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Original Article

Age-induced reduction of autophagy-related gene expression is associated with onset of Alzheimer's disease

Yasuhiro Omata[1], Young-Mi Lim[2], Yukihiro Akao[3], Leo Tsuda[2]

[1]*Department of Occupational and Environmental Health, Graduate School of Medicine, Nagoya University, Nagoya, Aichi, Japan;* [2]*Animal Models of Aging, CAMD, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan;* [3]*United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu City, Gifu, Japan*

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Abstract: Aging is a major risk factor for Alzheimer's disease (AD). Aggregation of amyloid beta (Aβ) in cerebral cortex and hippocampus is a hallmark of AD. Many factors have been identified as causative elements for onset and progression of AD; for instance, tau seems to mediate the neuronal toxicity of Aβ, and downregulation of macroautophagy (autophagy) is thought to be a causative element of AD pathology. Expression of autophagy-related genes is reduced with age, which leads to increases in oxidative stress and aberrant protein accumulation. In this study, we found that expression of the autophagy-related genes *atg1, atg8a, and atg18 in Drosophila melanogaster* was regulated with aging as well as their own activities. In addition, the level of *atg18* was maintained by *dfoxo (foxo)* and *dsir2 (sir2)* activities in concert with aging. These results indicate that some autophagy-related gene expression is regulated by *foxo/sir2*-mediated aging processes. We further found that reduced autophagy activity correlated with late-onset neuronal dysfunction caused by neuronal induction of Aβ. These data support the idea that age-related dysfunction of autophagy is a causative element in onset and progression of AD.

Keywords: Aging, macroautophagy, Alzheimer's disease (AD), amyloid beta (Aβ), neurodegeneration, *Drosophila melanogaster*

Introduction

Macroautophagy (autophagy) is the system for degradation and recycling of cellular waste in eukaryotic cells, and it is involved in cellular responses to stress including starvation, infection, and pathological accumulation of protein [1-4]. During autophagy, double-membrane vesicles called autophagosomes are formed to fuse with lysosomes, allowing the lysozymes to degrade their contents [5-7]. When autophagy is induced by starvation, newly synthesized amino acids and free fatty acids are provided that can be used for cellular energy [1, 8]. Autophagy also controls the quality of essential cellular components by eliminating damaged organelles and protein aggregates [1, 8].

In yeast, many autophagy-related genes have been identified. Most are essential for autophagosome formation [9]. Autophagosome formation occurs in the endoplasmic reticulum (ER) and involves a series of steps, including initiation, nucleation and expansion [7]. In mammals, yeast ATG1 homologues of unc-51-like kinase (ULK1/2), ATG13, ATG101 and focal adhesion kinase family interacting protein of 200 kDa (FIP200) form a complex with mTORC1 in nutrient status. ATG101 and FIP200 have been found only in mammals. In response to starvation, initiation step starts to dephosphorylate ULK1/2 rapidly. ULK1/2 is auto-phosphorylated and then phosphorylates ATG13 and FIP200. Alternatively, ULK1/2 is phosphorylated and activated by AMPK [10]. Nucleation involves formation of the isolation membrane (IM) from the source. Mammalian have four ATG18 homologues, WD-repeat protein interacting with phosphoinositides (WIPIs). In this process, WIPI2 (ATG18) and double-FYVE-containing protein (DFCP1) found only in mammals, which are autophagy-specific effectors for phosphatidylinositol 3-kinase (PI3K), are recruited to the IM

[11]. Finally, ATG16L complex induces microtubule associated protein light chain 3 (LC3) lipidation. IM having distinct localization of ATG protein expands within the omegasome and close to form autophagosome [12].

