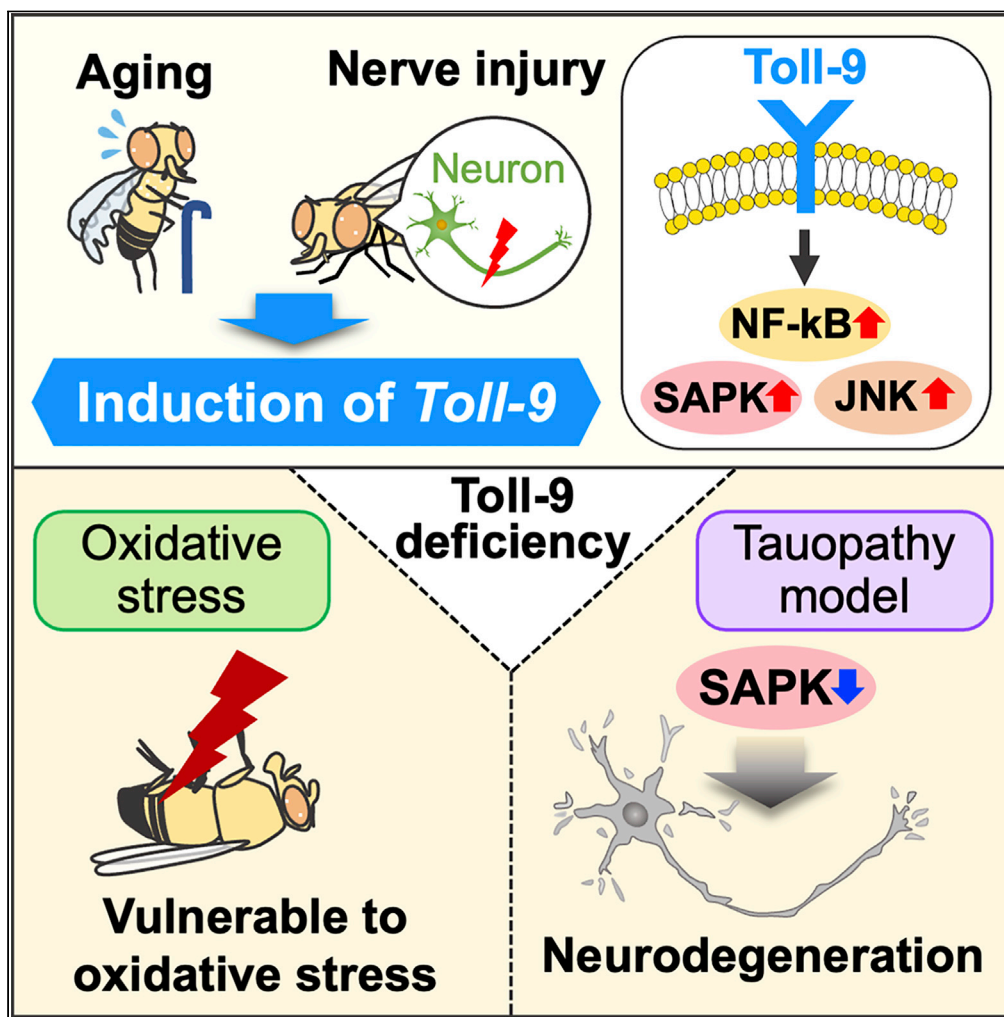


Article

Drosophila Toll-9 is induced by aging and neurodegeneration to modulate stress signaling and its deficiency exacerbates tau-mediated neurodegeneration



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Highlights

Toll-9 is induced in glia upon aging and modulates NF-kB, JNK and SAPK signaling

Toll-9 is dispensable for normal aging but affects lethality by oxidative stress

Toll-9 is induced upon acute nerve injury but does not affect glial phagocytosis

Toll-9 deficiency exacerbates neurodegeneration because of reduced SAPK signaling

Sakakibara et al., iScience 26, 105968
February 17, 2023 © 2023 The Author(s).
<https://doi.org/10.1016/j.isci.2023.105968>



Article

Drosophila Toll-9 is induced by aging and neurodegeneration to modulate stress signaling and its deficiency exacerbates tau-mediated neurodegeneration

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SUMMARY

***Drosophila Toll-9* is most closely related to mammalian Toll-like receptors; however, physiological functions of Toll-9 remain elusive. We examined the roles of Toll-9 in fly brains in aging and neurodegeneration. Toll-9 mRNA levels were increased in aged fly heads accompanied by activation of nuclear factor-kappa B (NF- κ B) and stress-activated protein kinase (SAPK) signaling, and many of these changes were modulated by Toll-9 in glial cells. The loss of Toll-9 did not affect lifespan or brain integrity, whereas it exacerbated hydrogen peroxide-induced lethality. Toll-9 expression was also induced by nerve injury but did not affect acute stress response or glial engulfment activity, suggesting Toll-9 may modulate subsequent neurodegeneration. In a fly tauopathy model, Toll-9 deficiency enhanced neurodegeneration and disease-related tau phosphorylation with reduced SAPK activity, and blocking SAPK enhanced tau phosphorylation and neurodegeneration. In sum, Toll-9 is induced upon aging and nerve injury and affects neurodegeneration by modulating stress kinase signaling.**

INTRODUCTION

Host immune responses against infection belong to either innate or adaptive immunity.^{1,2} Processes underlying adaptive immunity only exist in vertebrates,^{3,4} whereas innate immunity, a rapid and efficient defense mechanism that detects pathogen-associated molecular patterns through a set of pathogen recognition receptors,^{5,6} is well-conserved across animal species.^{7,8} In humans, ten Toll-like receptor (TLR) members (TLR1–10) function as pathogen recognition receptors against various microbial ligands, such as bacterial flagellin, lipopolysaccharide of Gram-negative bacteria, yeast zymosan, double-stranded RNA, and viral CpG-containing DNA.^{6,9–11} In *Drosophila*, the *Toll-1* gene was originally identified for its role in specifying dorsoventral polarity during embryogenesis^{12,13} and was later found to play essential roles in the production of antimicrobial peptides (AMPs).¹⁴

Unlike vertebrate TLRs, the Toll-1 receptor does not directly recognize microbial motifs. Instead, bacterial peptidoglycan is recognized by peptidoglycan recognition receptor SA and triggers a sequence of proteolytic cascade events leading to the cleavage of circulating immature cytokine pro-Spätzle into an active ligand.^{15,16} Mature Spätzle binds to the Toll-1 receptor and activates the highly conserved nuclear factor-kappa B (NF- κ B) signaling cascade^{17,18} to induce AMPs.^{19–21} In addition to canonical NF- κ B signaling, vertebrate TLRs and *Drosophila* Toll-1 activate noncanonical signaling, such as mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), and p38 stress-activated protein kinase (SAPK) pathways.^{22,23} For example, *Drosophila* Toll-1 promotes apoptosis through JNK signaling.^{24,25}

In addition to *Toll-1*, the *Drosophila* genome contains eight additional *Toll* genes: *Toll-2–9*.^{6,26} However, many of these Toll receptors do not play roles in immunity but rather in neurodevelopment.^{27–30} Among them, Toll-9 is most closely related to mammalian TLRs at a structural level,^{18,31} and according to the *Drosophila* RNAi Screening Center 20 (DRSC) Integrative Ortholog Prediction Tool (https://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl), TLR10 shows the highest similarity with fly Toll-9 among ten human TLRs. TLRs are type-I transmembrane receptors composed of an extracellular leucine-rich repeats domain, a single transmembrane domain, and an intracellular region called the Toll-interleukin receptor domain.^{32,33} Toll-9 is similar to vertebrate TLRs, both in its ectodomain and Toll-interleukin receptor

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domain. Unlike other Toll receptors, Toll-9 does not have multiple cysteine-rich motifs, which act as auto-inhibitory domains unique to *Drosophila* Toll receptors, in its ectodomain.^{18,34}

Several studies have shown that Toll-9 functions as a constitutively active receptor, and *Toll-9* overexpression triggers basal production of AMPs, including drosomycin and cecropin, in cultured cells and transgenic flies.^{35,36} However, a study with *Toll-9* knockout (KO) flies has shown that Toll-9 is dispensable for basal and immune-induced AMP production and for intestinal responses to oral bacterial infections.³⁵ More recently, activation of Toll-9/NF- κ B signaling has been shown to induce apoptosis to eliminate unfit cells from developing tissues during cell competition³⁷ and to promote a special form of compensatory proliferation of surviving cells.³⁸ However, physiological functions of Toll-9 in adult flies remain elusive.

A series of recent reports highlight critical roles of *Drosophila* Toll receptors in central nervous system (CNS) structural plasticity^{27,28} and cell death.^{24,25,37} In addition, mammalian TLRs are implicated in age-related neurodegenerative diseases.^{39,40} In this study, we systematically examined the roles of Toll-9 in the processes of aging and neurodegeneration.

