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Inhibition of JNK/dFOXO pathway and caspases rescues neurological impairments in *Drosophila* Alzheimer's disease model

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ABSTRACT

Amyloid-β-42 (Aβ42) has been implicated in the pathogenesis of Alzheimer's disease (AD). Neuronal *Aβ42* expression induces apoptosis and decreases survival and locomotive activity in *Drosophila*. However, the mechanism by which Aβ42 induces these neuronal impairments is unclear. In this study, we investigated the underlying pathway in theses impairments. JNK activity was increased in *Aβ42*-expressing brains, and the *Aβ42*-induced defects were rescued by reducing JNK or caspase activity through genetic modification or pharmacological treatment. In addition, these impairments were restored by *Drosophila forkhead box subgroup O* (*dFOXO*) deficiency. These results suggest that the JNK/dFOXO pathway confers a therapeutic potential for AD.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia in the world [1,2]. AD is characterized by amyloid plaques, neurofibrillary tangles, and loss of neurons [3]. Increased amyloid-β 42 (Aβ42), a major component of amyloid plaques in the AD brain, peptides and/or Aβ aggregation have been observed in AD patients [4]. In animal models, Aβ42 induces cell death, decrease survival rate, and locomotive dysfunction [5,6]. However, molecular mechanisms of the Aβ42-induced neurological impairments remain to be elucidated.

The c-Jun N-terminal kinase (JNK) is a major cellular stress response protein and leads to the induction of cell death [7]. The JNK signaling pathway is activated in human AD brains [8,9]. Many studies have shown that Aβ42 activates JNK [10–13]. This activation mediates Aβ42 neurotoxicity, and inhibition of JNK activity suppresses Aβ42 toxicity [10–12]. Nevertheless, limited studies have reported the role of JNK in the neurological phenotypes of AD model animals [14]. Thus, it has not been fully verified whether inhibition of JNK activity have therapeutic benefits for Aβ42-associated AD.

As a powerful genetic and cell biological system, *Drosophila* has been used to study AD [6]. Previously, we reported that the ectopic expression of *Aβ42* in *Drosophila* neurons induced apoptosis, decreased survivability, and locomotive dysfunction [13]. Additionally, *Drosophila* has well conserved disease-related signaling pathways, including the JNK pathway [15]. *Drosophila* genome contains most of the genes in the JNK signaling pathway components, and the cellular functions of JNK signaling pathway are well conserved in *Drosophila* [15].

In this study, we investigated the role of JNK/*Drosophila* forkhead box subgroup O (dFOXO) pathway and caspases in the neurological phenotypes of AD model animals. Reduction of JNK/dFOXO and caspase activity by genetic modification or pharmacological treatment strongly rescued *Aβ42*-induced neurological phenotypes. These results suggest that apoptosis induced by the JNK/dFOXO pathway is a major mediator of Aβ42-induced neurotoxicity, and that JNK/dFOXO pathway is a potential therapeutic target for treating AD.

2. Materials and methods

2.1. Fly strains

elav-GAL4 (pan-neuronal driver), *glass multimer reporter* (*GMR*)-GAL4 (eye driver), *UAS-Drosophila inhibitor of apoptosis protein 1* (*DIAP1*) and *basket*[1] (*bsk*[1]) were obtained from the Bloomington

Abbreviations: Aβ42, amyloid-β-42; AD, Alzheimer's disease; dFOXO, *Drosophila* forkhead box subgroup O; JNK, Jun-N-terminal kinase.

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Drosophila Stock Center. *hemipterous*^{1} (*hep*^{1}) was a gift from Dr. S. Noselli (CNRS, France). *GMR-Aβ42* was provided by Dr. Mary Konsolaki (Rutgers University, USA). *UAS-Aβ42* was provided by Dr. Damian C. Crowther (University of Cambridge, UK). *dFOXO*^{21} and *dFOXO*^{25} were gifts from E. Hafen (University of Zürich, Switzerland).