A growing number of studies have shown that expression levels of autophagy-related genes are tightly regulated by many types of transcription factors under different signaling pathways, including the insulin/growth factor pathway and nutrient-sensing signaling through the mTOR- and Akt-dependent pathways [13, 20, 39]. A previous study revealed that *atg1* expression is upregulated by *dfoxo* (the *Drosophila* homolog of *foxo*, which encodes forkhead box transcription factor class O), and that insulin signaling inhibits expression of autophagy-related genes by inactivating *foxo* in hepatic cells [14]. *dfoxo* is also necessary and sufficient to induce autophagy in the *Drosophila* larval fat body [15]. Moreover, *sirt1* is a sensor of CR to induce autophagy through deacetylation of *foxo* and also regulate the induction of autophagy through the inhibition of insulin signaling, which result in TOR inhibition [20]. The control of mRNA translation is a function of insulin/TOR pathway. 4E-BP is involved in cap-dependent mRNA translation. 4E–BP mutation is hypersensitive to starvation suggesting a coordination of translation and autophagy in response to insulin/TOR signaling [39].

Although the precise molecular mechanisms remain to be clarified, aging is an important regulator of autophagy function. In *Drosophila melanogaster*, expression of autophagy-related genes is reduced with aging [16-18]. Mutant *atg8* flies have a reduced lifespan and are sensitive to oxidative stress [16]. Conversely, increased expression of autophagy-related genes delays aging and extends lifespan [19, 20]. These results suggest that the activity of autophagy is closely associated with aging.

The suppression of neural autophagy leads to aberrant protein aggregation and neural degeneration. The expression level of WIPI4, a human homolog of *atg18*, is correlated with static encephalopathy of childhood with neurodegeneration in adulthood (SENDA), a subtype of neurodegenerative disease with iron accumulation in the brain [21]. Furthermore, many studies suggest a strong correlation between autophagy activity and Alzheimer's disease (AD), a highly epidemic cause of neuronal dysfunction in old age [22]. Defects in the lysosomal proteolysis machinery were detected in AD patients with a presenilin mutation [23]. In an APP-overexpressing mouse model, downregulation of Beclin-1, an essential regulator of autophagy initiation, caused accumulation of both intraneuronal and extracellular Aβ deposits followed by marked neurodegeneration [24].

In this report, we have revealed that the expression of autophagy-related genes is tightly regulated by many factors, including aging, sirtuin signaling, TOR signaling and autophagy activity itself. We further found that these regulatory systems may contribute to late-onset neuronal dysfunction in a *Drosophila* AD model induced by amyloid beta (Aβ) overexpression.

Materials and methods

Fly stocks

Flies were maintained in vials with SY medium at 25°C on a 12 h:12 h light/dark cycle. *dfoxo*, *dsir2* and *d4E-BP^{null}* lines were kindly provided by Drs. Hafen, Helfand and Lasko, respectively. The deficiencies *E44* and *ZP1* were obtained from the Bloomington Stock Center (Indiana University).

Human Aβ42 is a peptide cleaved from APP, a transmembrane protein, by β- and γ-secretase [25]. Although components required for γ-secretase activity are well conserved [26], β-secretase is absent or has quite low in *Drosophila* [27]. To generate transgenic lines as a AD model, rat pre-proenkephalin signal peptide was connected to Aβ42 by PCR using APP cDNA. Aβ42 E22G (arctic) and Aβ42 L17P constructs with a signal peptide were cloned into the pUAST vector [28]. Each amino acid substitution was generated by PCR starting with Aβ42 wild template individually. After embryonic injection of these vectors, transgenic flies were established using standard methods. UAS-Tg lines were crossed with *gmr-gal4* lines or *elav-gal4* lines. Several lines with high expression were used for experiments.

Histology

Plastic embedding and sectioning of eyes were carried out as described [29]. Images were captured by microscopy (Nikon).

Climbing assay

Twenty flies that had eclosed on the day of the assay were placed in empty plastic vials divid-

