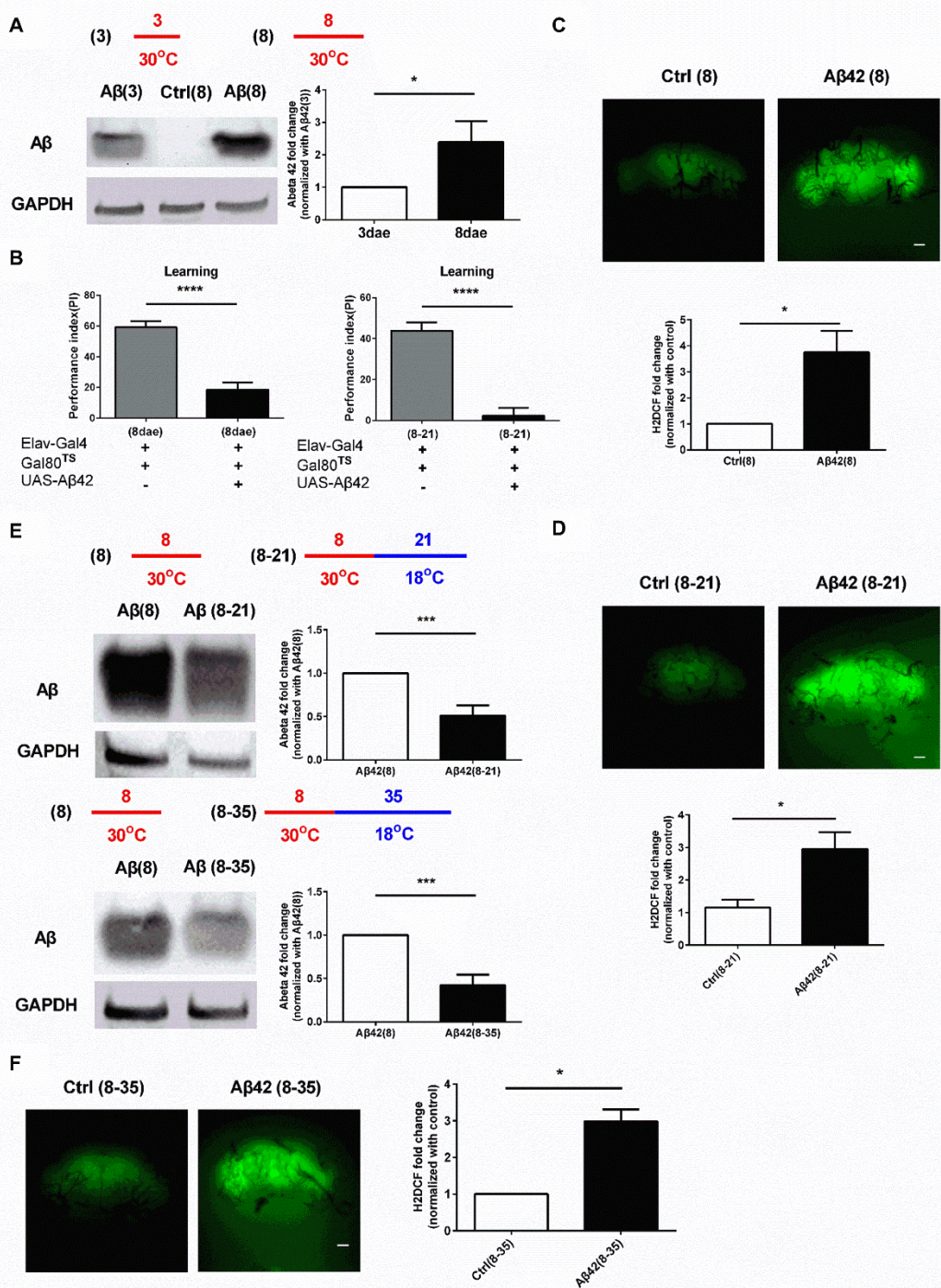


Figure 5. Prolonged A β induction causes learning impairment that cannot be reversed by reducing A β accumulation. (A) The level of A β accumulation in flies with 8 days induction was higher than that with 3 days induction, N = 3 respectively. * $p < 0.05$. (B) The learning impairment occurred in 830 $^{\circ}\text{C}$ A β flies was not improved in 830 $^{\circ}\text{C}$ -2118 $^{\circ}\text{C}$ A β flies, N = 6 respectively. **** $p < 0.0001$. (C) Increased ROS level was found in 830 $^{\circ}\text{C}$ flies. The quantitative data is shown at the bottom, N = 4 respectively. * $p < 0.05$. (D) Increased ROS level was also found in 830 $^{\circ}\text{C}$ -2118 $^{\circ}\text{C}$ and 830 $^{\circ}\text{C}$ -3518 $^{\circ}\text{C}$ A β flies. The quantitative data is shown on the right, N = 3 respectively. * $p < 0.05$. (E) The A β level in 830 $^{\circ}\text{C}$ -2118 $^{\circ}\text{C}$ and 830 $^{\circ}\text{C}$ -3518 $^{\circ}\text{C}$ A β flies was lowered by approximately 50% as compared to that in 830 $^{\circ}\text{C}$ flies. The quantitative data is shown on the right, N = 6 respectively. *** $p < 0.001$. (F) The ROS level in 830 $^{\circ}\text{C}$ -3518 $^{\circ}\text{C}$ A β flies was higher than that in the control flies. The quantitative data is shown at the bottom, N = 3 respectively. * $p < 0.05$. All Ctrl represents Elav-Gal4+Gal80^{TS}. White bar indicates 50 μm .

Prolonged A β induction-induced learning impairment and oxidative stress cannot be reversed by reducing A β accumulation

To understand if the prolonged A β induction-induced learning damage is reversible, we performed experiments with an extended A β induction period. Prolonged induction of A β induces learning deficit in flies [16, 27]. Here, flies with 8 days A β induction (*i.e.