RESULTS

Toll-9 mRNA levels are increased in fly heads during aging and modulate NF- κ B, JNK and SAPK signaling

To elucidate the roles of Toll-9 in the CNS during aging, we first examined whether mRNA levels of *Toll-9* and other *Toll* genes were altered upon aging in fly brains. Quantitative PCR (qPCR) analysis revealed that the expression of seven *Toll* receptors (*Toll-1*, -2, -4, -5, -6, -7, and -8) was readily detectable in young fly heads. Conversely, *Toll-9* expression was very low, and *Toll-3/MstProx* expression was under the detection limit. By contrast, *Toll-9* expression was strongly increased in aged fly heads (Figure 1A). In addition, *Toll-8/Tollo* expression was increased, whereas *Toll-4* expression was decreased during aging (Figure 1A). *Toll-1/TL*, *Toll-5/Tehao*, *Toll-6*, and *Toll-7* expression also showed a tendency to be increased; however, these changes did not reach statistical significance (Figure 1A). Next, to investigate how innate immune and stress kinase signaling is altered during aging, we examined the mRNA expression levels of genes downstream of NF- κ B, JNK, and SAPK pathways. These genes were significantly upregulated in aged fly heads (Figures 1B and 1C), as reported previously.^{41,42}

As *Toll-9* expression was most prominently increased during aging, we examined whether a loss of *Toll-9* has any effect on the observed age-dependent changes in gene expression. *Toll-9* KO flies were viable and developed normally.³⁵ We first examined the effects of *Toll-9* KO on the expression of other *Toll* genes in aged fly heads. Of interest, the mRNA expression levels of the *Tolls* that showed age-dependent increases in fly heads (Figure 1A) (*Toll-1/TL*, *Toll-5/Tehao*, *Toll-6*, *Toll-7*, and *Toll-8/Tollo*) were lower (Figure 1D), whereas that of *Toll-4* (which showed an age-dependent decrease in aged fly heads (Figure 1A)) was higher in *Toll-9* KO compared to wild-type (WT) aged fly heads (Figure 1D). These results suggest that *Toll-9* affects the expression of many *Toll* genes, except for *Toll-2*, in fly heads during aging. Next, we examined whether *Toll-9* KO affects genes downstream of NF- κ B, JNK, and SAPK signaling. We found that mRNA expression levels of many of these genes, which showed age-dependent increases in fly heads (Figures 1B and 1C), were significantly lower in *Toll-9* KO than WT aged fly heads (Figures 1E and 1F). Previous studies reported cross-regulation of the Toll and Imd pathways in innate immune response.^{43,44} Supporting this, a loss of *Toll-9* reduced expression of AMP genes known to be activated by the Imd as well as Toll signaling. These results suggest that *Toll-9* modulates NF- κ B, JNK and SAPK signaling during aging in fly brains.

Toll-9 knockdown in glial cells attenuates age-dependent upregulation of Toll family genes and genes downstream of NF- κ B, JNK and SAPK signaling

Glial cells play a central role in innate immunity via TLR-mediated signaling in the mammalian CNS.⁴⁵ We first examined whether *Toll-9* is expressed and functions in fly glial cells. In two independent fly lines carrying RNAi transgenes targeting different *Toll-9* regions, *Toll-9* knockdown in glial cells by the pan-glial repo-GAL4 driver significantly reduced *Toll-9* expression (Figure 2A, compare *Toll-9* RNAi #1 or *Toll-9* RNAi #2 and control *mCherry* RNAi with same genetic background), confirming both the expression of *Toll-9* in glial cells and knockdown efficiency of these RNAi lines. We next investigated whether *Toll-9* knockdown in glial cells attenuates age-dependent increases in the expression of *Toll* genes and genes downstream of NF- κ B, JNK and SAPK signaling. qPCR analysis revealed that the expression of many of

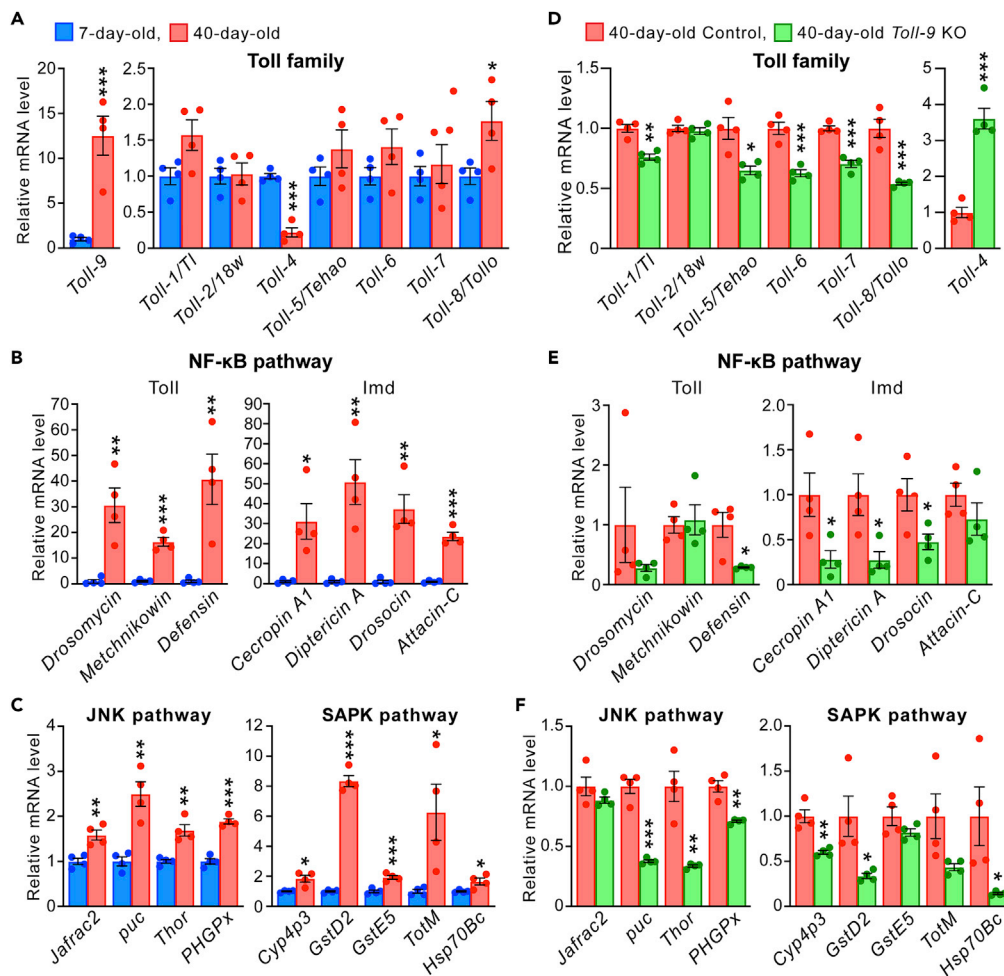


Figure 1. *Toll-9* mRNA levels are increased in fly heads during aging and modulate NF- κ B, JNK and SAPK signaling
(A–C) The mRNA expression levels of *Toll*-related genes in young (7-day-old) and aged (40-day-old) fly heads. The mRNA expression level of *Toll-9* was elevated in aged fly heads (A), and the genes downstream of NF- κ B (B), JNK and SAPK (C) pathways were upregulated in aged fly heads.
(D–F) The mRNA expression levels of *Toll*-related genes in aged *Toll-9* KO fly heads. A loss of *Toll-9* affected expressions of *Toll* family genes (D) as well as the genes downstream of NF- κ B (E), JNK and SAPK (F) pathways. The mRNA levels of *Toll*-related genes in fly heads were analyzed by qRT-PCR. Mean \pm SEM, n = 3–4; *p < 0.05, **p < 0.01 and ***p < 0.001 by Student's t test.

these genes was significantly lower in fly heads with *Toll-9* knockdown than in control aged fly heads (Figures 2B–2D). These results suggest that *Toll-9* in glial cells modulates gene expression downstream of NF- κ B, JNK and SAPK signaling.

The loss of *Toll-9* does not affect lifespan but slightly exacerbates hydrogen peroxide-induced lethality

A previous study reported that *Toll-9* was dispensable for antibacterial responses, whereas the loss of *Toll-9* shortened the lifespan of flies.³⁵ To minimize genetic background effects,^{46,47} we outcrossed *Toll-9* KO flies to our control w^{1118} background and examined the effects of *Toll-9* deficiency on age-related physiological changes. There were no significant differences between the lifespans of *Toll-9* KO and WT flies (Figure 3A, males and females). JNK and SAPK signaling is activated by environmental stressors, and thus we next investigated whether the loss of *Toll-9* affects resistance to oxidative stress. Exposure of flies to the oxidative stressor hydrogen peroxide (H_2O_2) reduced the survival of WT flies (Figure 3B, compare the survival of WT flies under 1% or 5% and Control), and *Toll-9* KO slightly worsened this