2.2. Acridine orange staining

The brains of L3 larvae were dissected in phosphate-buffered saline (PBS). The samples were incubated in 1.6×10^{-6} M solution of acridine orange (Aldrich, WI, USA) for 5 min at room temperature and rinsed briefly with PBS. The samples were examined under a fluorescent microscope (Carl Zeiss, Germany).

2.3. Western blots

Antibodies against JNK (1:1000 in TBST, Cell Signaling, MA, USA) and phospho-JNK (1:1000 in TBST, Cell Signaling, MA, USA) were used to detect JNK activation. Western blots were performed with standard procedures, using horseradish peroxidase-conjugated secondary antibodies (1:2000 in TBST, Cell Signaling, MA, USA).

2.4. Climbing assays

Climbing assay was performed as described previously [16]. Ten male flies of indicated lines were transferred into a test vial. After tapping the flies down to the bottom, the number of flies that climbed to the top of the vial within 8 s was counted. Ten trials were performed for each group. The experiment was repeated ten times. The climbing scores (percentage ratio of the number of climbed flies against the total number) were obtained for each test group, and the mean climbing score for at least ten repeated tests was compared to that of the control group. All experiments were carried out at 25 °C. We conducted a Student's *t* test for statistical analysis.

2.5. Analysis of Drosophila development

Fifty embryos of each genotype were collected on grape juice agar plates. After incubation for 2 days at 25 °C, the numbers of hatched larvae were counted. Then, the hatched larvae were transferred to standard media and aged at 25 °C in standard plastic vials. The numbers of pupae and enclosed flies were counted. The experiments were repeated at least five times, and statistically analyzed by a Student's *t* test.

2.6. JNK inhibitor treatment

Fifty embryos of each genotype were reared in standard plastic vials with DMSO (control) or 10 mM SP600125 (Sigma–Aldrich, MO, USA) containing media at 25 °C.

3. Results

3.1. Aβ42 induces apoptosis though JNK signaling in Drosophila eye and brain

Previously, we reported that the ectopic expression *Aβ42* in the *Drosophila* eye and larval brain strongly induced apoptosis [13]. Since hyper-activation of JNK signaling pathway has been implicated in apoptosis [7,16,17], we investigated whether JNK signaling pathway mediates *Aβ42*-induced apoptosis in *Drosophila*. First, we examined the level of phospho-JNK (pJNK), an active form of JNK, in the *Aβ42*-expressing brains. When *Aβ42* was ectopically expressed pan-neuronally, the pJNK level in the fly brains was elevated compared to control (Fig. 1A). Next, we tested if a reduction of JNK signaling rescues the *Aβ42*-induced defect in the developing eye. As previously reported [13,18], ectopically expressed *Aβ42* in the developing eyes resulted in destruction of the compound eye (Fig. 1B). This *Aβ42*-induced eye destruction was strongly suppressed by reducing the JNK signaling using mutation of *hemipterous* (*hep*), *Drosophila* JNK kinase coding gene, or *basket* (*bsk*), *Drosophila* JNK coding gene (Fig. 1B). Furthermore, as shown in Fig. 1C and D, *Aβ42*-induced cell death was also significantly reduced by *hep* or *bsk* deficiencies. The *Aβ42*-induced cell death was strongly suppressed by *Drosophila inhibitor of apoptosis protein 1* (*DIAP1*) (Fig. 1C and D), indicating that this cell death is caspase-dependent apoptosis. These results suggest that *Aβ42* induces apoptosis via activation of JNK signaling.

3.2. Reducing JNK signaling ameliorates Aβ42-induced neurological phenotypes

Previously, we found that the survival and motor activity of neuronal *Aβ42*-expressing flies had greatly deteriorated [13].