*, 8 $_{30}^{\circ}$ C A β flies) showed higher A β accumulation in the brain than flies with 3 days A β induction, as expected (Fig. 5A; $P = 0.0166$, unpaired Two-tailed T-test). However, similar levels of learning deficit were still observed in and between flies with prolonged A β induction (*i.e.*, 8 $_{30}^{\circ}$ C A β flies) only and those with the removal of A β after prolonged induction (8 $_{30}^{\circ}$ C-21 $_{18}^{\circ}$ C A β flies) (Fig. 5B; Right: $P < 0.0001$, unpaired Two-tailed T-test; Left: $P < 0.0001$, unpaired Two-tailed T-test). Results of the H2DCF staining revealed that the ROS levels remained high in both 8 $_{30}^{\circ}$ C A β flies and 8 $_{30}^{\circ}$ C-21 $_{18}^{\circ}$ C A β flies (Fig. 5C; $P = 0.0161$, unpaired Two-tailed T-test and 5d; $P = 0.0345$, unpaired Two-tailed T-test), suggesting that continuous accumulation of ROS (or fail to clear ROS) could play a role in learning damage in 8 $_{30}^{\circ}$ C-21 $_{18}^{\circ}$ C A β flies. Further examination in 8 $_{30}^{\circ}$ C-35 $_{18}^{\circ}$ C A β flies showed less than 10% of the accumulated A β was further reduced (the rest amount of A β in 8 $_{30}^{\circ}$ C-35 $_{18}^{\circ}$ C A β flies was 0.42 ± 0.15 v.s. 0.51 ± 0.17 in 8 $_{30}^{\circ}$ C-21 $_{18}^{\circ}$ C A β flies) and the levels of ROS of 8 $_{30}^{\circ}$ C-35 $_{18}^{\circ}$ C A β flies remain similar to 8 $_{30}^{\circ}$ C-21 $_{18}^{\circ}$ C A β flies (Fig. 5E; Top: $P = 0.0010$, unpaired Two-tailed T-test; Bottom: $P = 0.0009$, unpaired Two-tailed T-test and 5f; $P = 0.0273$, unpaired Two-tailed T-test). These results suggest that longer A β exposure might both increase A β accumulation to a level that cannot be fully cleared endogenously and induce ROS accumulation to a level that exceed the maximal ROS removal capacity of cells, leading to neuronal damage and learning impairment.