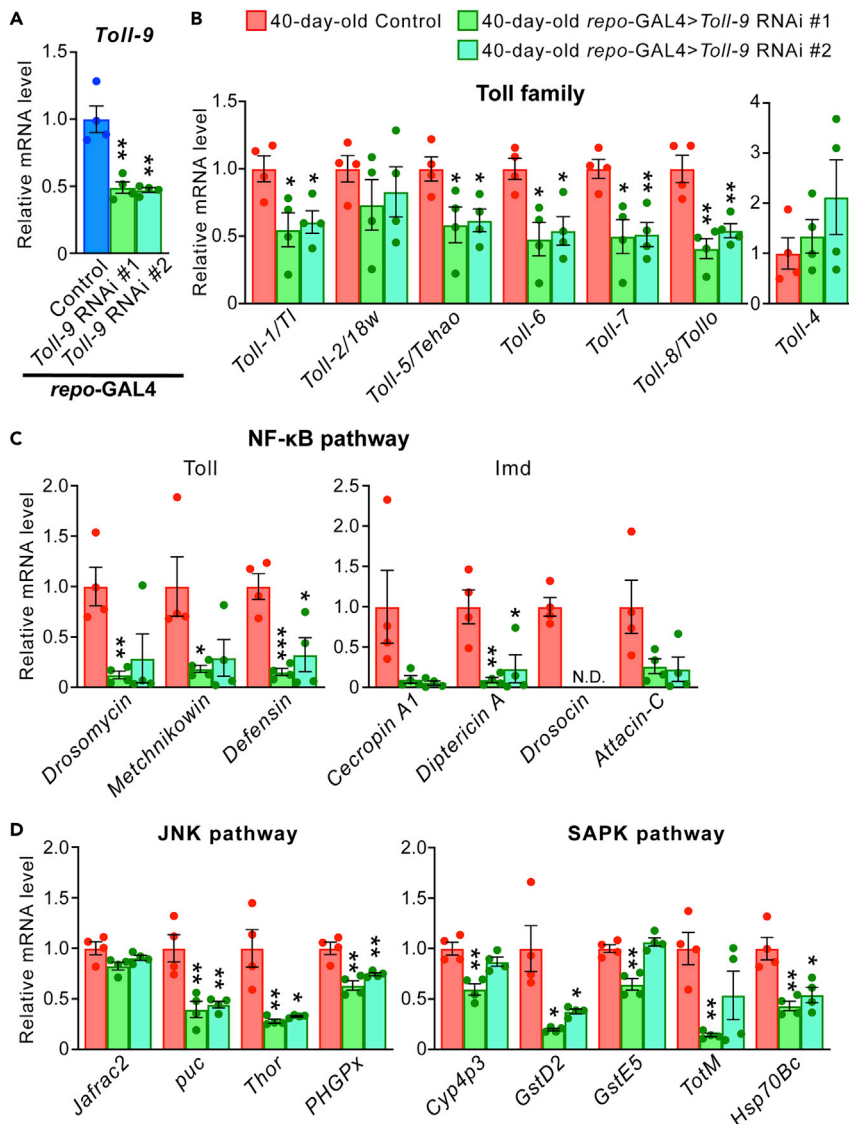


Figure 2. *Toll-9* knockdown in glial cells attenuates age-dependent upregulation of *Toll* genes and genes downstream of NF-κB, JNK and SAPK signaling

(A) Glial knockdown of *Toll-9* significantly decreased the mRNA expression level of *Toll-9* in fly head. The mRNA levels of *Toll-9* in heads of flies carrying the *repo-GAL4* driver (Control) or carrying both the *repo-GAL4* driver and *UAS-Toll-9* RNAi were analyzed by qRT-PCR. Mean \pm SEM, $n = 4$ (technical replicate); ** $p < 0.01$ by Student's *t* test versus Control.

(B–D) The mRNA expression levels of *Toll*-related genes in aged (40-day-old) fly heads expressing *Toll-9* RNAi in glial cells. Glial knockdown of *Toll-9* reduced the expression levels of *Toll* family genes (B) as well as the genes downstream of NF-κB (C), JNK and SAPK (D) pathways. The mRNA levels of *Toll*-related genes in fly heads were analyzed by qRT-PCR. Mean \pm SEM, $n = 3-4$; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's *t* test versus 40-day-old Control.

phenotype (Figure 3B, compare the survival of *Toll-9* KO and WT flies under 1% H_2O_2). These results suggest that the loss of *Toll-9* by itself does not affect lifespan but slightly exacerbates oxidative stress-induced lethality.

Neither *Toll-9* loss nor overexpression affects brain integrity during normal aging

To assess whether *Toll-9* plays any role in maintaining brain integrity during aging, we first investigated whether *Toll-9* KO affects spontaneous neurodegeneration. Neurodegeneration in fly brains is detected as vacuoles in neuronal cell body and neuropil regions.⁴⁸ The vacuole areas in several brain regions of

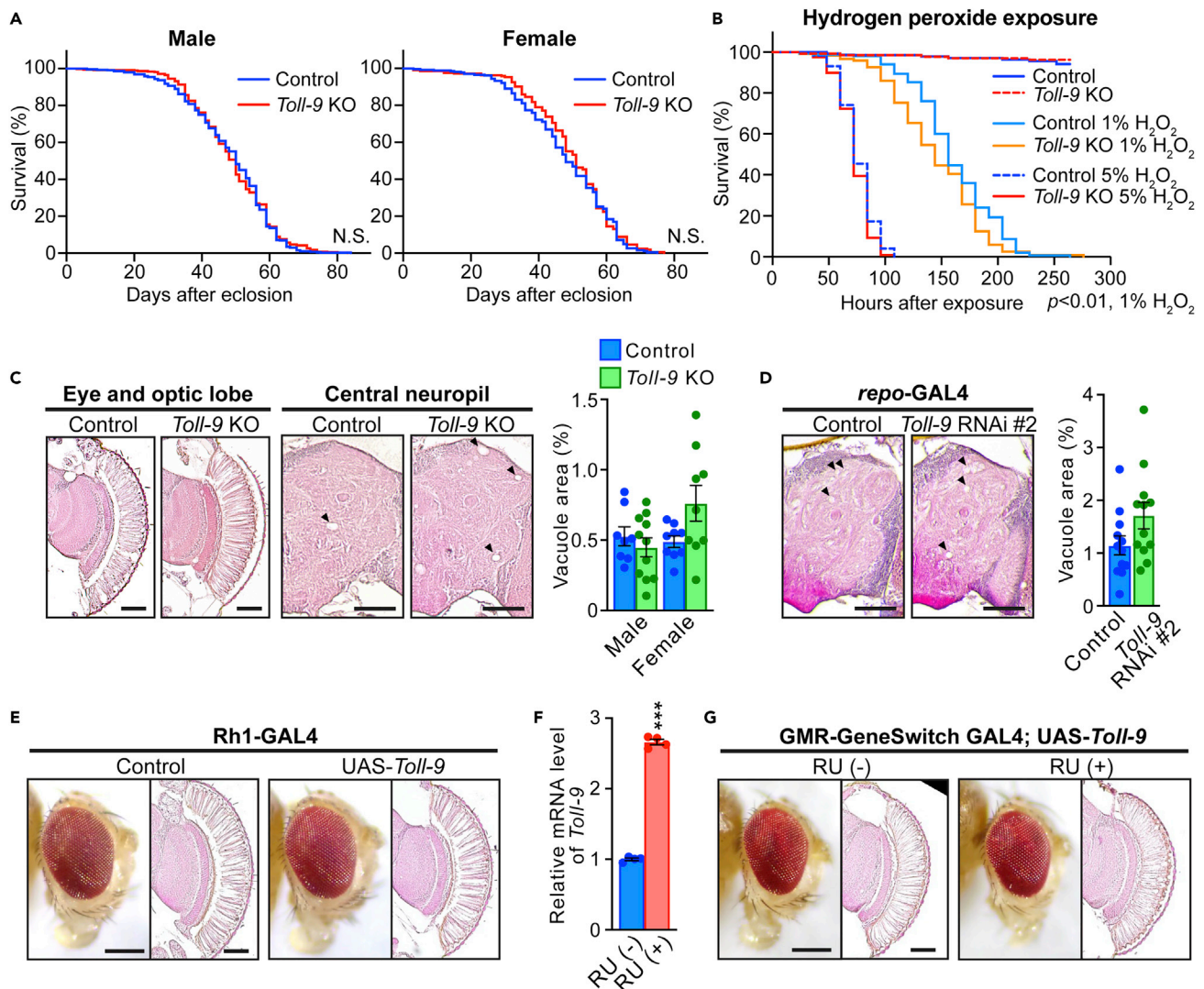


Figure 3. The loss of *Toll-9* does not affect lifespan and brain integrity during normal aging, but slightly exacerbates hydrogen peroxide-induced lethality

(A) A loss of *Toll-9* did not alter the lifespan in male (left panel) and female (right panel) flies (male; n = 464, control group or 383, *Toll-9* KO group; female; n = 266, control group or 214, *Toll-9* KO group).

(B) A loss of *Toll-9* significantly reduced survival time under 1% H₂O₂ exposure compared with that of Control flies (n = 135, Control with 10% sucrose only, n = 131, *Toll-9* KO with 10% sucrose only, n = 150, Control with 1% H₂O₂ exposure, n = 121, *Toll-9* KO with 1% H₂O₂, n = 174, Control with 5% H₂O₂ exposure, n = 119, *Toll-9* KO with 5% H₂O₂). The lifespans or survival times after H₂O₂ exposure of flies were determined by Kaplan-Meier survival analysis with logrank test. p < 0.01; Control versus *Toll-9* KO in 1% H₂O₂, not significant; Control versus *Toll-9* KO in 5% H₂O₂.

(C–G) Neither *Toll-9* loss nor overexpression affects brain integrity during normal aging.

(C) Eyes and central neuropils from paraffin-embedded brain sections with hematoxylin and eosin (HE) staining from 55-day-old control and *Toll-9* KO female flies are shown (left panel). Arrowheads indicate vacuoles. A loss of *Toll-9* did not cause age-dependent neurodegeneration in the central neuropil (right panel). Percentages of vacuole areas in the central neuropil from 55-day-old male and female flies were analyzed. Mean ± SEM, n = 8–11 hemispheres, not significant by Student's t test.

(D) RNAi-mediated glial knockdown of *Toll-9* did not affect age-dependent neurodegeneration. Central neuropils from 50-day-old male flies carrying the *repo-GAL4* driver (Control) or carrying both the *repo-GAL4* driver and UAS-*Toll-9* RNAi are shown (left panel). Percentages of vacuole areas in the central neuropil from 50-day-old male flies were analyzed (right panel). Mean ± SEM, n = 12 hemispheres, not significant by Student's t test.

(E) Post-developmental overexpression of *Toll-9* by the Rh1-GAL4 driver did not cause retinal degeneration. Eyes of female flies carrying the Rh1-GAL4 driver alone (Control) or carrying both the Rh1-GAL4 driver and UAS-*Toll-9* are shown.

(F–G) Overexpression of *Toll-9* in adult stage was induced by RU-486 feeding combined with the GeneSwitch GMR-GAL4 driver.