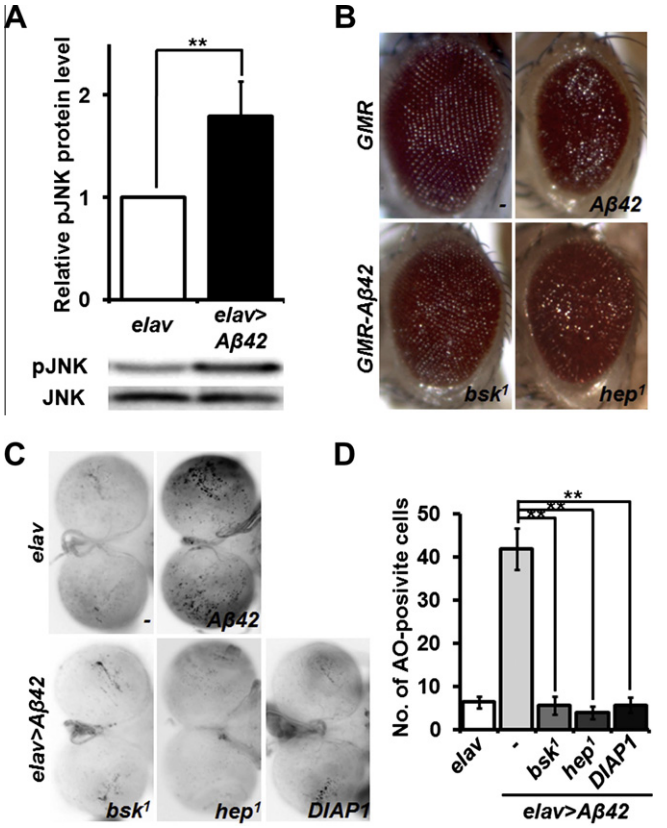

Fig. 1. *Aβ42* induces apoptosis through the activation of JNK signaling pathway in the *Drosophila* neurons. (A) The level of phospho-JNK (pJNK) in the control (*elav*) and pan-neuronally *Aβ42*-expressing (*elav>Aβ42*) fly heads. Relative phospho-JNK level was obtained as a relative band intensity of phospho-JNK to JNK ($n = 5$). The genotypes of the samples were *elav* (*elav-GAL4*/*elav-GAL4*), *elav > Aβ42* (*UAS-Aβ42*/*UAS-Aβ42*; *elav-GAL4*/*elav-GAL4*). (B) Genetic interactions of *Aβ42* with *bsk*^{1} and *hep*^{1} in the developing eye. The genotypes of the samples were *GMR* (*GMR-GAL4*/+), *GMR-Aβ42* (*GMR-Aβ42*/+; *GMR-Aβ42*/*GMR-Aβ42*), *bsk*^{1} (*GMR-Aβ42*/*bsk*^{1}; *GMR-Aβ42*/*GMR-Aβ42*), and *hep*^{1} (*hep*^{1}/Y; *GMR-Aβ42*/+; *GMR-Aβ42*/*GMR-Aβ42*). (C and D) The effect of reduction of JNK signaling on *Aβ42*-induced cell death. (C) Representative images of acridine orange (AO)-stained larval brains of indicated groups. (D) Graph showing the mean number of AO-positive cells in the larval brains of indicated groups ($n = 10$). The genotypes of the samples were *elav* (*elav-GAL4*/+), *elav > Aβ42* (*UAS-Aβ42*/+; *elav-GAL4*/+), *bsk*^{1} (*UAS-Aβ42*/*bsk*^{1}; *elav-GAL4*/+), *hep*^{1} (*hep*^{1}/Y; *UAS-Aβ42*/+; *elav-GAL4*/+) and *DIAP1* (*UAS-Aβ42*/+; *elav-GAL4*/*UAS-DIAP1*). Data from (A) and (D) are expressed as means ± s.e. ($^{**}P < 0.001$, Student's *t*-test).