Early A β exposure makes cell more vulnerable to the oxidative stress in later life

As we found that early A β accumulation decreases XBP1 expression in 3 $_{30}^{\circ}$ C-45 $_{18}^{\circ}$ C A β flies, we hypothesized that reducing the expression of catalase and dPrx5 might make cells more vulnerable to the oxidative stress. Here, we sought to understand the XBP1 expression pattern and determine the ROS accumulation in different stage of A β flies. Overexpression of A β increased the expression of the spliced XBP1 (XBP1s) in 3 $_{30}^{\circ}$ C A β flies (Fig. 6A;

XBP1s: $P = 0.0061$, unpaired Two-tailed T-test; XBP1t: $P = 0.9859$, unpaired Two-tailed T-test), suggesting early A β accumulation promotes ER stress response. Results of the confocal microscopy showed that the levels of ROS were higher in 3 $_{30}^{\circ}$ C A β flies than to control flies (Fig. 6B; $P = 0.0161$, unpaired Two-tailed T-test). This result suggests that the presence of A β increases oxidative stress and causes ER stress activation to promote XBP1 splicing. This result was consistent with our previous report [16]. Interestingly, the expression of XBP1s was decreased back to the basal level and the levels of ROS in 3 $_{30}^{\circ}$ C-21 $_{18}^{\circ}$ C A β flies were similar to that in the control flies (Fig. 6C; XBP1s: $P = 0.9481$, unpaired Two-tailed T-test; XBP1t: $P = 0.8721$, unpaired Two-tailed T-test and 6d; $P = 0.0010$, unpaired Two-tailed T-test). However, the levels of ROS in 3 $_{30}^{\circ}$ C-45 $_{18}^{\circ}$ C A β flies were higher than that in control flies (Fig. 3G). Therefore, we hypothesized that decreased XBP1 and antioxidants in the aged A β flies make animal less resistant to the ROS stress. To confirm our hypothesis, we challenged the flies with two different conditions, starvation and paraquat challenge. Results from the starvation challenge experiment showed that the survival rate of 3 $_{30}^{\circ}$ C-45 $_{18}^{\circ}$ C A β flies was similar to the age-matched control flies (Fig. 6e; $P = 0.7994$, Log-rank test). Intriguingly, paraquat feeding reduced the life-span of 3 $_{30}^{\circ}$ C-45 $_{18}^{\circ}$ C A β flies, as compared to the control group (Fig. 6F; $P < 0.0001$, Log-rank test). These results confirmed that 3 $_{30}^{\circ}$ C-45 $_{18}^{\circ}$ C A β flies are highly vulnerable to the oxidative stress but not to the starvation stress. In addition, results of the H2DCF staining showed that early induction of XBP1 reduced the accumulation of ROS in 3 $_{30}^{\circ}$ C-45 $_{18}^{\circ}$ C A β flies (Fig. 4D), further supporting that excessive ROS is one of the main factors to trigger the development of the pathological behaviors. Longevity assay showed that not only the learning performance was impaired in the early A β induced flies, but also the life-span. As shown in Fig. 6G ($P < 0.0001$, Log-rank test), early A β exposure caused life-span shortening in flies. Our previous study showed that overexpression of XBP1 extends the life-span of A β flies [16]. To understand the role of XBP1 in early A β exposure-induced pathology, the life-span and cell viability were examined. Our results showed that early induction of XBP1 extended the life span of the early A β exposed flies (Fig. 6H; Ctrl vs. A β 42: $P < 0.0001$; A β 42 vs. A β 42+XBP1: $P = 0.0119$, Log-rank test) and improved the cell viability in 3 $_{30}^{\circ}$ C-45 $_{18}^{\circ}$ C A β flies. The vacuolated area in the cell body region of mushroom body was quantified and examined by propidium iodide staining (Fig. 6I; $P = 0.0019$, $F(2, 12) = 11.05$, One-way ANOVA).

