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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].

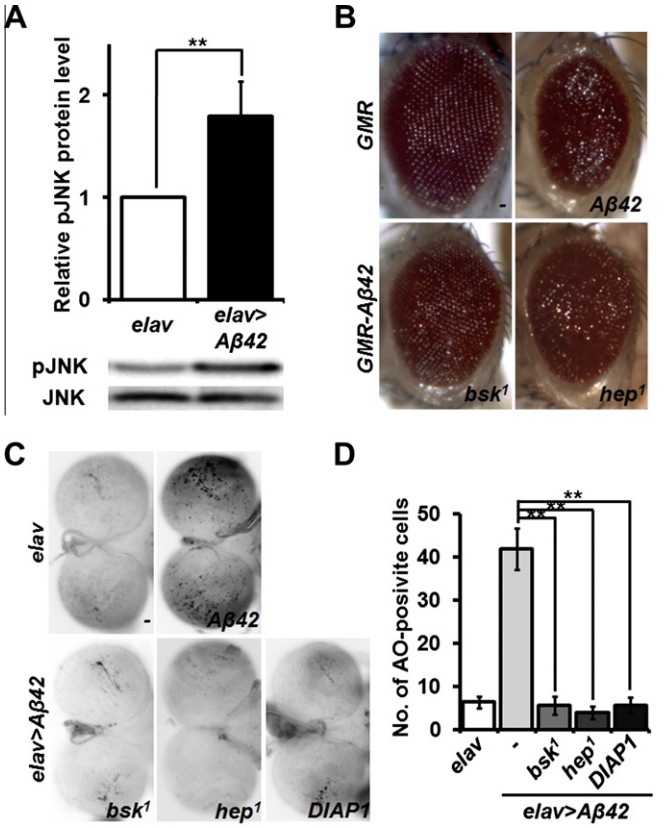

Fig. 1. *Aβ42* induces apoptosis through the activation of JNK signaling pathway in the *Drosophila* neurons. (A) The level of phospho-JNK (pJNK) in the control (*elav*) and pan-neuronally *Aβ42*-expressing (*elav>Aβ42*) fly heads. Relative phospho-JNK level was obtained as a relative band intensity 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).