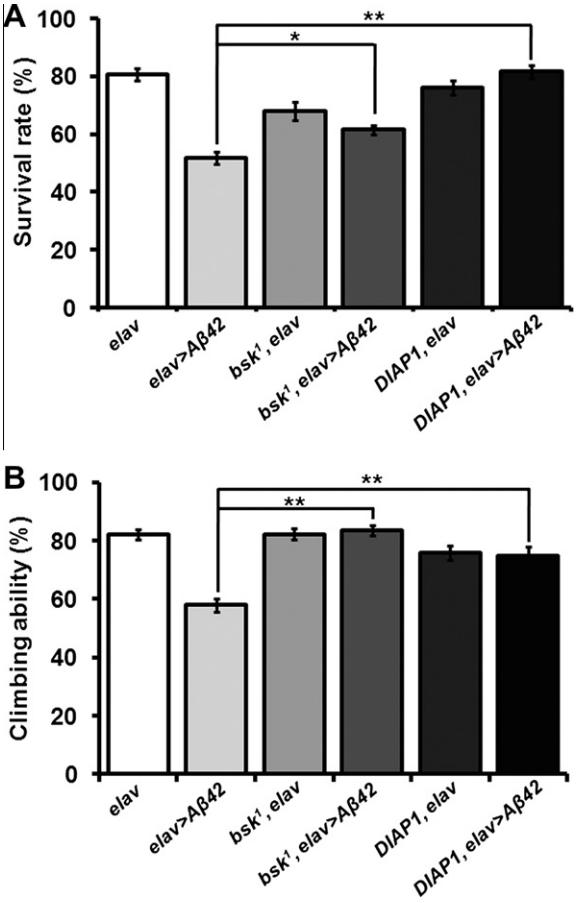

Fig. 2. Reduction of *JNK* signaling ameliorates *Aβ42*-induced neurological phenotypes. Comparisons of survival rates (A, $n \geqslant 7$) and climbing abilities (B, $n \geqslant 10$) of pan-neuronal *Aβ42*-expressing flies containing JNK deficiency (*bsk*^{1}, *elav* > *Aβ42*) or caspase inhibitor overexpression (*DIAP1, elav* > *Aβ42*) with control (*elav* > *Aβ42*). The effects of JNK deficiency (*bsk*^{1}, *elav*) or caspase inhibitor overexpression (*DIAP1, elav*) in the control background (*elav*) on the survival rate were also shown. Data from (A) and (B) are expressed as means ± s.e. ($^*P < 0.05$; $^{**}P < 0.001$, Student's *t*-test). The genotypes of the samples were *elav* (*elav-GAL4/+*), *elav* > *Aβ42* (*UAS-Aβ42/+*; *elav-GAL4/+*), *bsk*^{1}, *elav* (*bsk*^{1}*/+*; *elav-GAL4/+*), *bsk*^{1}, *elav* > *Aβ42* (*UAS-Aβ42/bsk*^{1}; *elav-GAL4/+*), *DIAP1, elav* (*elav-GAL4/UAS-DIAP1*) and *DIAP1, elav* > *Aβ42* (*UAS-Aβ42/+*; *elav-GAL4/UAS-DIAP1*).

Therefore, we investigated whether these neurological phenotypes mediated in JNK signaling by examining the effect of *bsk* mutation on these phenotypes. Although *bsk* mutation decreased survivability in the control group (*bsk*^{1}, *elav*), presumably because of a disrupted function of JNK during development, a reduced JNK level significantly suppressed *Aβ42*-induced lethality (Fig. 2A). Moreover, the impaired motor activity in the *Aβ42*-expressing flies was significantly restored by *bsk* mutation (Fig. 2B). These neurological phenotypes of *Aβ42*-expressing flies were also rescued by co-expression of *DIAP1* (Fig. 2A and B), indicating that apoptosis is crucial for these phenotypes.

3.3. SP600125 beneficially affects Aβ42-induced neurological phenotypes

Since *Aβ42*-induced neurological phenotypes are strongly associated with JNK activity (Figs. 1 and 2), we assessed the effect of a JNK inhibitor on the neurological phenotypes induced by *Aβ42*. First, we examined the effect of SP600125, a specific inhibitor of JNK [19], on the *Aβ42*-induced apoptosis. As expected,

