Tau Accumulation *via* Reduced BAG3-mediated Autophagy Is Required for GGGGCC Repeat Expansion-Induced Neurodegeneration

Running title: G4C2 Induces Neurodegeneration *via* Autophagy and Tau

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SUMMARY

Expansions of trinucleotide or hexanucleotide repeats lead to several neurodegenerative disorders including Huntington disease (HD, caused by the expanded CAG repeats (*CAGr*) in the *HTT* gene) and amyotrophic lateral sclerosis (ALS, could be caused by the expanded GGGGCC repeats (*G4C2r*) in the *C9ORF72* gene), of which the molecular mechanisms remain unclear. Here we demonstrate that loss of the *Drosophila* orthologue of tau protein (dtau) significantly rescued *in vivo* neurodegeneration, motor performance impairments, and shortened life-span in *Drosophila* models expressing mutant HTT protein with expanded *CAGr* or the expanded *G4C2r*. Importantly, expression of human tau (htau4R) restored the disease-relevant phenotypes that were mitigated by the loss of dtau, suggesting a conserved role of tau in neurodegeneration. We further discovered that *G4C2r* expression increased dtau accumulation, possibly due to reduced activity of BAG3-mediated autophagy. Our study reveals a conserved role of tau in *G4C2r*-induced neurotoxicity in *Drosophila* models, providing mechanistic insights and potential therapeutic targets.

INTRODUCTION

An expansion mutation of the GGGGCC repeat (*G4C2*) within intron 1 of *C9orf72* is a common mutation associated with of sporadic amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (DeJesus-Hernandez et al., 2011; Renton et al., 2011). ALS is a fatal neurodegenerative disorder primarily affecting motor neurons, whereas FTD is causing neurodegeneration primarily in the frontal, insular, and anterior temporal cortex. The aggregation-prone dipeptides synthesized from the expanded *G4C2 via* repeat-associated non-ATG (RAN) translation and/or the *G4C2* RNA foci / membraneless granules originated *via* phase separation are likely the cause of neurodegeneration, but the downstream molecular mechanisms remain unclear. Abnormalities of the microtubule-binding protein tau play a central role in several neurodegenerative diseases termed tauopathies, including

Alzheimer's disease (AD), Progressive supranuclear palsy (PSP) and tau-positive FTD with parkinsonism (FTDP-17), etc.. Among them, FTDP-17 is a type of FTD caused by aberrant splicing of tau, and similar mechanisms mediate the pathology in Huntington disease (HD), another disease caused by nucleotide repeat expansion (the CAG repeat expansion in exon1 of the *HTT* gene) (Fernandez-Nogales et al., 2014). Thus, we investigated whether tau may mediate *G4C2*-induced neurotoxicity, which might share molecular commonalities in their pathological mechanisms with CAG repeat expansion diseases.

Drosophila models have been widely used to study genetic neurodegenerative disorders, especially CAG expansion and GGGGCC expansion diseases, probably because of their monogenetic nature. Many of the genetic or chemical modifiers identified in these models have been validated in patient cells and mammalian models. In this study, we first characterized the pathophysiological function of *Drosophila* orthologue of tau (dtau) in HD models. We confirmed a conserved role of dtau in HD neurotoxicity by showing that the loss of dtau significantly rescued phenotypes in HD *Drosophila* models expressing human mutant HTT protein (mHTT) exon1 fragment, consistent with the observation in an HD mouse model expressing the mHTT exon1 fragment (Fernandez-Nogales et al., 2014). We then investigated the potential role of dtau in *G4C2*-induced neurotoxicity in *Drosophila* models, and explored tau-relevant pathogenic mechanisms.

RESULTS

dtau knockout rescued HD-relevant phenotypes including *in vivo* neurodegeneration, behavioral deficits and shortened life-span in *Drosophila* models.

In order to capture the neuronal morphology relatively clearly in the *Drosophila* brain *in vivo*, we utilized a simple *UAS-GAL4* system to express membrane GFP markers (mCD8GFP) in a very small group of neurons by *GMR61G12-GAL4*, so that their morphology could be clearly observed. We detected the *in vivo* HD-relevant neurodegeneration by observing the loss of major axon bundles in

the GFP labeled by neurons induced by expression of human mHTT but not wtHTT exon1 fragment (*HTT.ex1.Q72* versus *HTT.ex1.Q25*, Fig. S1A). The neurodegeneration occurs in both dendrites and axons (Figure S2).