(F) The mRNA expression level of *Toll-9* was induced in male fly heads by RU-486 feeding for 30 days. The mRNA levels of *Toll-9* in fly heads were analyzed by qRT-PCR. Mean ± SEM, n = 4 (technical replicates); ***p < 0.001 by Student's t test versus RU (–).

(G) Induction of *Toll-9* expression in fly eyes during adult stage did not cause retinal degeneration. Eyes of male flies carrying both the GeneSwitch GMR-GAL4 driver and UAS-*Toll-9* with vehicle (RU (–)) and RU-486 (RU (+)) feeding are shown. All scale bars represent 200 μm.

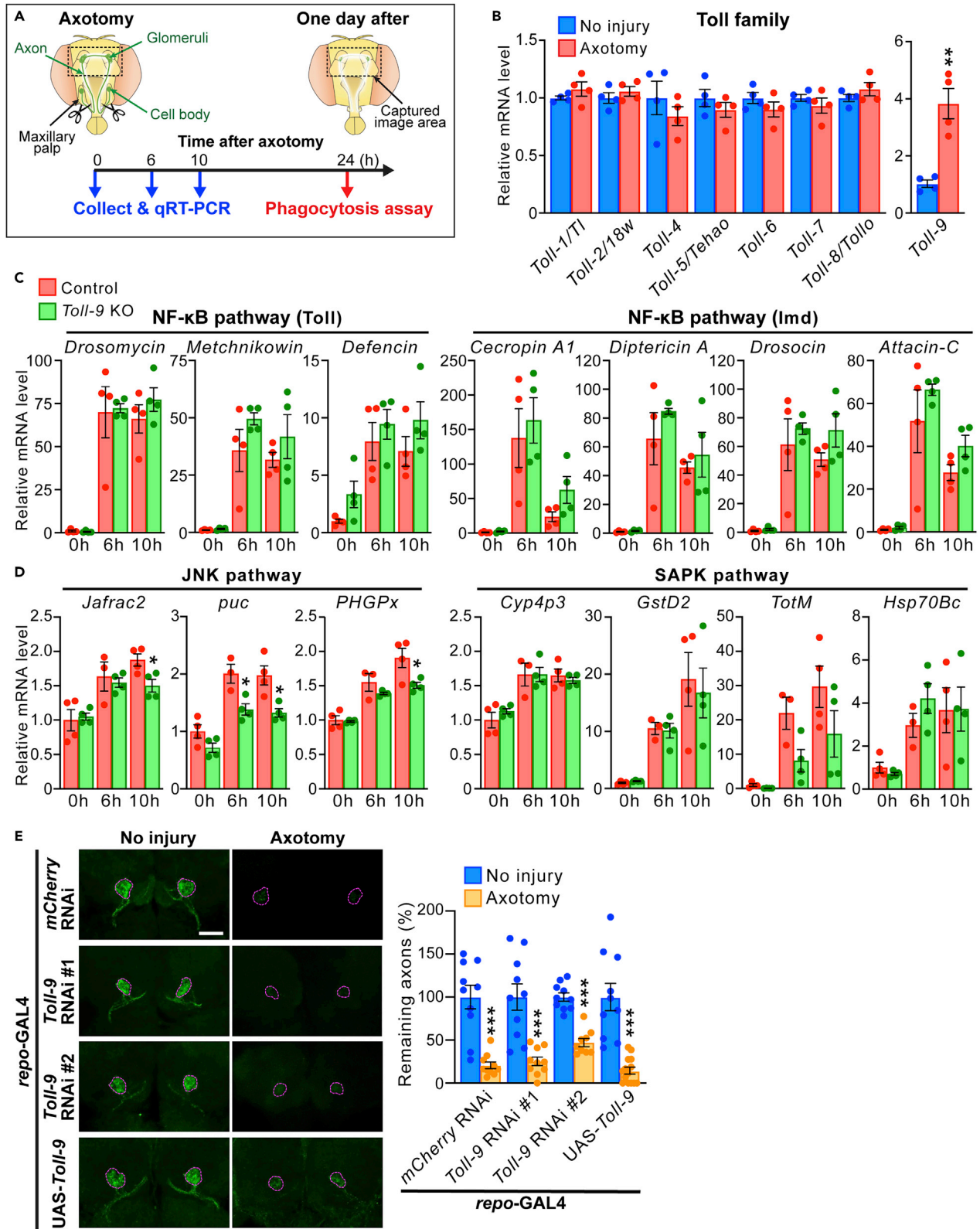


Figure 4. *Toll-9* mRNA expression levels are increased in response to acute nerve injury and modulate JNK signaling

(A) Schematic representation of axotomy treatment. Adult flies have a pair of the maxillary palps as an olfactory organ. The olfactory receptor neurons in the antennal lobe of fly brain have their cell bodies at the tip of the maxillary palps and extend long axons into the antennal lobe. Axotomy is induced by severing maxillary palps and 6, 10 or 24 h after axotomy, flies are subjected to qRT-PCR or phagocytosis assay.

(B) The mRNA expression levels of *Toll* family genes in fly heads at 0 (No injury) or 6 (Axotomy) hours after axotomy. Mean \pm SEM, n = 4; **p < 0.01 by Student's t test versus No injury.

(C–D) The mRNA expression levels of genes downstream of NF- κ B (C), JNK and SAPK (D) pathways in Control and *Toll-9* KO fly heads at 0, 6 and 10 h after axotomy. Mean \pm SEM, n = 3–4; *p < 0.05 by Student's t test versus Control.

(E) Neither knockdown nor overexpression of *Toll-9* in glial cells affects glial engulfment of degenerating neurons in fly brains 24 h after axotomy.

Representative z stack projections of OR85e GFP + axons (dotted line) from the groups with No injury or Axotomy of flies carrying UAS-*mCherry* RNAi, UAS-*Toll-9* RNAi #1, UAS-*Toll-9* RNAi #2, and UAS-*Toll-9* under the repo-GAL4 driver are shown (left panels). Scale bar represents 25 μ m. The right graph shows the percentages of GFP fluorescence normalized by the value of fluorescence intensity from the group with No injury. Mean \pm SEM, n = 9–14 (*mCherry* RNAi: No injury n = 10, Axotomy n = 10; *Toll-9* RNAi #1: No injury n = 10, Axotomy n = 10; *Toll-9* RNAi #2: No injury n = 10, Axotomy n = 9; UAS-*Toll-9*: No injury n = 10, Axotomy n = 14); ***p < 0.001 by Welch's t-test versus No injury.

55-day-old WT and KO flies exhibited no statistically significant differences, although there was a tendency that neurodegeneration was slightly increased in female *Toll-9* KO flies (Figure 3C). We also examined the effects of glial knockdown of *Toll-9* on neurodegeneration in aged fly brains and found that there was a tendency that *Toll-9* knockdown slightly worsened neurodegeneration, although the difference did not reach statistical significance (Figure 3D). Previous studies have shown that *Toll-9* promotes apoptosis during development,³⁷ and that developmental *Toll-9* overexpression causes abnormal tissue development and lethality.³⁰ Consistent with these reports, developmental overexpression of *Toll-9* in eye imaginal discs by the GMR-GAL4 driver caused glazed eyes due to malformation of the internal structure of the retina (Figure S1). We next examined whether post-developmental induction of *Toll-9* induces neurodegeneration in adult fly brains. To avoid hypoplasia and lethality due to developmental *Toll-9* overexpression, we first used the Rh1-GAL4 driver to express *Toll-9* in differentiated photoreceptor neurons.^{49,50} *Toll-9* induction by Rh1-GAL4 did not cause developmental abnormalities or degeneration of photoreceptor neurons in flies up to 60 days old (Figure 3E). We also utilized the GMR-GAL4 driver combined with the chemically inducible GeneSwitch system to drive *Toll-9* induction in the retina in adult flies. Flies carrying UAS-*Toll-9* and the GeneSwitch GMR-GAL4 driver developed normally, and *Toll-9* expression was induced by feeding adult flies with RU-486 (mifepristone), as measured by qPCR (Figure 3F). *Toll-9* induction during the adult stage did not cause retinal degeneration in flies up to 55 days old (Figure 3G). These results suggest that *Toll-9* does not affect brain integrity under normal aging conditions in flies.

***Toll-9* mRNA expression levels are increased in response to nerve injury, but a loss of *Toll-9* does not either affect acute stress response or glial engulfment activity**

To examine the roles of *Toll-9* under neurodegenerative conditions, we investigated whether mRNA levels of *Toll-9* and other *Toll* genes are altered in response to acute neurodegeneration. For this purpose, we utilized a well-established nerve injury model.⁵¹ Olfactory receptor neurons in the antennal lobe of the fly brain have their cell bodies at the tips of the maxillary palps and extend long axons into the antennal lobe⁵² (Figure 4A). Severing the maxillary palps removes the cell bodies, causes axonal degeneration in the antennal lobe, and triggers host defense responses and tissue repair programs, such as the engulfment of cellular debris by surrounding glial cells.^{51,53} Using this model, we compared mRNA levels of *Toll-9* and other *Toll*-related genes in the fly brain before and 6 h after acute neurodegeneration. *Toll-9* expression was specifically and significantly increased after severing the maxillary palps (Figure 4B). We also examined the mRNA levels of genes downstream of NF- κ B, JNK, and SAPK signaling in fly brains 6 and 10 h after severing the maxillary palps and found that all these genes were upregulated (Figures 4C and 4D).

Next, we examined whether *Toll-9* KO affects the observed gene expression changes after acute nerve injury. We found that although the induction of genes downstream of the JNK pathway was slightly attenuated in *Toll-9* KO flies, mRNA levels of many genes downstream of NF- κ B, JNK and SAPK signaling were similarly induced in both WT and *Toll-9* KO flies (Figures 4C and 4D). These results suggest that a loss of *Toll-9* does not affect acute stress response induced by nerve injury in fly brains.