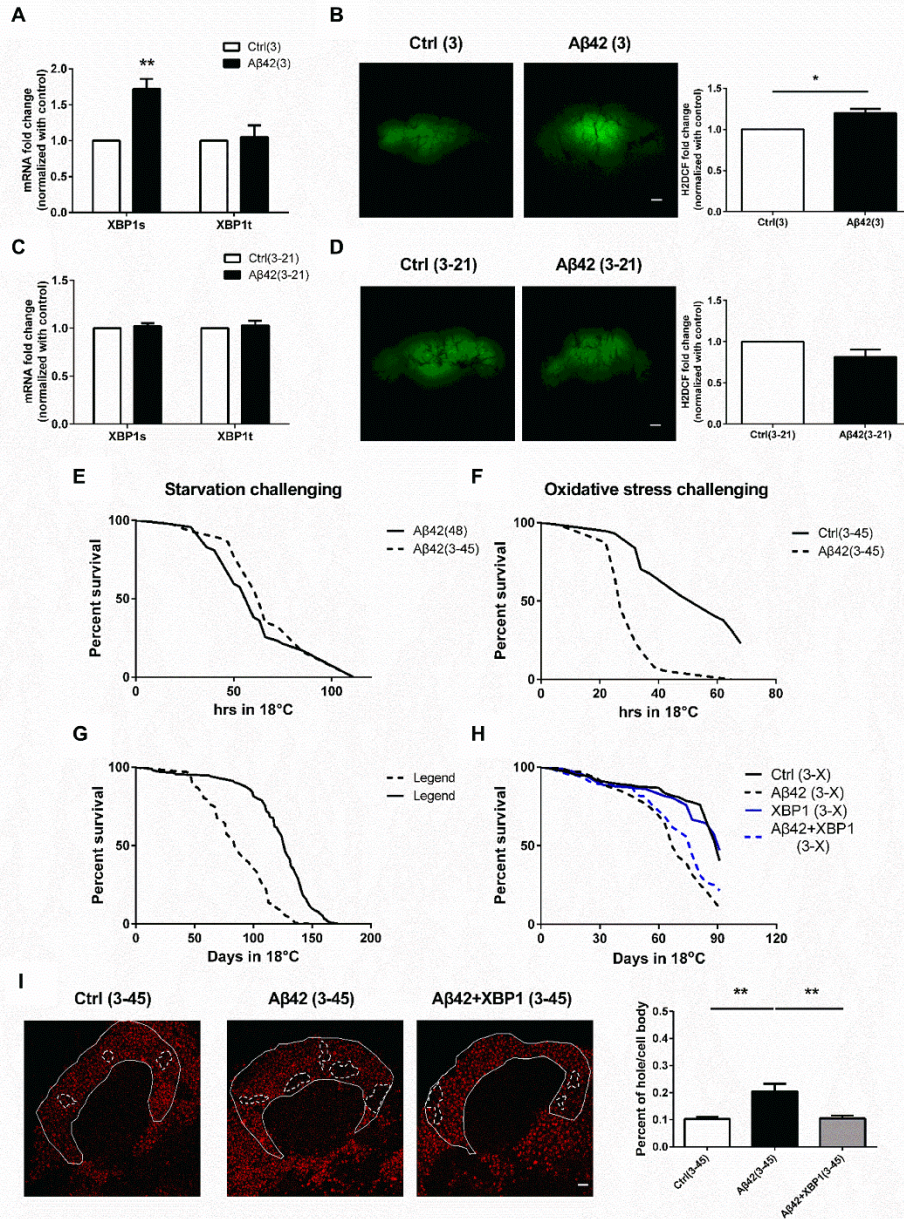


Figure 6. Early XBP1 expression improves the resilience to oxidative stress and prevents cells death in later life. (A) The XBP1s, but not XBP1t, mRNA level was increased in the brain of 3₃₀^{°C} A β flies, N = 3 respectively. ***p* < 0.01. (B) Increased ROS level was found in 3₃₀^{°C} A β flies. The quantitative data is shown at the bottom, N = 4 respectively. **p* < 0.05. White bar indicates 50 μ m. (C) Both XBP1t and XBP1s mRNA level had not been significantly changed in the brain of 3₃₀^{°C} -21₁₈^{°C} A β flies, N = 3 respectively. (D) The ROS in 3₃₀^{°C} -21₁₈^{°C} A β flies was similar to control flies. The quantitative data is shown at the bottom, N = 8 respectively. ****p* < 0.001. White bar indicates 50 μ m. (E) The survival rate of 3₃₀^{°C} -45₁₈^{°C} A β flies was similar to that of the age matched control (48₁₈^{°C}) flies upon starvation challenge. *n* = 70-75 flies for each genotype. (F) The survival rate of 3₃₀^{°C} -45₁₈^{°C} A β flies was reduced after paraquat treatment. *n* = 70-75 flies for each genotype. (G) Early A β accumulation induced early death. Both the A β and the control group were housed under 30^{°C} for 3 days after eclosion and then transferred to 18^{°C} for life-span analysis. *n* = 110-120 flies for each genotype. (3-X) represents (3₃₀^{°C} -X₁₈^{°C}) (H) Early XBP1 overexpression improved the life-span. *n* = 110-120 flies for each genotype. (I) Early XBP1 overexpression prevented cell loss induced by early A β expression in the brain, as examined by the propidium iodide staining (*F* (2, 12) = 11.05, *p* = 0.0019; Ctrl (3-45) vs. A β 42 (3-45): *p* = 0.0039, respectively; A β 42 (3-45) vs. A β 42+XBP1 (3-45): *p* = 0.0043, respectively, *n* = 5). Areas around calyx were quantified and the quantification was done under the double-blind procedure. White circles represent the vacuolated area. The quantitative results are shown on the right. White bar indicates 5 μ m. ***p* < 0.01. All Ctrl represents Elav-Gal4+Gal80^{TS}.