At the whole animal level, expressing mutant HTTexon1 (*HTT.ex1.Q72*) by the pan neural driver *elav-GAL4* caused motor performance impairments and shortened life-span compared to ones expressing the wild-type version (*HTT.ex1.Q25*, Figure S1B-C), recapitulating HD-relevant phenotypes in patients.

We then investigated the potential role of tau in HD by testing the knockout of the *Drosophila tau* gene to eliminate the expression of dtau. *Drosophila tau* is the homolog of human *MAPT*, the gene expressing the tau protein. The dtau knockout line we utilized was previously reported to present a specific deletion of the genetic region spanning from exon 2 to exon 6, which codes for the microtubule-binding region of dtau (Burnouf et al., 2016). Interestingly, loss of dtau significantly rescued HD-relevant *in vivo* neurodegeneration, behavioral deficits and shortened life-span phenotypes, suggesting that dtau mediates mHTT neurotoxicity, at least partially (Figure S1A-C). The observation was consistent with the study using the mouse R6/2 HD model, confirming a conserved role of dtau in neurodegeneration. Expressing human tau (htau4R) restored HD-relevant phenotypes in the dtau KO *Drosophila* (Figure S1C-D), further validating the conservation of dtau function in neurodegeneration.

dtau knockout rescued expanded *G4C2*-induced ALS-relevant phenotypes including *in vivo* neurodegeneration, behavioral deficits and shortened life-span.

The study using HD models demonstrated a conserved role of dtau in neurodegeneration, and thus we investigated potential functions of dtau in expanded *G4C2*-induced neurotoxicity in a well-characterized and widely used *Drosophila* model expressing 30 repeats of *G4C2* ($(G4C2)_{30}$) (Xu et al., 2013). Similar as the HD *Drosophila* model, *UAS-mCD8GFP-GAL4* driven $(G4C2)_{30}$ expression

led to *in vivo* neurodegeneration (Figure 1A). In addition, $(G4C2)_{30}$ expression in the motor neurons driven by *UAS-mCD8GFP-GAL4* led to significant reduction of neuro-muscular junctions (NMJs), which were detected by the NMJ marker Brp and axon marker HRP (Figure 1B). At the whole animal level, neuronal expression of $(G4C2)_{30}$ driven by *elav-GAL4* led to motor performance impairments and shortened life-span (Figure 1C-D). Loss of dtau significantly mitigated all these disease-relevant phenotypes (Figure 1A-D), suggesting that dtau may mediate neurotoxicity induced by expanded *G4C2* as well.

We then examined whether dtau and human tau are conserved in mediating the expanded *G4C2*-induced toxicity by expressing htau4R in the neurons driven by *elav-GAL4*. htau4R expression restored $(G4C2)_{30}$ induced neurodegeneration, deficient motor performance and the shortened life-span phenotypes in the dtau KO *Drosophila* (Figure 1C-D), confirming that tau plays a conserved role in the expanded *G4C2*-induced neurodegeneration as well.

The expanded *G4C2* increased tau protein levels in *Drosophila* and human cells

We then explored potential mechanisms regarding how dtau influenced the expanded *G4C2r*-induced toxicity. There are two major possibilities: 1. dtau is an upstream regulator of expanded *G4C2r* expression; 2. dtau is a downstream factor that is modulated by expanded *G4C2r* and mediates its neurotoxicity. Thus, we first examined whether dtau knockout lowered the expanded *G4C2r* RNA, which is likely the fundamental source of neurotoxicity in this model (Xu et al., 2013). Since it is extremely difficult to amplify the *G4C2* repeat region *per se* by quantitative PCR, we chose the upstream and downstream sequences for qPCR measurements (Figure S3A). We observed no significant change in expanded *G4C2r* mRNA levels in the dtau heterozygous knockout flies, suggesting that dtau is not an upstream regulator of expanded *G4C2r* expression (Figure S3B).