Upon removal of olfactory neuron cell bodies by severing the maxillary palps, degenerating axons in the antennal lobe are efficiently engulfed by surrounding glial cells through activation of *draper*, a mammalian ortholog of *MEGF10/11*.^{51,54} We investigated whether inducing *Toll-9* promotes glial clearance of degenerating axons. By labeling a subset of olfactory neurons with GFP, we assessed glial engulfment activity by quantifying

fluorescent axonal debris in the antennal lobe 24 h after axotomy. As expected, glial engulfment activity was lost in *drpr* mutant flies (Figure S2), whereas degenerating axons were removed in flies with glial *Toll-9* knockdown (Figure 4E, compare *Toll-9* RNAi #1 or *Toll-9* RNAi #2 and control *mCherry* RNAi) and heterozygous *Toll-9* KO (Figure S2, compare *Toll-9*^{+/-} and Control). Moreover, *Toll-9* overexpression in glial cells did not affect the removal of GFP-labeled degenerated axons by glial cells (Figure 4E).

Taken together, these results indicate that *Toll-9* expression was induced by nerve injury but did not either affect acute stress response or glial engulfment activity, suggesting that induction of *Toll-9* may be involved in subsequent neurodegenerative process.

Toll-9 deficiency exacerbates disease-related tau phosphorylation and neurodegeneration accompanied by reduced SAPK activity in a fly tauopathy model

Activated TLR signaling and dysregulated kinases including JNK and SAPK have been implicated in several neurodegenerative diseases.⁵⁵⁻⁵⁷ To investigate whether *Toll-9* KO affects neurodegeneration, we utilized a transgenic *Drosophila* that expresses human microtubule-associated protein tau, which is related to tauopathy.^{58,59} Ectopic expression of human tau under the control of the eye-specific GMR-GAL4 driver causes retinal degeneration, resulting in disorganized ommatidia structures and age-dependent and progressive degeneration of photoreceptor axons in the lamina.^{60,61}

We found that heterozygous *Toll-9* KO significantly worsened tau-induced axonal degeneration in the lamina (Figure 5A). Similarly, RNAi-mediated *Toll-9* knockdown significantly worsened tau-mediated neurodegeneration, whereas *Toll-9* knockdown alone did not affect eye structures (Figure 5B). As tau is hyperphosphorylated in diseased brains, and tau phosphorylation is often associated with toxicity,⁶² we examined whether *Toll-9* deficiency increases phosphorylation levels of tau. Western blot analysis using phosphorylation-specific antibodies revealed that heterozygous *Toll-9* KO significantly increased the levels of tau phosphorylated at AD-related AT8 (Ser202/Thr205/Thr208) and Thr217 sites and decreased the levels of non-phosphorylated tau at disease-related sites (Figure 5C). The levels of total tau protein, which are detected as multiple bands due to posttranslational modifications including varying degrees of phosphorylation, were not altered, suggesting that tau phosphorylation levels were increased by heterozygous *Toll-9* KO (Figure 5C).

In *Drosophila*, a major kinase responsible for tau phosphorylation is *Shaggy*, a homologue of mammalian glycogen synthase kinase (GSK) 3 α / β .^{62,63} The activity of GSK3 α / β is negatively regulated by Akt-mediated phosphorylation in vertebrates⁶⁴ as well as in *Drosophila*,^{65,66} and activation of Toll signaling antagonizes insulin signaling in the fat body via Akt inhibition.⁶⁷ In mammals, Akt is activated by phosphorylation at Ser473.⁶⁸ We examined whether heterozygous *Toll-9* KO reduced phosphorylation of *Drosophila* Akt at Ser505,^{68,69} equivalent to Ser473 of mammalian Akt, in flies expressing human tau. Western blot analysis revealed that Akt phosphorylation levels were not affected by heterozygous *Toll-9* KO (Figure 5D).

As JNK and SAPK are also reported to be involved in tau phosphorylation and toxicity,^{70,71} we next examined whether *Toll-9* deficiency alters JNK and/or SAPK activity in flies expressing tau. Western blot analysis revealed that heterozygous *Toll-9* KO significantly reduced levels of phosphorylated SAPK, but not JNK (Figure 5D), suggesting that reduced SAPK activity may affect tau phosphorylation and toxicity.

Blocking SAPK function exacerbates tau phosphorylation and neurotoxicity

Previous studies have shown that SAPK plays both protective and detrimental roles in neurodegenerative processes.^{72,73} As our results suggest that reduced activity of fly SAPKs is associated with exacerbated neurodegeneration (Figure 5), we examined whether blocking SAPK function enhances tau phosphorylation and/or neurodegeneration in a fly tauopathy model.

The *Drosophila* genome contains three SAPK-related genes (p38a, p38b, and p38c^{74,75}), and p38b is most ubiquitously expressed in fly tissues, including the CNS, and is an ortholog of human MAPK14/p38 α and MAPK11/p38 β .^{72,74} To assess whether reduced SAPK activity exacerbates neurodegeneration, we examined the effects of inactivation of fly SAPK on tau phosphorylation and tau-mediated neurodegeneration. By utilizing a transgenic line carrying the dominant negative form of p38b (DN-p38b),⁷⁶ which was generated by replacing Thr183 of the MAPKK target site with Ala, we co-expressed DN-p38b or control luciferase protein with human tau in the retina under the control of the GMR-GAL4 driver. qPCR confirmed significant increases in DN-p38b mRNA levels in fly heads (Figure S3). Flies co-expressing DN-p38b exhibited

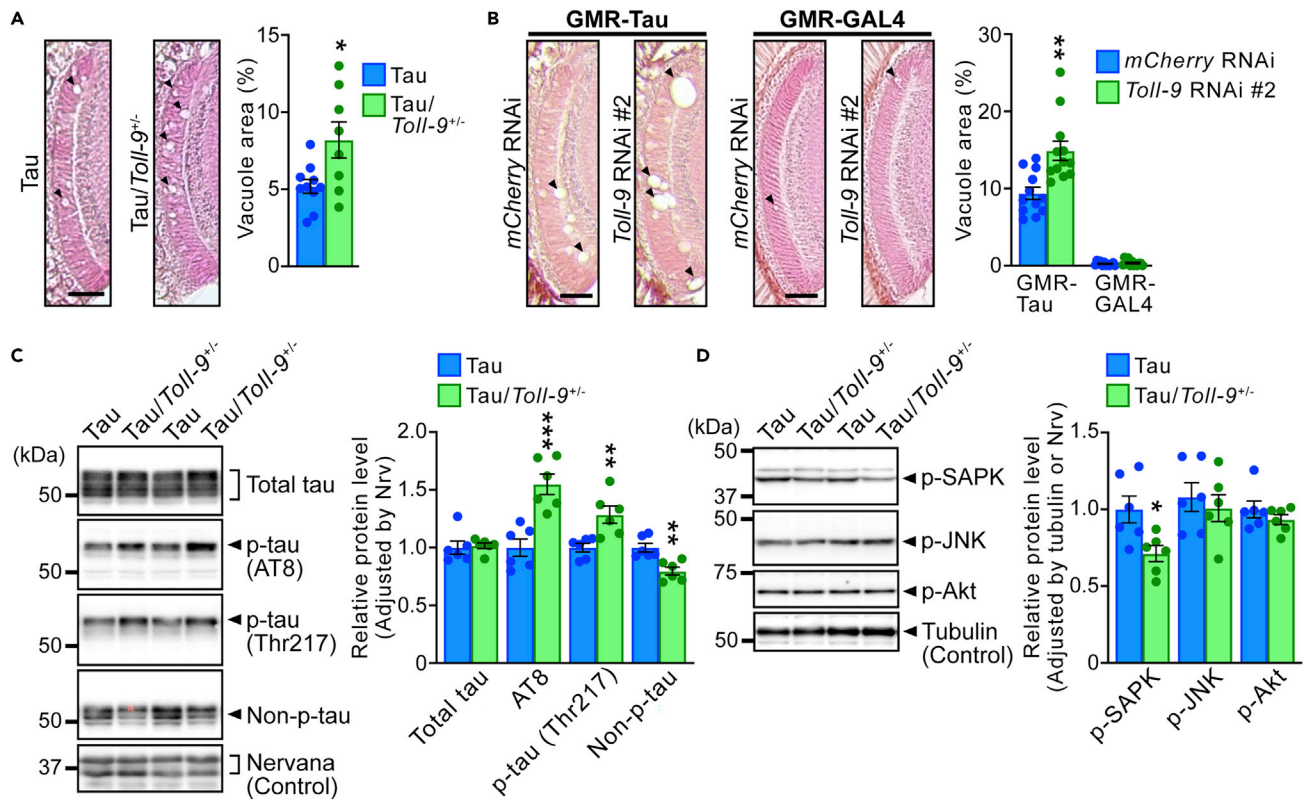


Figure 5. *Toll-9* deficiency exacerbates disease-related tau phosphorylation and neurodegeneration via reduced SAPK activity in a fly model of tauopathy

(A) The heterozygous KO of *Toll-9* exaggerated axon degeneration in the lamina caused by overexpression of human tau in fly eyes. Hematoxylin and eosin (HE) staining of the paraffin-embedded head section containing the lamina from 7-day-old flies expressing human tau under control background (Tau) and heterozygous KO of *Toll-9* (Tau/*Toll-9*^{+/-}) are shown (left panels). Percentages of vacuole areas in the lamina (indicated by arrowheads in the images) are shown. Mean ± SEM, n = 8–10 hemispheres, *p < 0.05 by Student's t test versus Tau.