DISCUSSION

The current study aims to understand the molecular-pathological changes occurred after the clearance of A β accumulation and to determine the role of these changes in the development of learning impairment in later life. Findings of the current study reveal that an endogenous A β clearance system is present in flies for the removal of A β . However, the reduction of A β accumulation only temporarily recovers the memory deficit. At the molecular level, early A β exposure reduces the expression of XBP1, catalase and dPrx5 in later life and affects animal stress response. This study, for the first time, confirms that early-transient A β exposure, even being removed soon afterwards, has a pathological impact on the animal's life.

Existence of an endogenous A β degradation system in fruit fly

The clearance of A β in the brain can be carried out through two different mechanisms - the "active" and the "passive" clearance pathways. A β is actively removed by degrading enzymes, cellular degradation systems, or be taken up by glia cells. Degrading enzymes including neprilysin and insulin-degrading enzyme (IDE) has been demonstrated to play an important role in this active protein removal process in flies [37, 38]. Cellular degradation systems have also been shown to degrade A β in the fruit fly [39]. Glia cells in the brain of flies provide a protection to neurons from A β toxicity [40]. On the other hand, the passive mechanism includes diffusion of A β from brain to the peripheral [41, 42]. In the current study, we did not identify which mechanism is responsible for the clearance of A β and the recovery of the pathological behaviors. It would be of great interest to identify the major A β degradation/removal system in flies in the future.