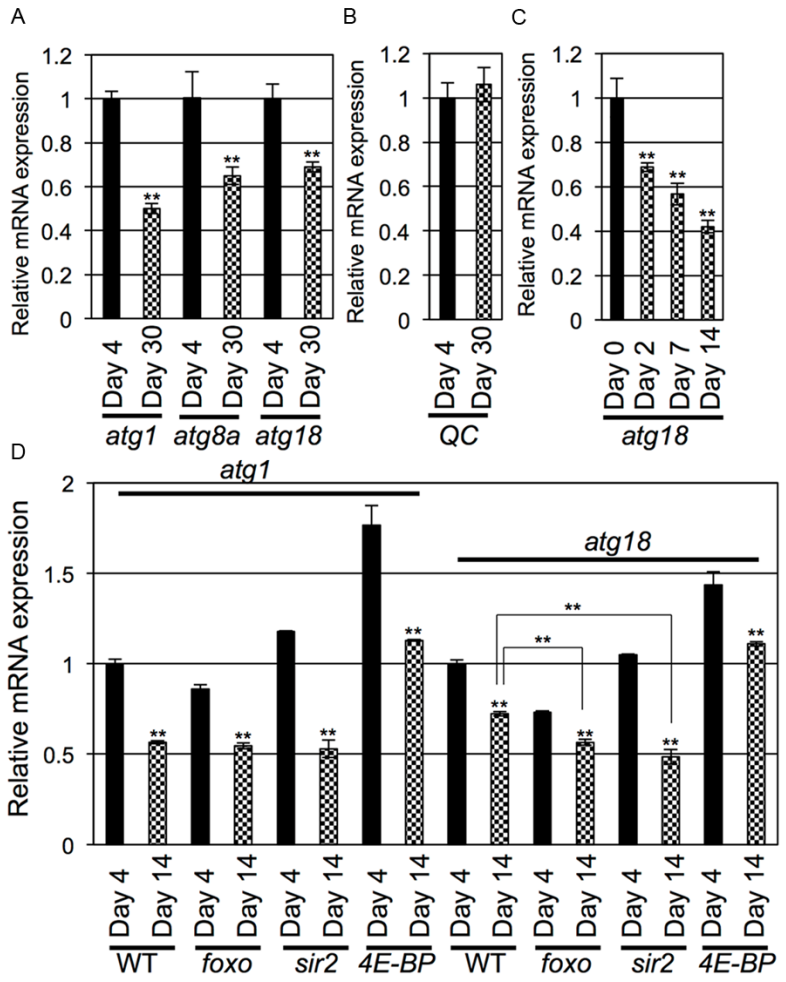

Figure 1. Downregulation of *atg1*, *atg8a* and *atg18* mRNA in heads of aged *D. melanogaster*. A. *atg1*, *atg8a* and *atg18* mRNA expression in wild-type (WT) flies at 4 and 30 days (means±SD, n=4); B. *Glutaminyl cyclase* (QC) mRNA expression in wild-type flies at 4 and 30 days (means±SD, n=4). C. *atg18* mRNA expression in wild-type flies at 0 to 14 days (means±SD, n=4). D. *atg1* and *atg18* expression in *foxo*, *sir2 and 4E-BP* mutants at 4 and 14 days (means±SD, n=4). Statistical significance of each expression levels in older flies against younger flies was analyzed by student t-test (**$P < 0.01$).

ed into bottom, middle and top areas. Vials were tapped on a table to make the flies drop to the bottom, and then placed on the table to allow the flies to climb up. The number of flies in each area was counted after 1 min. The assay was repeated every 5 days for 50 days. Males and females were tested separately. Each assay was repeated three times and average numbers were calculated. Averaged data of them was shown since there was no significant difference between males and females.

Quantitative PCR (qPCR)

Total RNA was extracted from 30 fly heads using ISOGEN (Nippon Gene). cDNA was synthesized using the PrimeScript RT reagent kit (Takara). SYBR Premix Ex-Taq II (Takara) was used for amplification. PCR amplification and data analysis was performed on a Thermal Cycler Dice (Takara) under previously described conditions [30]. RP49 was used for a control of mRNA level.

Oligonucleotides for real-time PCR analysis were follows:

atg1: Sense; 5'- CATTCGTTGGTGCAAAAGTG -3', Antisense; 5'- CATGCTGCTGCAATATGCTC -3'; *atg8a:* Sense; 5'- CATTCCACCAACATCGGCTA -3', Antisense; 5'- GCCGTAAACATTCTCATCGG -3'; *atg18:* Sense; 5'-AACCAGAACATAACTTCCCTGGC -3', Antisense; 5'- TCGTGTGGTAGATCTTGTCCAG -3'; *QC:* Sense; 5'- AGGTCCATTTCAACCGCAC -3', Antisense; 5'-TTCAGCGATTGGACCAGGTA -3'. The sequence data for *rp49* was as described previously [30].