(B) RNAi-mediated knockdown of *Toll-9* exaggerated axon degeneration in the lamina caused by overexpression of human tau in fly eyes. The lamina of flies expressing human tau and *mCherry* RNAi (GMR-Tau, *mCherry* RNAi), flies expressing human tau and *Toll-9* RNAi (GMR-Tau, *Toll-9* RNAi #2), flies expressing *mCherry* RNAi alone (GMR-GAL4, *mCherry* RNAi) or flies expressing *Toll-9* RNAi alone (GMR-GAL4, *Toll-9* RNAi #2) are shown (left panel). Percentages of vacuole areas in the lamina are shown in right panel. Mean ± SEM, n = 12 hemispheres, *p < 0.05 by Student's t test versus *mCherry* RNAi.

(C) Heterozygous KO of *Toll-9* increased the levels of tau phosphorylated at AD-related AT8 (Ser202/Thr205/Thr208) and at Thr217 sites. In contrast, the level of non-phosphorylated tau was decreased in heterozygous KO of *Toll-9*.

(D) Heterozygous KO of *Toll-9* significantly reduced the level of phosphorylated SAPK. Fly heads from Tau and Tau/*Toll-9*^{+/-} were subjected to western blotting with anti-tau, anti-phospho tau, anti-non-phospho tau, anti-phospho SAPK (p38), anti-phospho JNK and anti-phospho Akt antibodies. Nervana, a fly ortholog of ATPase Na⁺/K⁺ transporting subunit β1, or tubulin was used as the loading control. For (C) and (D), mean ± SEM, n = 6, **p < 0.01 by Student's t test versus Tau. Scale bars represent 100 μm.

significantly enhanced tau-induced axon degeneration in the lamina compared with flies co-expressing luciferase, whereas expression of DN-p38b alone did not cause neurodegeneration (Figure 6A). In addition, western blot analysis revealed that DN-p38b co-expression significantly increased the levels of tau phosphorylated at the AD-related AT8 (Ser202/Thr205/Thr208) site (Figure 6B). The total tau protein level was not altered, suggesting that tau phosphorylation levels were increased by DN-p38b (Figure 6B). These results indicate that blocking SAPK function enhances tau phosphorylation and neurodegeneration in a *Drosophila* model of tauopathy, and suggest that *Toll-9* deficiency exacerbates tau-mediated neurodegeneration at least in part due to reduced SAPK signaling.

DISCUSSION

Innate immune responses and anti-stress signaling are altered during aging and in neurodegenerative diseases. TLRs are evolutionarily conserved across animal species and play a central role in host defense mechanisms by activating the NF-κB and MAPK pathways.^{9,11} In *Drosophila*, *Toll-9* is most closely related

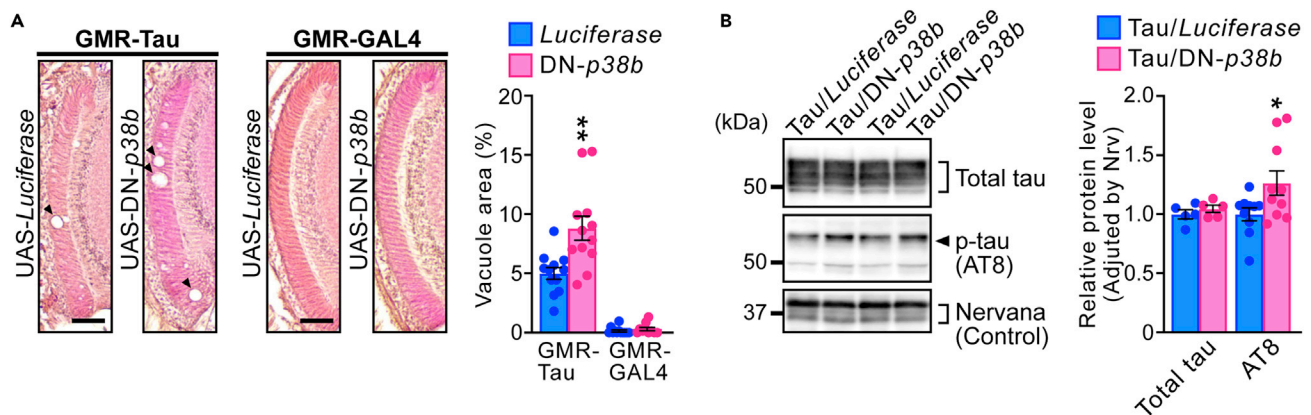


Figure 6. Blocking SAPK function by using dominant negative form of p38b exacerbates phosphorylation and neurotoxicity of tau

(A) The lamina of flies expressing human tau and control luciferase protein (GMR-Tau, UAS-Luciferase) or expressing human tau and dominant negative form of p38b (GMR-Tau, UAS-DN-p38b), flies expressing *Luciferase* alone (GMR-GAL4, UAS-Luciferase) or flies expressing DN-p38b alone (GMR-GAL4, UAS-DN-p38b) are shown (left panel). Percentages of vacuole areas in the lamina are shown in right panel. Mean \pm SEM, n = 10–12 hemispheres, **p < 0.01 by Student's t test versus *Luciferase*.

(B) Overexpression of DN-p38b significantly increased the levels of tau phosphorylated at AD-related AT8 site. Fly heads expressing Tau and control luciferase protein (Tau/*Luciferase*) or expressing Tau and DN-p38b (Tau/DN-p38b) were subjected to western blotting. Mean \pm SEM, n = 5–10, *p < 0.01 by Student's t test versus Tau/*Luciferase*. Scale bars represent 100 μ m.

to mammalian TLRs at a structural level^{18,31}; however, the physiological functions of *Toll-9* in adult flies remain elusive. In this study, we systematically examined the roles of *Toll-9* in the CNS during aging and under neurodegenerative conditions. Our results suggest that *Toll-9* is involved in neurodegenerative process by modulating stress kinase signaling in adult *Drosophila* brains.

Altered innate immunity is a general hallmark of aging and conserved across species.^{77–79} In humans, innate immunity is up- or down-regulated, and TLR signaling is generally activated due to neuroinflammation in aged brains.⁸⁰ AMP gene expression levels downstream of NF- κ B signaling are upregulated in aged *Drosophila* brains.^{41,42} Consistent with these reports, genes downstream of the Toll pathway, including those involved in NF- κ B, JNK, and SAPK signaling, were increased in aged fly heads (Figures 1B and 1C). Among nine Toll receptors, *Toll-9* expression was most prominently increased in aged fly heads, and many genes related to NF- κ B, JNK and SAPK pathways were attenuated in aged flies with *Toll-9* KO and glial *Toll-9* knockdown (Figures 1D–1F and 2B–2D). Our data suggest that *Toll-9* is induced as one of the downstream genes of innate immune and/or anti-stress signaling in aged fly brains to modulate activity of NF- κ B, JNK and SAPK pathways. Of interest, human TLR10 is the best ortholog of the *Drosophila* *Toll-9* gene, and recent reports have shown that TLR10 modulates signaling of other TLRs.^{81,82}

A previous study reported that the loss of *Toll-9* shortened the lifespan of flies, suggesting that *Toll-9* plays a protective role during aging.³⁵ In this study, we did not find significant differences in lifespan between WT and *Toll-9* KO flies (Figure 3A). These inconsistent results may be because of different genetic backgrounds of the flies used in the studies: Narbonne-Reveau et al. used *yw* flies as a control background,³⁵ whereas we outcrossed *Toll-9* KO flies to our control *w¹¹¹⁸* background. In addition, differences in experimental conditions and environmental factors might affect the lifespan of flies. In support of this, H₂O₂-induced lethality was slightly exacerbated in *Toll-9* KO flies (Figure 3B), suggesting that *Toll-9* plays a protective role against oxidative stress.

This study also revealed that *Toll-9* expression was induced in the model of acute nerve injury.^{51,54} In *Drosophila*, degenerated axons were removed by glial cells through activation of *draper*, a mammalian ortholog of MEGF10/11.^{51,54} More recently, *Toll-6/dSarm* signaling in glial cells was shown to promote *draper*-mediated phagocytic clearance of apoptotic neurons during development.⁸³ By contrast, our data suggest that *Toll-9* does not affect glial phagocytotic activity (Figures 4E and S2). In response to nerve injury, *Toll-9* as well as genes downstream of NF- κ B, JNK and SAPK signaling were acutely (6 and 10 h after nerve injury) induced in fly heads (Figures 4C and 4D). However, in contrast to the context of aging (Figures 1E and 1F), *Toll-9* KO did not affect the induction of genes downstream of NF- κ B, JNK and SAPK signaling induced by nerve injury (Figures 4C and 4D). These results suggest that basal, low expression level of *Toll-9* does not

play a role in acute activation of NF- κ B and stress kinase signaling upon neurodegeneration. Rather, *Toll-9* modulates these signaling pathways only when it is induced under stressed condition, which may explain why *Toll-9* lacks an autoinhibitory domain and acts as constitutive active receptor.³⁶

Accumulating evidence indicates that TLR signaling is actively involved in the pathogenesis of many neurodegenerative diseases, including Alzheimer's disease (AD) and tauopathy.^{84,85} Several studies reported that neuroinflammation due to microglial activation exacerbates tau pathology.^{86–89} Induction of intra-cerebral or systemic inflammation by lipopolysaccharide, a powerful pro-inflammatory component, promotes microglial activation through TLR4, which exacerbates hyperphosphorylated tau aggregation in both rTg4510 and 3xTg-AD mouse models.^{90,91} In addition, TLR3 activation by double-stranded RNA increases disease-related tau phosphorylation in cultured human neuronal cells.⁹² By contrast, activation of TLR signaling also plays neuroprotective roles against tau pathology: chronic and mild stimulation of TLR4 by lipopolysaccharide reduces tau phosphorylation in P301S-Tg mice,⁹³ and stimulation of TLR9 signaling with CpG oligodeoxynucleotides reduces tau pathology in 3xTg-AD mice.⁹⁴ To investigate the potential role of *Toll-9* in neurodegeneration, we utilized a fly tauopathy model that expresses human tau proteins,⁹⁵ and found that *Toll-9* loss significantly exacerbated disease-related tau phosphorylation and neurodegeneration (Figure 5). Our results contribute another line of evidence for the neuroprotective role of Toll-related signaling in tauopathy-related neurodegeneration.