The effects of transient and prolonged A β exposure on cell's functions are different

Despite A β peptides are degradable, endogenously, at different stage; the result of behavioral recovery is different. Memory damage was recovered in 330°C-2118°C A β flies, while learning deficit was not reversed in 830°C-2118°C A β flies. Why the learning damage cannot be recovered? Multiple mechanisms underlying this phenomenon can potentially be existed and two of the possible mechanisms are: 1) Impairment of the endogenous A β degradation system. The amount of A β left in 830°C-2118°C A β flies could not be further degraded or removed as in 830°C-3518°C A β flies, suggesting the amount of A β accumulated in cells exceeds the capacity of the endogenous A β degradation system and 2)

Continuous accumulation of ROS. Our experiment results showed that oxidative stress still remained high in 830°C-2118°C A β flies, even though about half of the accumulated A β was soon being removed after the prolonged A β induction, suggesting the assault of oxidative stress was not ceased. This data suggests that prolonged A β exposure and excessive A β accumulation would damage cells and these damages cannot be easily recovered endogenously. Therefore, we hypothesize that the quantity (*i.e.*, the amount of A β accumulated in cells), and the quality (*i.e.*, the duration of A β accumulation) are both important for the development of A β -induced toxicity. Our data suggests that short-term A β exposure-caused functional changes can be recovered and are reversible if A β is soon removed by the endogenous A β clearing system but prolonged A β exposure-induced cellular functional changes (*e.g.*, degradation system malfunction) and is difficult to recover as the amount of A β accumulated is larger and it is more difficult to remove the accumulated A β by the endogenous A β clearing system.

Differential mechanisms involved in the post A β clearance-induced toxicity and the continuous A β accumulation-induced toxicity

Our results revealed that the mechanism involved in the development of pathological behaviors in flies with the removal of A β after A β accumulation and those with continuous A β accumulation is different. Many cellular signaling pathways have been shown to play a role in the abnormal learning and memory behaviors development in flies with continuous A β expression. In our current study, manipulating these pathways failed to prevent the development of behavioral damage in early A β exposed flies, suggesting another mechanism is presence in mediating the neurological (*i.e.* pathological) changes in later life. Notably, the ROS accumulation-induced learning impairment was found only in the aged animal. The ROS level was increased following A β induction (as shown in 330°C A β flies), returned to the normal soon after (*i.e.*, 21 days) the initiation of the removal of A β (as shown in 330°C-2118°C A β flies), but increased again awhile (*i.e.* 45 days) after the removal of A β (as shown in 330°C-4518°C A β flies). This finding suggests that the aging process may also contribute to the development of learning and memory impairment in animals experienced with the early A β exposure. Decreased tolerance to the oxidative stress, declined behavioral performance and increased amount of the dysfunctional ER have all been documented as the features of aging [27, 43]. We hypothesize that the ability to resist oxidative stress decreased as animals getting older (*i.e.*, aged), especially for those with cells that had experienced A β exposure in early life. In addition, the increasing ROS accumulation

during aging further increases the cells' burden in maintaining the balance of the ROS levels, making cells more difficult in carrying out various physiological processes. Could early A β exposure facilitate the aging process? Or early A β exposure amplifies the effects of aging? Our results suggest that early A β exposure partially amplifies the aging effects, especially on the oxidative stress response. It would be of great interest for further study to reveal the relation between A β exposure and the aging process.

Oxidative stress triggers learning impairment in later life

Oxidative stress has long been suggested involving in the aging process and the development of various diseases including AD. As aging is the major risk factor for AD, the role of ROS in the development of AD and aging has attracted scientist's attentions [44]. Current study suggests that A β exposure induced XBP1 dysregulation disrupts the expression of various oxidative stress response molecules in later life, which decreases cells' ability to clear ROS and subsequently leads to the induction of behavioral damage.