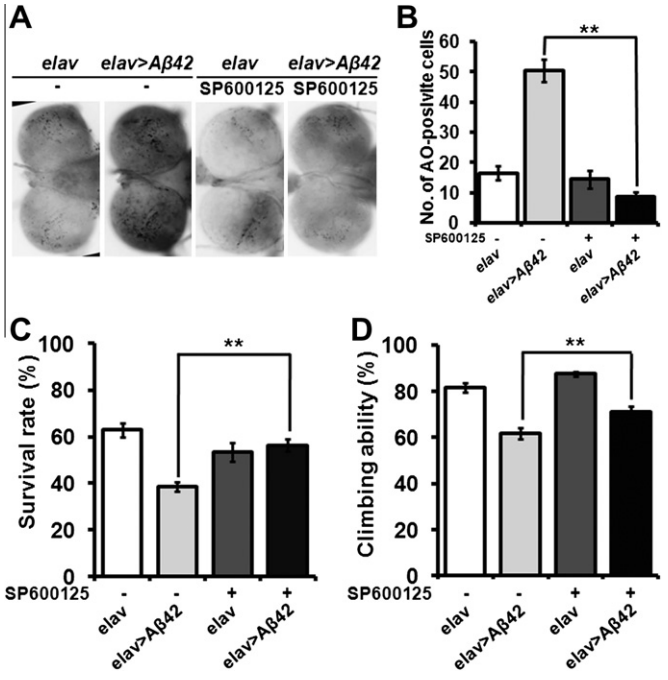

Fig. 3. SP600125 suppresses *Aβ42*-induced apoptosis and neurological phenotypes. (A) Representative images of AO-stained larval brains of SP600125-fed or DMSO-fed larva, with (*elav* > *Aβ42*) or without (*elav*) *Aβ42* expression. (B) Graph showing the mean number of AO-positive cells in the larval brains of each group ($n = 10$). (C and D) Survival rates (C, $n = 13$) and climbing abilities (D, $n = 10$) of SP600125-fed or DMSO-fed flies with (*elav* > *Aβ42*) or without (*elav*) *Aβ42* expression. Data from (B), (C), and (D) are expressed as means ± s.e. ($^{**}P < 0.001$, Student's *t*-test). The genotypes of the samples were *elav* (*elav-GAL4/+*) and *elav* > *Aβ42* (*UAS-Aβ42/+*; *elav-GAL4/+*).

Aβ42-induced apoptosis was strongly suppressed by SP600125 (Fig. 3A and B), confirming again that JNK signaling is a critical mediator of this process. Next, we checked the effect of SP600125 on the neurological phenotypes of *Aβ42*-expressing flies. Coincident with above data, we showed that the *bsk* mutation decreased survivability in the control group (Fig. 2A). SP600125 feeding decreased the survivability of flies (Fig. 3C). However, SP600125 increases the survival rate of *Aβ42*-expressing flies and improves the motor activity of *Aβ42*-expressing flies (Fig. 3C and D). These results suggest that the pharmacological inhibition of JNK activity could protect neurons and rescue neurological phenotypes of *Aβ42*-expressing flies.

3.4. dFOXO is required for Aβ42-induced neurological phenotypes

Previously, dFOXO has been reported to be a downstream factor of JNK signaling under stress conditions [20]. Thus, we tested if dFOXO activity is required for the induction of apoptosis by *Aβ42*. The *Aβ42*-induced eye destruction and apoptosis in the larval brain was strongly suppressed by the *dFOXO* mutant alleles, *dFOXO*^{21}, and *dFOXO*^{25} (Fig. 4A, B, and C), suggesting that dFOXO is involved in the *Aβ42*-induced apoptosis.

Next, to investigate whether dFOXO was also implicated in *Aβ*42-induced neurological phenotypes, we examined the effect of *dFOXO* deficiency on the neurological phenotypes of *Aβ42*-expressing flies. As shown in Fig. 4D and E, the *Aβ42*-induced neurological phenotypes were strongly suppressed by *dFOXO* deficiencies, while *dFOXO* mutations in the control group did not show neurotoxicity and locomotive defects. Collectively, these results suggest that dFOXO mediates *Aβ42*-induced apoptosis and neurological phenotypes.