The evidence above suggested that dtau is more likely a downstream mediator of the expanded *G4C2r*-induced toxicity. We first examined potential missplicing of tau transcripts. Alternative splicing

of exon 10 of *MAPT* gene results in tau isoforms containing either three or four microtubule-binding repeats (3R and 4R, respectively) (Liu and Gong, 2008). Similar as the human tau, dtau proteins expressed from different *tau* transcripts (Figure S3C) contain either three or four putative tubulin-binding repeats (MTBDs) that are homologous with mammalian MTBDs (Heidary and Fortini, 2001). In addition, all dtau proteins have an extra MTBD that is homologous with *Caenorhabditis elegans* but not mammalian ones. Thus, dtau have the 4R versus 3R isoforms due to alternative splicing (or 5R versus 4R if we consider the extra MTBD), corresponding to 4R versus 3R isoforms in human tau. We thus measured different *dtau* transcript levels and the 3R/4R ratio in fly brains with $(G4C2)_{30}$ expression versus the control (no repeat or $(G4C2)_{3}$), and observed no significant changes in either the transcript levels or the 4R/3R ratio (Figure S3D), suggesting that the expanded *G4C2r* may not induce tau missplicing.

Increased level of tau protein is another possible driver of neurodegeneration. We thus investigated whether tau protein level is increased in the expanded *G4C2r* expressing *Drosophila* model.

Expression of expanded *G4C2r* ($(G4C2)_{30}$) led to increased tau protein levels in tissue samples from 3rd stage larvae, pupae and adult *Drosophila* (Figure 2A-C), suggesting that the expanded *G4C2r* expression increases tau accumulation. Consistent with the observation in *Drosophila*, exogenous expression of the expanded *G4C2r* ($(G4C2)_{25}$) in HEK293T cells also resulted in tau protein accumulation (Figure 2D). We further investigated levels of tau aggregates, which is likely a toxic biomarker associated with tau accumulation. Consistently, HeLa cells with expanded *G4C2r* expression showed drastic increase of tau aggregates (Figure 2E), suggesting that expanded *G4C2r* is capable of enhancing aggregated form of tau as well.

We further investigated the levels of phosphorylated tau (p-tau), which is considered as a main toxic species that causes neurodegenerations. Western-blots of brains of adult *Drosophila* expressing expanded *G4C2r* showed an increase of certain forms of phosphorylated tau (p-tau) as detected by

the p-tau antibody ps262 or AT8 (Figure S4A). Consistently, the p-tau increase by expanded *G4C2r* expression was observed in HEK293T cells as well (Figure S4B). The p-tau increase is likely a subsequence of increase tau accumulation (Figure 2A-E), and may also contribute to the neurotoxicity induced by the expanded *G4C2r* expression.

Expanded *G4C2r* expression decreased *BAG3* (*starvin*) mRNA levels and impaired the autophagy flux

The tau protein is known to be degraded *via* autophagy in the cells (Zhu et al., 2017), and thus we hypothesized that expanded *G4C2r* expression impaired autophagy function, resulting in tau accumulation as a consequence. We assayed autophagy flux *in vivo* by testing the cleavage of GFP-mCherry-atg8a. atg8a (autophagy-related protein 8a) in *Drosophila* is the homolog of the mammalian protein LC3, which is the key autophagosome protein that gets cleaved in lysosomes. Thus, GFP-atg8a or GFP-LC3 cleavage is a widely used assay for measurements of the autophagy flux (Kimura et al., 2007). By expressing *GFP-mCherry-atg8a* driven by *OK371-GAL4* in *Drosophila*, the autophagy flux in the motor neurons (expressing *OK371*) could be measured by the levels of free GFP, the cleaved product of the GFP-mCherry-atg8a protein. The free GFP band essentially disappeared in all the $(G4C2)_{30}$ expressing *Drosophila* brains tested compared to the ones from $(G4C2)_3$ expressing brains (Figure 2F), indicating a drastic impairment of autophagy flux. Consistently, the two other functional readout for autophagy flux including the levels of ubiquitinated proteins in the lysate precipitations and the ref(2)P protein (the *Drosophila* orthologue of *SQSTM1/p62* in mammals (DeVorkin and Gorski, 2014)) were significantly increased by $(G4C2)_{30}$ expression compared to the $(G4C2)_3$ expressing or the wild-type (*w1118*) controls (Figure S5), further demonstrating that the autophagy flux was impaired by the expanded *G4C2r* expression**.**

We then explored the potential molecular mechanisms of autophagy impairment. Studies have found that BAG3 (BAG family molecular chaperone regulator 3), a key autophagy modulator (Kathage et al.,