Western blotting

Twenty fly heads were homogenized in RIPA lysis buffer containing proteinase inhibitors; RIPA lysis buffer is described in [31]. Homogenate was sonicated on a Bioruptor (CosmoBio), then ultracentrifuged at 189,000 x g, 4°C, for 30 min. This first supernatant was collected as the SDS-soluble fraction. The pellet was dissolved in 70% formic acid, and then centrifuged at 13,000 rpm, 4°C, for 20 min. This second supernatant was dried for 1 h at room temperature by speed vacuum, resuspended with 1 M Tris-HCl (pH 6.8), and kept as the SDS-insoluble fraction. Each fraction was separated on 15-20% Tris-tricine gel (Wako) at room temperature, and then transferred to a 0.22-μm nitrocellulose membrane (Whatman) for 1 h at 4°C. The membrane was blocked with 5% skim milk (Difco). Antibodies against Aβ (6E10 from Covance; 82E1 from

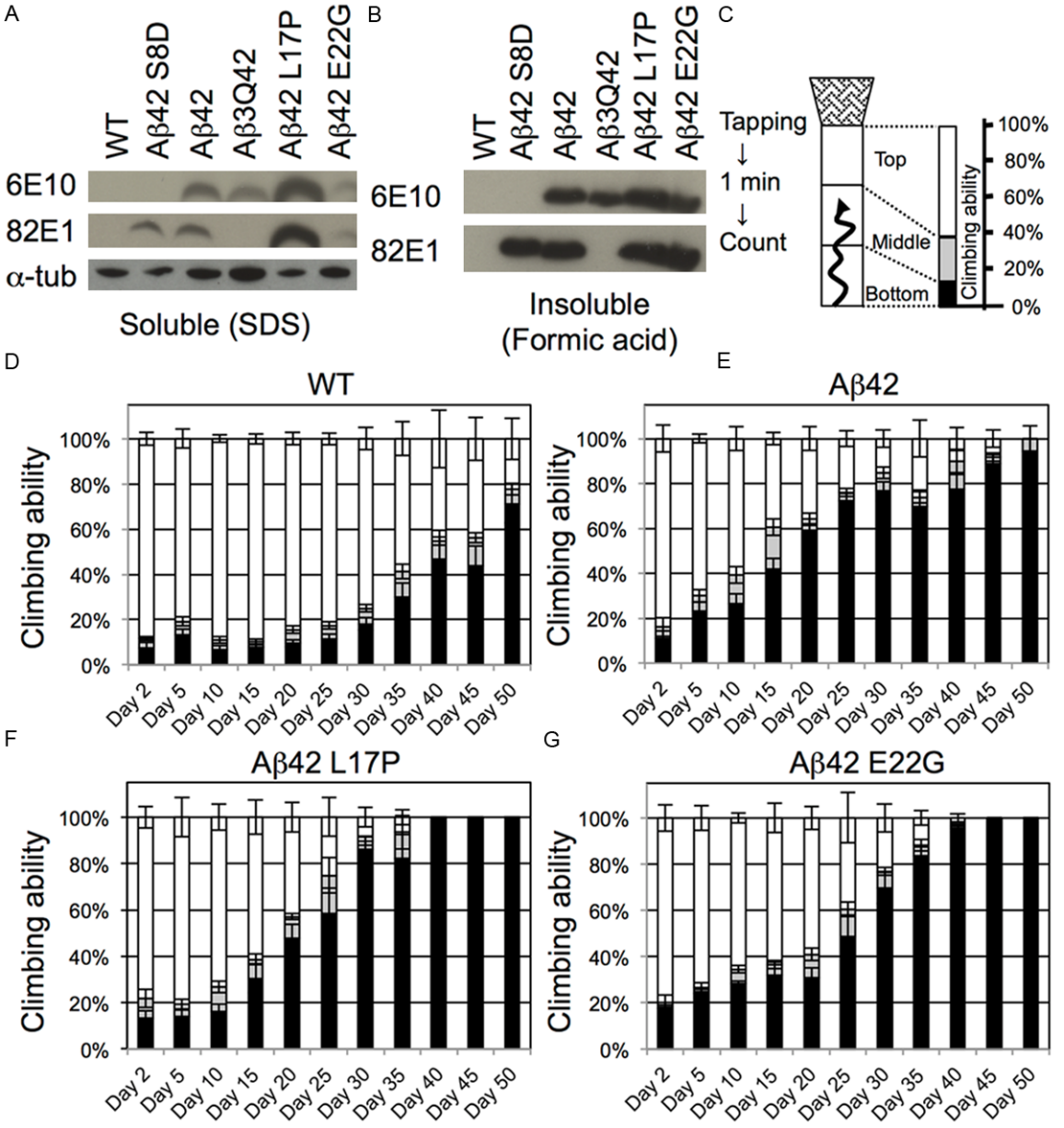

Figure 2. Aβ transgenic flies produce Aβ42 and exhibit reduced locomotor activity. (A, B) Western blots of soluble (A) and insoluble fractions (B) in 30 days WT and transgenic flies. Epitope of 6E10 is 3-8 of Aβ42 and 82E1 is 1-5 of Aβ42. (C-G) Climbing assay. Twenty flies were placed in an empty vial marked to define three equal areas (C). Climbing ability was