As a mechanism by which *Toll-9* deficiency exacerbates neurodegeneration, we demonstrated the involvement of impaired activation of SAPK signaling (Figures 5 and 6). Several studies have indicated potential links between p38 MAPK/SAPK, tau phosphorylation, and neurodegeneration.⁷² p38 MAPK/SAPK can directly phosphorylate tau *in vitro*,⁹⁶ and a neuropathological study on AD patient brains has shown that p38 α MAPK/SAPK colocalizes with tau,⁹⁷ suggesting that p38 MAPK/SAPK may be related to tau pathology. Studies using mouse tauopathy models, including hTau and rTg4510 mice, have reported that inhibition of p38 α MAPK/SAPK with selective inhibitors reduces tau pathology and cognitive deficits by suppressing inflammation.^{98,99} By contrast, recent studies reported that activation of p38 γ MAPK/SAPK in neurons ameliorated cognitive deficits by reducing tau toxicity in mouse AD models.^{100,101}

Our results demonstrated that *Toll-9* deficiency significantly enhanced tau phosphorylation and toxicity (Figures 5A–5C) accompanied by reduced p38 MAPK/SAPK activity (Figure 5D). Moreover, inactivation of p38 MAPK/SAPK exacerbated disease-related tau phosphorylation and neurodegeneration (Figure 6), suggesting that *Toll-9* deficiency promotes tau-mediated neurodegeneration due to impaired activation of p38 MAPK/SAPK signaling. However, the mechanisms by which p38 MAPK/SAPK inactivation enhances tau phosphorylation and toxicity have yet to be addressed. Tau phosphorylation at many disease-related sites is mediated by GSK3 β ,^{102,103} and a recent report revealed that p38 MAPK/SAPK can directly phosphorylate and inactivate GSK3 β .¹⁰⁴ In addition, activation of p38 MAPK/SAPK signaling upregulates the expression of several anti-stress genes, which prevent oxidative stress and protein misfolding. Impairment of these signaling may also contribute to the enhancement of tau phosphorylation and toxicity. Further study is required to unravel neuroprotective mechanisms of p38 MAPK/SAPK.

In conclusion, this study demonstrates that *Toll-9* is induced upon aging and neurodegeneration to modulate stress signaling, and *Toll-9* deficiency exacerbates neurodegenerative process in a fly model of tauopathy.

Limitations of the study

Although this study demonstrates that *Toll-9* is induced to modulate NF- κ B and stress kinase signaling in aged fly heads and suggests a potential role of *Toll-9* in aging, a single knockout of *Toll-9* gene does not show prominent effects on either lifespan or brain integrity during normal aging. This may be due to compensatory functions of other Toll family genes. Among them, *Toll-4* is of particular interest, because mRNA expression level of *Toll-4* is significantly upregulated in *Toll-9* KO flies (Figure 1D). To address this possibility, generation and analysis of *Toll-4* single KO and *Toll-4/Toll-9* double KO flies will be important. In addition, roles of SAPK signaling in tau-mediated neurodegeneration need to be validated in mammalian systems in future study.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2023.105968>.

ACKNOWLEDGMENTS

We thank Dr. T. Aigaki at Tokyo Metropolitan University, Dr. K. Akagi at Toyama University, Dr. T. Awasaki at Kyorin University, Dr. M.R. Freeman at Oregon Health & Science University, Dr. J. Royet at Aix-Marseille Université, Dr. Y. Yagi at Nagoya University, and TRiP at Harvard Medical School (NIH/NIGMSR01-GM084947) and the Bloomington stock center for fly stocks. This study was supported by the Research Funding for Longevity Science from National Center for Geriatrics and Gerontology, Japan, Grant No. 21-13 to K.M.I. and M.S., No. 19-49 to M.S. and JSPS KAKENHI Grant No. JP16K08637, JP20K20672, JP20H03571, JP22K19763 to K.M.I., No. JP22H02963 to M.S. and No. JP20J15563 to R.Y.

AUTHOR CONTRIBUTIONS

Conceptualization, Y.S., R.Y., M.S., and K.M.I.; Investigation, Y.S., R.Y., S.C., Y.H., Y.T., R.N., K.T., and M.S.; Writing (original draft), Y.S., R.Y., M.S., and K.M.I.; Writing (review and editing), Y.S., R.Y., M.S., and K.M.I. and all authors; Supervision, M.S. and K.M.I.; Funding acquisition, R.Y., M.S., and K.M.I.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: November 14, 2022

Revised: December 6, 2022

Accepted: January 10, 2023

Published: February 17, 2023

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-tau antibody (clone Tau12)	Merck Millipore	Cat# MAB2241; RRID: AB_11211810
Mouse monoclonal anti-tau-1 antibody (clone PC1C6)	Merck Millipore	Cat# MAB3420; RRID: AB_94855
Mouse monoclonal anti-phospho tau (Ser202/Thr205/Thr208) antibody (clone AT8)	Thermo Fisher Scientific	Cat# MN1020; RRID: AB_223647
Rabbit polyclonal anti-phospho tau (Thr217) antibody	Thermo Fisher Scientific	Cat# 44-744; RRID: AB_2533741
Rabbit polyclonal anti-phospho p38 MAPK (Thr180/Tyr182) antibody	Cell Signaling Technology	Cat# 9211S; RRID: AB_331641
Rabbit monoclonal anti-phospho JNK (Thr183/Tyr185) antibody	Cell Signaling Technology	Cat# 4668S; RRID: AB_823588
Rabbit polyclonal anti-phospho <i>Drosophila</i> Akt (Ser505) antibody	Cell Signaling Technology	Cat# 4054S; RRID: AB_331414
Mouse monoclonal anti- <i>Drosophila</i> nervana protein antibody	Developmental Studies Hybridoma Bank	Cat# Nrv5F7; RRID: AB_528395
Mouse monoclonal anti- α -tubulin antibody	Sigma-Aldrich	Cat# T9026; RRID: AB_477593
Chicken polyclonal anti-GFP antibody	Aves Labs	Cat# GFP-1020; RRID: AB_10000240
Alexa Fluor 488 AffiniPure Goat Anti-Chicken IgY (IgG) (H + L)	Jackson ImmunoResearch	Cat# 103-545-155; RRID: AB_2337390
Chemicals, peptides, and recombinant proteins		
Hydrogen peroxide	Fujifilm Wako Chemicals	Cat# 081-04215
Sucrose	Fujifilm Wako Chemicals	Cat# 196-00015
Mifepristone	Tokyo Chemical Industry	Cat# M1732
Critical commercial assays		
ECL Prime Western Blotting Detection Reagents	GE Healthcare	Cat# RPN2236
Hematoxylin Solution, Mayer's	Sigma-Aldrich	Cat# MHS16
Eosin Y solution	Sigma-Aldrich	Cat# HT110132
TRIZol Reagent	Thermo Fisher Scientific	Cat# 15596018
PrimeScript RT reagent Kit with gDNA Eraser	TaKaRa Bio	Cat# RR047A
Thunderbird SYBR qPCR Mix	Toyobo	Cat# QPS-201
SlowFade Gold	Invitrogen	Cat# S36936
Experimental models: Organisms/strains		
<i>D. melanogaster</i> : UAS-human tau	Sekiya et al., 2017 ⁹⁵	N/A
<i>D. melanogaster</i> : Canton-S	Bloomington <i>Drosophila</i> Stock Center	BDSC: 64349; FlyBase: FBst0064349
<i>D. melanogaster</i> : Toll-9 KO	Narbonne-Reveau et al., 2011 ³⁵	N/A
<i>D. melanogaster</i> : UAS-Toll-9	Yagi et al., 2010 ³⁰	N/A
<i>D. melanogaster</i> : γ^1w^{67c23}	Yagi et al., 2010 ³⁰	N/A
<i>D. melanogaster</i> : Or85e-mCD8::GFP	MacDonald et al., 2006 ⁵¹	N/A
<i>D. melanogaster</i> : repo-GAL4 (X)	Lai et al., 2006 ¹⁰⁵	N/A
<i>D. melanogaster</i> : <i>drpr</i> ⁴⁵	Awasaki et al., 2006 ¹⁰⁶	N/A
<i>D. melanogaster</i> : GMR-GAL4	Bloomington <i>Drosophila</i> Stock Center	BDSC: 1104; FlyBase: FBst0001104
<i>D. melanogaster</i> : Rh1-GAL4	Bloomington <i>Drosophila</i> Stock Center	BDSC: 8688; FlyBase: FBst0008688

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>D. melanogaster</i> : GeneSwitch GMR-GAL4	Bloomington Drosophila Stock Center	BDSC: 6759; FlyBase: FBst0006759
<i>D. melanogaster</i> : repo-GAL4 (III)	Bloomington Drosophila Stock Center	BDSC: 7415; FlyBase: FBst0007415
<i>D. melanogaster</i> : UAS- <i>mCherry</i> RNAi	Bloomington Drosophila Stock Center	BDSC: 35785; FlyBase: FBst0035785
<i>D. melanogaster</i> : UAS- <i>Toll-9</i> RNAi #1	Bloomington Drosophila Stock Center	BDSC: 30535; FlyBase: FBst0030535
<i>D. melanogaster</i> : UAS- <i>Toll-9</i> RNAi #2	Bloomington Drosophila Stock Center	BDSC: 34853; FlyBase: FBst0034853
<i>D. melanogaster</i> : UAS-DN- <i>p38b</i>	Bloomington Drosophila Stock Center	BDSC: 59005; FlyBase: FBst0059005
<i>D. melanogaster</i> : attP2	Bloomington Drosophila Stock Center	BDSC: 36303; FlyBase: FBst0036303
<i>D. melanogaster</i> : UAS- <i>Luciferase</i>	This paper	N/A
Oligonucleotides		
qRT-PCR primers for <i>D. melanogaster</i> , see Table in STAR Methods	This paper	N/A
Software and algorithms		
Prism 9	GraphPad	https://www.GraphPad.com
Excel	Microsoft	https://www.microsoft.com
ImageJ	NIH	https://imagej.nih.gov/ij/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Koichi M. Iijima (iijimakm@ncgg.go.jp).