Activation of XBP1 has been suggested to protect cells from oxidative stress. XBP1-deficient cells show decreased catalase expression and increased ROS production [45]. Moreover, it has been shown that knockdown of XBP1 reduces antioxidant genes expression in the human RPE cells [46]. Accumulated evidence has suggested XBP1 activation is cell's early response during the development of AD. The XBP1 expression is found transiently increased in the APP/PS1 and 5xFAD mice and also in the human AD cases [17]. In supporting this, findings of the current and other studies using A β flies as a model also suggest that XBP1 splicing is a cellular early response to protect cells from A β toxicity [15, 16]. Of note, current study further demonstrated that XBP1 dysregulation affects learning performance in later life. Our results showed that early A β exposure leads to the reduction of both the total and the spliced forms of XBP1 in 3₃₀^{°C}-45₁₈^{°C} A β flies. Ectopic expression of XBP1 during the 3 days A β induction period prevents the learning damage in 3₃₀^{°C}-45₁₈^{°C} A β flies. Further analysis indicated the involvement of proteasome and autophagy in memory impairment was unlikely in 3₃₀^{°C}-45₁₈^{°C} A β flies (Fig. 3). Collectively, findings of this study suggest that oxidative stress is the major factor that contributes to the development of behavioral damage, which is affected by XBP1 expression. 1) Although increased oxidative stress after 3 days of A β induction was reduced after A β accumulation is reduced in 3₃₀^{°C}-21₁₈^{°C} A β flies, increased oxidative stress was observed, again, in 3₃₀^{°C}-45₁₈^{°C} A β flies. 2) Co-expression of XBP1 with

A β for 3 day induction prevents the oxidative stress increase in 3₃₀^{°C}-45₁₈^{°C} A β flies. 3) Co-expression of dPrx5 with A β for 3 days induction prevents the behavioral damage in 3₃₀^{°C}-45₁₈^{°C} A β flies. 4) Reduction of dPrx5 expression in 3₃₀^{°C}-45₁₈^{°C} A β flies is prevented if XBP1 is co-expressed with A β for 3-day induction. 5) Antioxidant treatment after A β clearance can prevent learning damage in 3₃₀^{°C}-45₁₈^{°C} A β flies.

Various clinical and pre-clinical AD studies showed that persistent stress decreases XBP1 splicing and ER stress [16, 17]. Here, we propose that transient A β exposure is already strong enough to weakening the ER stress response and hampering cell's ability to neutralize the cellular stress, even after the accumulated A β has been removed.

Maintaining cognitive function after A β clearance

It remains unclear on why early TBI experience increases the chance of dementia and neurodegeneration in later life. A recent meta-analysis showed that TBI increases the rate of AD about 1.5 times [47]. A study from military veterans shows that TBI increases the risk of having Parkinson's disease by approximately 56% [48]. Although a number of studies have demonstrated a transient increase of A β accumulation in the region of brain injury [5, 49], the effect of early A β accumulation in the late development of neurodegeneration and dementia is largely unknown. Results of the current study confirmed that early-transient A β expression/accumulation promotes the development of learning and memory deficits together with cell death in later life, partially resembling the situations of having TBI in humans. Our data suggest that increased ROS and decreased ER function are two major causes of neuronal dysfunction in later life and the application of antioxidants can prevent the development of learning and memory deficits and cell function in animals with early A β exposure. Further our results also propose that the combination of using current developed small molecular to facilitate A β clearance and antioxidant treatment would benefit the cognitive function in the AD. Although more studies are needed to further uncover the detailed underlying molecular mechanisms, results of this study suggest that the A β fly model is suitable for use to study the effects of post A β toxicity.

Decades of studies on A β -induced pathologies mainly focus on the effects of the continuous A β accumulation. Our study, for the first time, showed the toxic effects of the early-transient A β accumulation. We demonstrated that the mechanism underlying its toxic effect is different to that of the continuous A β accumulation and further revealed the pathological role of the dysregulated ER stress response and ROS balance induced by the early-

transient A β accumulation. Previous studies show that reduced A β accumulation in the brain does not accompany with cognitive improvement. These observations not only raise a question on the role of A β during AD disease progression but also challenge the authentic of A β hypothesis. The current results not only link early A β accumulation to the post A β clearance but also give further evidence for supporting the A β hypothesis.

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Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2021.1015.

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