reported as percentage of flies climbing to each area (black=bottom, gray=middle, white=top) in 1 min. The assay was performed using WT (D) and transgenic Aβ42 (E), Aβ42 L17P (F) and Aβ42 E22G (G) flies. Each experiment was performed three times and average results are shown. Aβ42=elav > Aβ42, Aβ42 L17P=elav > Aβ42 L17P, Aβ42 E22G=elav > Aβ42 E22G.

IBL) and α-tubulin (Sigma) were used for detection. Goat α-mouse HRP (Santa Cruz Biotechnology) was used as secondary antibody. Signals were visualized using Immunostar LD (Wako) to be exposed to Bio Max MR film (Sigma). The films were developed using CEPROS SV (Fujifilm, Japan).

Results

Expression of autophagy-related genes is reduced with aging

It has previously been implicated that aging affects expression of autophagy-related genes

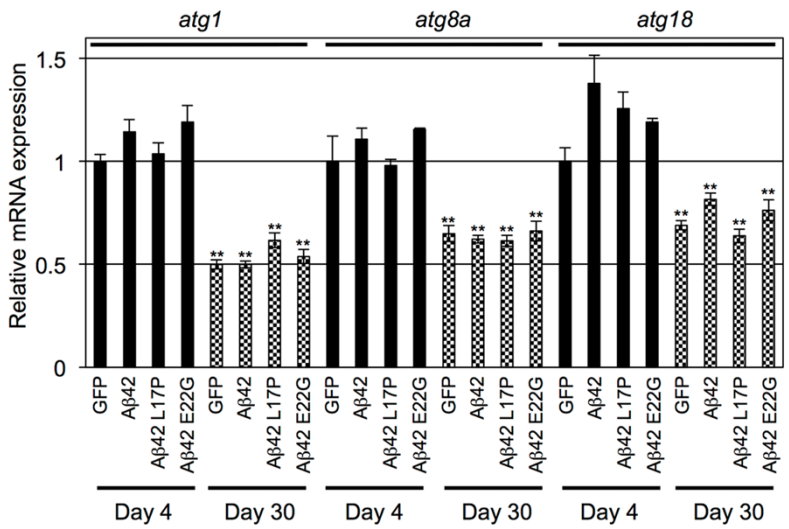

Figure 3. Overexpression of Aβ42 does not affect expression of autophagy-related genes. Relative expression of *atg1*, *atg8a* and *atg18* in transgenic flies with pan-neuronal expression of Aβ42, Aβ42 L17P and Aβ42 E22G (means±SD, n=4). Statistical significance of old flies against young flies was analyzed by student t-test (**$P < 0.01$). Aβ42=elav > Aβ42, Aβ42 L17P=elav > Aβ42 L17P, Aβ42 E22G=elav > Aβ42 E22G.