Materials availability

The materials underlying this article will be shared upon request to the [lead contact](#).

Data and code availability

This study did not generate original code. All data produced in this study are included in this article and supplementary information. Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila genetics

Flies were maintained in standard cornmeal media at 25°C. Transgenic fly line carrying UAS-human tau was previously described.⁹⁵ Canton-S line is a kind gift from Dr. K. Akagi (Toyama University). *Toll-9* KO fly is a kind gift from Dr. J. Royet (Aix-Marseille Université).³⁵ UAS-*Toll-9* and the background strain y^1w^{67c23} line (used as a control for UAS-*Toll-9*) are kind gifts from Dr. Y. Yagi (Nagoya University).³⁰ Or85e-mCD8::GFP fly is a kind gift from Dr. M.R. Freeman (Oregon Health & Science University).⁵¹ Repo-GAL4 (X) driver line and *drpr^{Δ5}* mutant fly are kind gifts from Dr. T. Awasaki (Kyorin University).^{105,106} The GMR-GAL4 (#1104), Rh1-GAL4 (#8688), GeneSwitch GMR-GAL4 (#6759), repo-GAL4 (III) (#7415), UAS-*mCherry* RNAi (#35785), UAS-*Toll-9* RNAi #1 (#30535), UAS-*Toll-9* RNAi #2 (#34853), UAS-DN-*p38b* (#59005) and attP2 background line (#36303) were obtained from the Bloomington Stock Center. The UAS-*Luciferase* transgenic flies were generated by PhiC31 integrase-mediated transgenesis systems (Best Gene Inc.). Experiments were performed using age-matched male or female flies. Genotypes and ages of all flies used in this study are described in [Table S1](#).

METHOD DETAILS

Histological analysis

Preparation of paraffin sections, hematoxylin and eosin staining, and analysis of neurodegeneration were performed as described previously.^{60,61,107} Heads of male or female flies were fixed in 4% paraformaldehyde for 24h at 4°C and embedded in paraffin. Serial sections (6 μm thickness) through the entire heads

were prepared, stained with hematoxylin and eosin (Sigma-Aldrich), and examined by bright-field microscopy. Images of the sections were captured with AxioCam 105 color (Carl Zeiss). For quantification, we focused on analyzing sections covering the central neuropil or lamina regions. We selected a section with the most severe neurodegeneration in each individual fly and the area of vacuoles was measured using ImageJ (NIH). Images of the external eye structures were captured with AxioCam 208 color (Carl Zeiss).

Lifespan analysis

Lifespan analysis was performed as described previously.⁶⁰ Food vials containing 25 male and female flies were placed on their sides at 25°C under conditions of 70% humidity and a 12:12-h light:dark cycle. Food vials were changed every 2–3 days, and the number of dead flies was counted each time. At least four vials for each genotype were prepared.

Hydrogen peroxide exposure assays

As described previously,⁶⁰ flies were starved with 2% agar medium for 2 h before hydrogen peroxide (H₂O₂) treatment. Two round and one rectangle filter papers were placed in empty vial, and 750 μL of 1% and 5% H₂O₂ (Fujifilm Wako Chemicals) in 10% sucrose (Fujifilm Wako Chemicals) solution was added to the filter papers. The dead flies were scored every 12 h. At least 119 male flies (7-day-old) per genotype were used to perform this experiment. For control experiment, at least 131 male flies (7-day-old) per genotype were administered with 750 μL of 10% sucrose solution.

RU feeding

As described previously,⁹⁵ flies carrying GeneSwitch GMR-GAL4 driver and UAS-*Toll-9* were fed the food containing 500 μM mifepristone (Tokyo Chemical Industry) or vehicle (ethanol; final concentration 1%) from the day after eclosion. These food vials were changed every 3–4 days. The male flies were collected at 30 days and 55 days after feeding for qPCR and histological analysis, respectively.

Western blotting

Western blotting was performed as described previously.^{60,61} More than ten fly heads for each genotype were homogenized in Tris-Glycine SDS sample buffer, and the same amount of the lysate was loaded to each lane of 10% Tris-Glycine gels and transferred to nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk, blotted with the antibodies described below, incubated with appropriate secondary antibody, and developed using ECL Prime Western Blotting Detection Reagent (Cytiva). The membranes were also probed with anti-nervana or anti- α -tubulin and used as the loading control for other blots in each experiment. Mouse monoclonal anti-tau (clone Tau12) (Merck Millipore, Cat#. MAB2241), mouse monoclonal anti-tau-1 (clone PC1C6) (Merk Millipore, Cat#. MAB3420) used for detection of non-phospho tau, mouse monoclonal anti-phospho tau (Ser202/Thr205/Thr208) (clone AT8) (Thermo Fisher Scientific, Cat#. MN1020), rabbit polyclonal anti-phospho tau (Thr217) (Thermo Fisher Scientific, Cat#. 44–744), rabbit polyclonal anti-phospho p38 MAPK (Thr180/Tyr182) (Cell Signaling Technology, Cat#. 9211S), rabbit monoclonal anti-phospho JNK (Thr183/Tyr185) (Cell Signaling Technology, Cat#. 4668S), rabbit polyclonal anti-phospho *Drosophila* Akt (Ser505) (Cell Signaling Technology, Cat#. 4054S), mouse monoclonal anti-*Drosophila* nervana protein (Developmental Studies Hybridoma Bank, Cat#. Nrv5F7) and mouse monoclonal anti- α -tubulin (Sigma-Aldrich, Cat#. T9026) antibodies were purchased. Imaging was performed with ImageQuant LAS 4000 (GE Healthcare Life Sciences), and the signal intensity was quantified using ImageJ (NIH).

RNA extraction and quantitative real time PCR analysis

Quantitative real time PCR analysis was performed as described previously.^{60,61} More than 25 flies for each genotype were collected and frozen. Heads were mechanically isolated, and total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol with an additional centrifugation step (16,000 × g for 10 min) to remove cuticle membranes prior to the addition of chloroform. Total RNA was reverse-transcribed using PrimeScript RT-PCR kit (TaKaRa Bio), and quantitative RT-PCR was performed using Thunderbird SYBR qPCR Mix (Toyobo) on a CFX96 real time PCR detection system (Bio-Rad Laboratories). The average threshold cycle value was calculated from at least three replicates per sample. Expression of genes of interest was standardized relative to *Gapdh1*. Primer sequences used in this study were described in [Table S2](#).

Axotomy treatment

Axotomy was induced by maxillary palp ablations as previously described.^{51,52} Maxillary nerves were severed by removing maxillary palps with forceps under CO₂ anesthesia. Flies were allowed to recover in fresh food vial and maintained at 25°C. Six or 10 h after axotomy, flies were frozen for qRT-PCR analysis.

Phagocytosis assay

One day after axotomy treatment, flies were subjected to whole mount immunostaining as described previously.¹⁰⁷ Briefly, fly brains were dissected in phosphate-buffered saline (PBS), and then fixed in 4% paraformaldehyde in PBS for 50 min. Brains were washed with 0.5% TritonX-100/PBS and blocked in 10% normal goat serum in 1% TritonX-100/PBS for 10 min. Chicken anti-GFP antibody (1:500; Aves Labs, Cat#. GFP-1020) was added and incubate for overnight at 4 °C. The next day, the brains were washed three times with 0.5% TritonX-100/PBS and incubated with anti-chicken Alexa488 secondary antibody (1:1,000; Jackson ImmunoResearch, Cat#. 103-545-155) for 2 h at room temperature. Brains were washed three times with 0.5% TritonX-100/PBS and rinsed with PBS, and then mounted on glass coverslip with antifade mounting media (SlowFade™ Gold, Invitrogen).

Images were acquired using either a LSM780 or LSM800 confocal laser-scanning microscope (Carl Zeiss) fitted with 20× objective. For all experiments, the entire antennal lobe was imaged in 1 μm steps. Severed and non-severed brains from the same genotype were imaged on the same day with the same confocal microscope settings. Quantification of fluorescence intensity was performed on single z sections in each side of the relevant glomerulus using ImageJ (NIH). For representative images in [Figures 4E](#) and [S2](#), z stack confocal images were reconstructed with a maximum intensity projection.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics

All results were expressed as mean ± SEM. Unpaired Student's t test (Excel, Microsoft) was used to determine statistical significance as indicated in the figure legends. Kaplan-Meier survival analyses with log-rank tests (GraphPad Prism 9, GraphPad Software) were used to determine statistical significance for lifespan analysis. Welch's t test (GraphPad Prism 9, GraphPad Software) was used to determine statistical significance for phagocytosis assay. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ throughout the manuscript.