2017), is negatively associated with tau protein levels (Ji et al., 2019; Lei et al., 2015). We thus hypothesized that the expanded *G4C2r* expression may reduce BAG3 levels, leading to impaired autophagy function and tau accumulation. Consistent with this hypothesis, $(G4C2)_{30}$ expression in *Drosophila* significantly reduced the mRNA levels of *starvin* (*stv*), the *Drosophila* orthologue of BAG3 (Arndt et al., 2010), compared to the $(G4C2)_3$-expressing controls (Figure 2G). Due to a lack of anti-starvin antibody, we examined potential BAG3 protein level changes in HEK293T cells, and observed consistent BAG3 lowering by the expanded *G4C2r* expression (Figure 2H). To further confirm that BAG3 may functionally modulate tau protein levels, we knocked-down BAG3 in HEK293T cells co-expressing $(G4C2)_{25}$ and tau, and observed increased the tau level by lowering BAG3 expression (Figure S6A). Consistently, overexpressing BAG3 reduced tau levels (Figure 2I). Taken together, the expanded *G4C2r* expression decreased BAG3 levels, leading to impaired autophagy function and increased tau levels, or at least making a partial contribution of tau increase.

DISCUSSION

Taken together, our study revealed a functional role of tau in *G4C2* mediated neurotoxicity. While some of the previous studies suggest a potential link between tau and *G4C2* expansion, there has been no study demonstrating tau as a potential mediator of neurotoxicity induced by *G4C2* expansion. Thus, we have made a novel discovery that tau may mediated the expanded *G4C2r*-induced neurotoxicity by showing that knockout of dtau mitigated *in vivo* neurodegeneration, motor performance deficits and shortened lifespan in the *Drosophila G4C2* model (Figure 2).

While our study was mainly performed in the *Drosophila* model, tau's function in *G4C2*-induced neurotoxicity is likely conserved between human and flies. dtau has previously been cloned and demonstrated to displays microtubule-binding properties (Heidary and Fortini, 2001), but its potential role in neurodegeneration has been controversial. Studies using either *Drosophila* RNAi lines or tau hypomorphic/deficiency lines led to contradictory results regarding the potential detrimental or

beneficial effects of dtau removal, possibly because of off-target effects or incomplete removal of dtau, and there was no disease phenotype “restoration” experiments expressing human tau (htau) to test the functional similarity between dtau and htau. Meanwhile, dtau knockout did not trigger major effects in terms of fly survival and fly climbing ability (Figure S1 & Figure 1), and it did not influence the toxic effects on fly survival related to the expression of human Aβ, suggesting that dtau may mediate neurodegeneration in certain context, and not in a nonspecific manner. On the other hand, our study demonstrated that expression of htau restored most phenotypes that were rescued by the dtau knockout (Figure S1C-D & 1B-D). In addition, the expanded *G4C2r* expression increased tau levels in both *Drosophila* and mammalian cells (Figure 2A-E). Finally, dtau knockout rescued HD-relevant phenotypes in *Drosophila* expressing mHTT proteins (Figure S1), consistent with the observation in a mouse model (Fernandez-Nogales et al., 2014). These pieces of cross-species evidence suggest a conserved role of dtau in mediating neurodegeneration, and justify using dtau for mechanistic studies of tauopathies, providing a much faster and more convenient model for research in this field compared to mouse models.

BAG3-mediated autophagy may play a key role in maintaining cellular homeostasis under stress as well as during aging (reviewed by (Behl, 2016)), when the increased demand for protein degradation enhances BAG3 expression, followed by a functional switch from BAG1-mediated proteasomal to BAG3-mediated autophagic pathway. Meanwhile, potential changes or functional roles of BAG3 in *G4C2* models was previously unknown, and our study revealed a potential role of the conserved BAG3-autophagy pathway in the expanded *G4C2r*-induced neurotoxicity.

In summary, our study revealed an evolutionarily conserved BAG3-mediated autophagy-dependent mechanism between the expanded *G4C2r*-induced ALS and tauopathies, providing entry points for targeting these diseases.

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Conflict of Interest

The authors claim no conflicts of interest.