in *D. melanogaster* [16-18]. To confirm this, qPCR was performed to compare expression of *atg1*, *atg8a* and *atg18* in fly heads at 4 and 30 days after eclosion. Expression of all three genes was reduced with aging processes (**Figure 1A**). In contrast, expression of *glutaminyl cyclase* (QC), an enzyme responsible for converting the N terminus of glutamine or glutamate into pyroglutamate [38], did not change with age (**Figure 1B**), indicating that the effect of aging on *atg* gene expression was specific. To refine this temporal change, we compared expression of *atg18* at 0, 2, 7 and 14 days after eclosion. Expression of *atg18* decreased to ~70% by 7 days after eclosion, and appeared to reach a plateau at 14 days (**Figure 1C**). These results suggest that expression of autophagy-related genes may be regulated by early aging processes.

Expression of atg18 is reduced in dfoxo and dsir2 mutants

To further assess the relationship between aging and expression of autophagy-related genes, we examined expression levels of *atg1* and *atg18* in *dfoxo* and *dsir2* mutants. Expression of *atg18* at day 4 was reduced in *dfoxo* mutants compared to wild type (WT, **Figure 1D**), possibly due to a positive effect of *dfoxo* on autophagy-related gene expression, as reported in fat body [15]. Expression of *atg18* at day 14 was reduced in *dfoxo* and *dsir2* compared to WT (**Figure 1D**). Although *dsir2* showed almost the same expression of *atg1* and *atg18* as WT at day 4, expression of *atg18* was significantly reduced at day 14 compared to WT (**Figure 1D**). These results suggest that *dfoxo* and *dsir2* activity may be required to maintain *atg18* expression during aging.

Initiation factor 4E-binding protein (4E-BP) negatively regulates autophagy-related gene expression

Many studies suggest that 4E-BP acts downstream *foxo* in the regulation of aging [39]. Therefore, we examined the effect of *4E-BP* on expression of *atg1* and *atg18*. Surprisingly, expression of both genes was higher in *4E-BP* mutants than in WT at days 4 and 14, but was also reduced with age (**Figure 1D**). These results suggest that *4E-BP* negatively regulates autophagy-related gene expression.

Development of a transgenic fly model for AD

Because our data indicate that expression of autophagy-related genes is tightly regulated during aging, and it has previously been shown that autophagy is involved in development of age-related neuronal disorders such as AD, we developed a fly model of AD to further test this relationship. We cloned human Aβ42 and generated mutant forms of S8D, L17P, E22G and 3Q42 using a rat pre-proenkephalin signal peptide sequence, as reported [31]. Substitution mutations such as S8D and E22G or N-terminal truncated Aβ 3Q42 have been identified from patients and those mutations in Aβ42 facilitate aggregation in fly and mouse [32-36]. L17P mutation inhibits Aβ aggregation in PC12 cells [37]. We established transgenic flies using each of these constructs, and confirmed their biochemical properties by western blotting of protein extracts from heads of 30 days flies. Expression of Aβ was detected in SDS-soluble