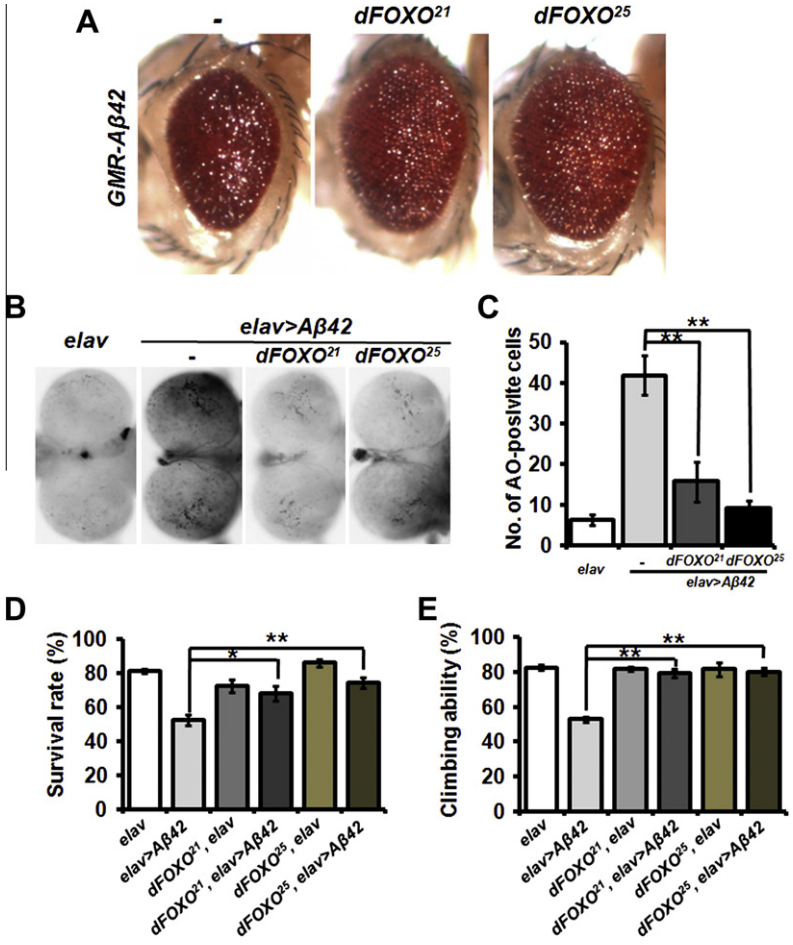

Fig. 4. dFOXO mediates *Aβ42*-induced apoptosis and neurological phenotypes. (A) Genetic interactions of overexpressed *Aβ42* with $dFOXO^{21}$ or $dFOXO^{25}$ in the developing eye. The genotypes of the samples were *GMR-Aβ42* (*GMR-Aβ42/GMR-Aβ42*; *GMR-Aβ42/+*), $dFOXO^{21}$ (*GMR-Aβ42/GMR-Aβ42*; *GMR-Aβ42/*$dFOXO^{21}$), and $dFOXO^{25}$ (*GMR-Aβ42/GMR-Aβ42*; *GMR-Aβ42/*$dFOXO^{25}$). (B and C) Representative images of AO-stained larval brains of each group (B) and graph showing the mean number of AO-positive cells in the larval brains of each group (C, $n = 10$). (D and E) *dFOXO* deficiencies increased the survival rate (D) and motor activity (E) of *Aβ42*-expressing flies (D, $n \geqslant 10$; E, $n \geqslant 10$). The genotypes of the samples were *elav* (*elav-GAL4/+*), *elav > Aβ42* (*UAS-Aβ42/+*; *elav-GAL4/+*), $dFOXO^{21}$, *elav* (*elav-GAL4/* $dFOXO^{21}$), $dFOXO^{21}$, *elav > Aβ42* (*UAS-Aβ42/+*; *elav-GAL4/*$dFOXO^{21}$), $dFOXO^{25}$, *elav* (*elav-GAL4/*$dFOXO^{25}$), and $dFOXO^{25}$, *elav > Aβ42* (*UAS-Aβ42/+*; *elav-GAL4/*$dFOXO^{25}$). Data from (C–E) are expressed as means ± s.e. ($^{*}P < 0.05$; $^{**}P < 0.001$, Student's *t*-test).