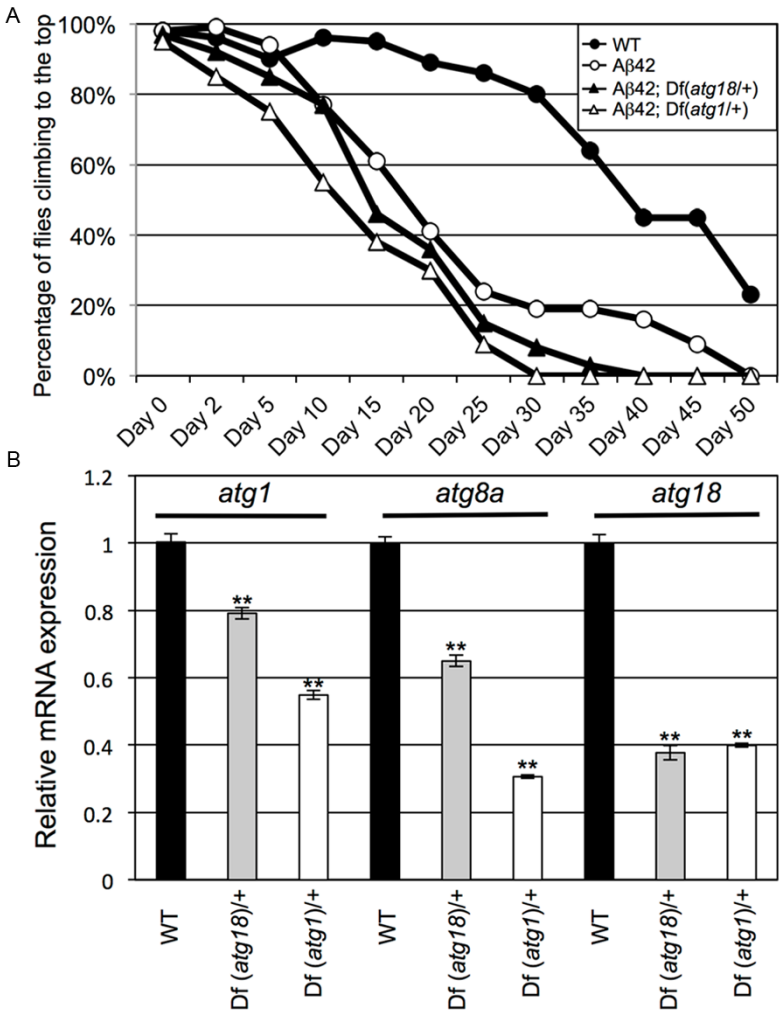

Figure 4. Expression of autophagy-related genes correlates with neuronal toxicity caused by Aβ42. A. Locomotor activity of *Aβ42*, *Aβ42; Df(atg18)/+* and *Aβ42; Df(atg1)/+* flies. Aβ42=elav > Aβ42; B. Expression of *atg1*, *atg8a* and *atg18* in *Df(atg1)/+* and *Df(atg18)/+* flies was quantified by qPCR (means±SD, n=4). Statistical significance of *Df atg18/+* or *Df atg1/+* against WT was analyzed by student t-test (**$P < 0.01$). *Df(atg1)=E44*, *Df(atg18)=ZP1*.

fractions (**Figure 2A**). Aggregation of Aβ was confirmed in SDS-insoluble formic acid fractions in transgenic flies (**Figure 2B**).

To test the transgenic flies for Aβ-dependent neuronal toxicity, we overexpressed Aβ and its two mutants using a pan-neuronal dependent Gal4 line, *elav-Gal4*. We used a climbing assay to monitor the locomotor activity of newly eclosed flies every 5 days for 50 days (**Figure 2C**). Aβ42, Aβ42 E22G, and Aβ42 L17P flies demonstrated 70-100% climbing disability by 40 days, whereas WT flies showed only 40% disability at 40 days (**Figure 2D-G**). These data support previous reports that Aβ accelerates age-induced locomotor dysfunction.

Expression of autophagy-related genes affects development of AD

It has been thought that the neuronal toxicity of AD is mediated by regulation of autophagy-related gene expression by Aβ [41]. To confirm this possibility, we analyzed the expression of autophagy-related genes *atg1*, *atg8a* and *atg18* in Aβ42 transgenic flies at days 4 and 30. We found that expression of all three genes in Aβ42 transgenic flies was reduced with age, similar to the reduced expression in WT flies (**Figure 3**). These results indicate that Aβ42 did not affect the level of autophagy-related gene expression. Aβ42 mutations such as L17P and Aβ42 E22G also did not affect the expression, although they showed climbing disability.