4. Discussion

Among the signaling pathway, JNK has been extensively implicated in AD [21]. Our study has shown that a misexpression of *Aβ42* in the neurons increases the level of JNK phosphorylation, indicating that JNK signaling is activated. Downregulation of JNK signaling strongly suppresses *Aβ42*-induced cytotoxicity in both the eye and the brain. Furthermore, a reduction of JNK singling also rescues the decreased survival rate and locomotive defect of the *Aβ42*-expressing flies. During our preparation of this manuscript, another group reported that the JNK pathway is implicated in *Aβ42*-induced eye degeneration [18]. Collectively, these findings suggest that hyper-activation of the JNK is a major cause of neuronal impairments in *Drosophila Aβ42*-expressing AD models.

Apoptosis is one of the important downstream events of JNK activation in the neurodegeneration [11]. Previously, we have shown that misexpression of *Aβ42* in the developing eye induced cell death [13]. Tare et al. (2011) have reported that the Aβ42-induced cell death is partially inhibited by a co-expression of the caspase inhibitor baculovirus p35. These suggest that Aβ42-induced cell death is through both p35-sensitive caspases-dependent and -independent pathways. In contrast to p35, we have shown that DIAP1, an inhibitor of all caspases, almost completely suppresses Aβ42-induced cell death and neurological phenotypes, indicating that Aβ42 kills the cells through DIAP1-sensitive caspase-dependent apoptosis. Previous studies have shown that Dronc, an initiator caspase, is the only DIAP1-sensitive caspase that is not suppressed by p35 [22–24]. Therefore, our results in combination with previous reports, suggest that Dronc is an important mediator of the *Aβ42*-induced cell death.

JNK activates apoptosis via regulating various downstream effectors, including FOXO [11,20]. As a transcription factor, dFOXO mediates transcriptional activation of *head involution defective* (*hid*), a pro-apoptotic gene, by JNK signaling [17,20]. FOXO activation has been implicated in oxidative stress and neurodegeneration [17,25–27]. However, the role of FOXO in pathogenesis of AD is little known. We have shown that down-regulation of dFOXO strongly rescues *Aβ42*-induced cell death and neurological phenotypes, suggesting the critical role of dFOXO in the *Aβ42*-induced pathogenesis. Based on the relationship between dFOXO and JNK, dFOXO would mediate JNK signaling by regulating apoptosis in the *Aβ42*-expressing neurons.

As JNK plays an important role in the neurodegeneration, JNK inhibitors have been applied to treat disease-like phenotypes in various neurodegenerative disease models [28]. Recent studies have proven that SP600125 reduces APP-induced neurodegeneration [12], and reverses memory deficit induced by Aβ42 in Aβ-injected rats [29]. Our study has consistently shown that Aβ42-

induced apoptosis and neurological phenotypes rescue by oral uptake of SP600125. These results suggest potential use of JNK inhibitors as a therapeutic drug against AD. However, when flies were fed with SP600125 during development, their survival rate was slightly decreased compared to the control group. This might possibly be the result of a disrupted developmental role of JNK. Indeed, *Drosophila* JNK has been implicated in various developmental processes [30]. The mice that lacked both JNK1 and JNK2 showed embryonic lethality with developmental defects [31,32]. Therefore, to devise the strategy to inhibit JNK signaling for treating AD, possible side effects might have to be seriously considered. Studies to find proper time and dose for treatment are urgently needed.

In conclusion, our results suggest that *Aβ42*-induced JNK activation, which leads to cell death via caspase-dependent apoptosis, is a major event responsible for the neuronal phenotypes of the *Drosophila* AD model. The pharmacological inhibition of JNK activity in a proper period might be a useful therapeutic strategy to treat AD.

Acknowledgments

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