Next, we analyzed the relationship between expression of autophagy-related genes and the toxic effect of Aβ42 on locomotor activity. To reduce the expression level of autophagy-related genes, we used two lines, *E44* and *ZP1*, which carry the deficiencies *Df(atg1)* and *Df(atg18)*, respectively. We found that *Df (atg1)/+* heterozygotes demonstrated strongly enhanced neuronal toxicity caused by Aβ42 (**Figure 4A**). *Df(atg18)/+* heterozygotes also demonstrated an enhanced phenotype, although it was less severe than in *Df(atg1)/+* flies. These results suggest that expression of *atg1* has an important role in the neuronal toxicity of Aβ42 during aging.

Autophagy-related genes have a positive autofeedback effect

To confirm the expression levels of autophagy-related genes in these deficiency backgrounds, we performed qPCR analysis on mRNA from heterozygote fly heads. We found that *Df (atg1)/+* decreased the expression not only of

atg8a and *atg18*, but of *atg1* itself as well (**Figure 4B**). *Df(atg18)/+* showed a similar tendency, although the effect was less strong (**Figure 4B**). These results suggest that there may be auto-feedback machinery that controls autophagy-related genes, and that *atg1* may play a central role in maintaining expression of autophagy-related genes.

Discussion

In this report, we have shown that expression of autophagy-related genes was regulated by age-related signaling. *dsir2* (a *Drosophila* SIRT1 homolog) and *dfoxo* were required to maintain *atg18* expression during aging (**Figure 1**), suggesting that, among autophagy-related genes, this gene specifically is regulated by *foxo/sir2* activity. Interestingly, aging seemed to affect expression of all autophagy-related genes tested, suggesting that aging and *foxo/sir2* may act at different levels to regulate autophagy-related gene expression.

Previous studies have shown that *sir2*, *foxo* and *4E-BP* are involved in regulating the *Drosophila* lifespan [40]. Our data, however, indicate that *4E-BP* antagonized expression of autophagy-related genes (**Figure 1D**). *4E-BP* is believed to be controlled by TOR signaling [39, 42]. Therefore, the negative effect of *4E-BP* on autophagy-related gene expression may be mediated through the effect of TOR signaling pathway, which also seems to antagonize autophagy-related gene expression.

Autophagy is highly correlated with lysosomal activity, and the autophagy-lysosome pathway is thought to be involved in many cellular processes [5, 43]. Recent studies indicate that lysosomal activity affects expression of autophagy-related genes. The lysosome nutrient sensing (LYNUS) machinery is responsible for sensing whether there are sufficient nutrients [44]. Under a sufficient nutrient status, the mammalian target of rapamycin complex 1 (mTORC1, a member of the LYNUS machinery) phosphorylates transcription factor EB (TFEB) on the lysosomal surface and inhibits its nuclear localization [45]. In this way, TFEB is unable to induce expression of lysosomal and autophagy-related genes under nutrient sufficient conditions. These results suggest that the level of autophagy-related genes might be regulated by the state of lysosome formation and autophagy itself. Here, expression of autophagy-related genes was affected by the activity of other autophagy-related genes as well as their own activity, suggesting that auto-feedback regulation is part of the mechanism used to maintain expression of autophagy-related genes in *Drosophila*.

In this study, we observed that reducing the expression of autophagy-related genes strongly enhanced the neuronal toxicity caused by Aβ expression. Furthermore, reducing *atg1* expression using the *Df(atg1)/+* heterozygote showed a more severe enhancement of Aβ-dependent neuronal toxicity than reducing *atg18* expression using the *Df(atg18)/+* heterozygote (**Figure 4**). Interestingly, *atg1* also demonstrated strong auto-feedback regulation, as reducing expression of *atg1* resulted in further defects in expression of *atg* genes. Therefore, it is possible that a drastic reduction in expression of many *atg* genes may contribute to the neuronal toxicity of Aβ42, and that aging and autophagy may be determinants of AD onset.

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Disclosure of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Address correspondence to: Dr. Leo Tsuda, Animal Models of Aging, Center for development of Advanced Medicine for Dementia (CAMD), National Center for Geriatrics and Gerontology, Morioka 7-430, Obu, Aichi, Japan 474-8511. Tel: +81-562-46-2311; Fax: +81-562-46-3157; E-mail: ltsuda@ncgg.go.jp

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