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FIGURE LEGENDS

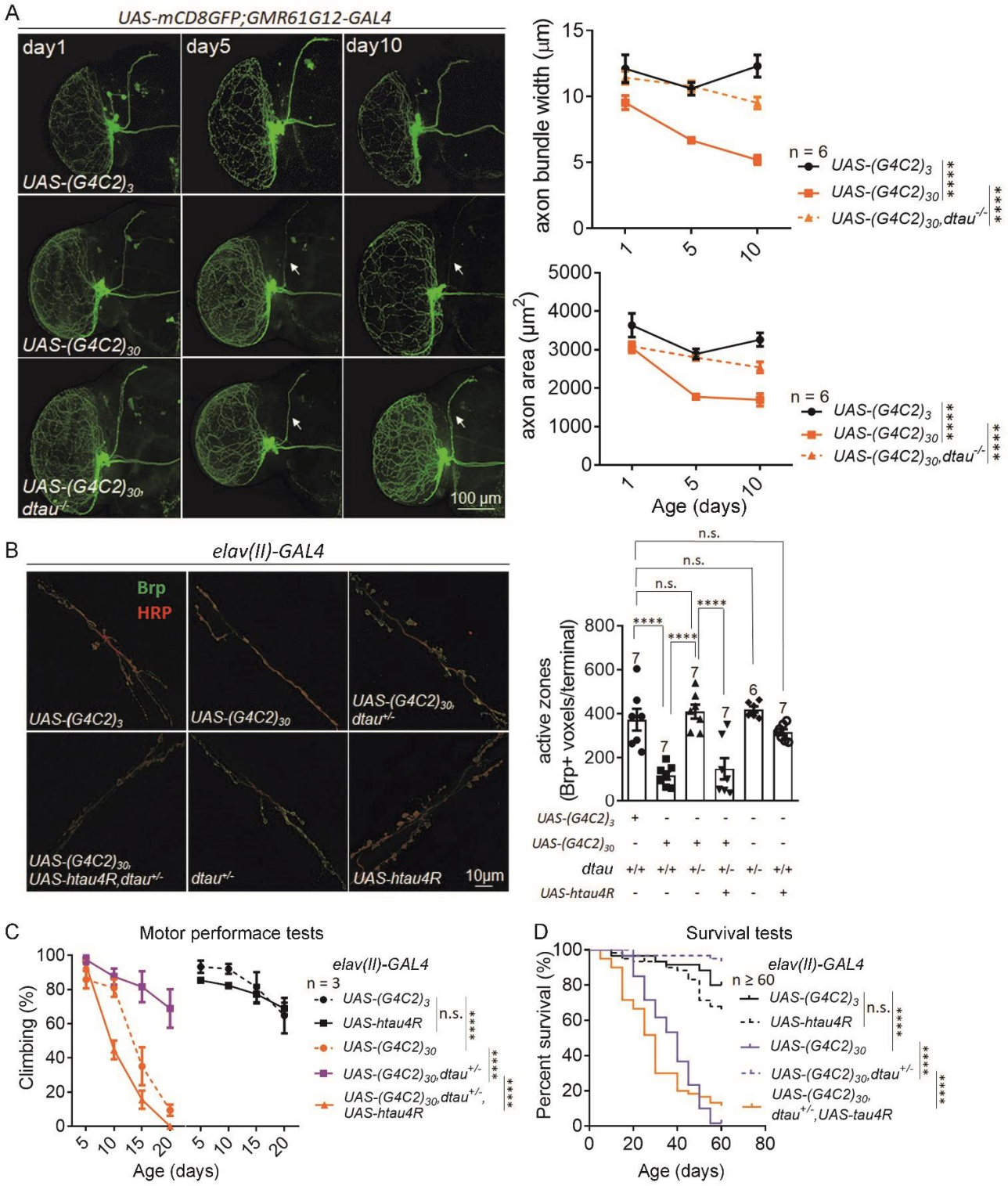

Figure 1. *dtau* knockout rescued *in vivo* neurodegeneration, motor function deficits, and shortened lifespan in *G4C2r* expressing flies.

A) Similar as Figure 1A-B, but testing the expanded *G4C2r* induced toxicity. *dtau* KO significantly rescued *in vivo* neurodegeneration induced by $(G4C2)_{30}$ expression.

B) Representative images and quantifications of neurodegeneration in the neuromuscular junctions (NMJs) in flies with the indicated genotypes. NMJs of muscle 6/7 in abdominal segment A2 and A3 of third instar larvae were stained with anti-HRP (neuronal axon marker, red channel) and anti-Bruchpilot (Brp) (active zone marker, green channel). The Brp-positive active zones were reduced by $(G4C2)_{30}$ expression, and rescued by *dtau* KO. The white arrows indicated the buttons of NMJs. *Scale* bars, 10 μm.

C-D) Similar as Figure 1C-D, but for *G4C2* flies. *dtau* KO significantly rescued motor function deficits and shortened lifespan induced by $(G4C2)_{30}$ expression.

For all plots in A-C, error bars indicate mean ± SEM. Asterisks denote statistically significant differences (*p≤0.05, **p≤0.01, ***p≤0.001， ****p≤0.0001).

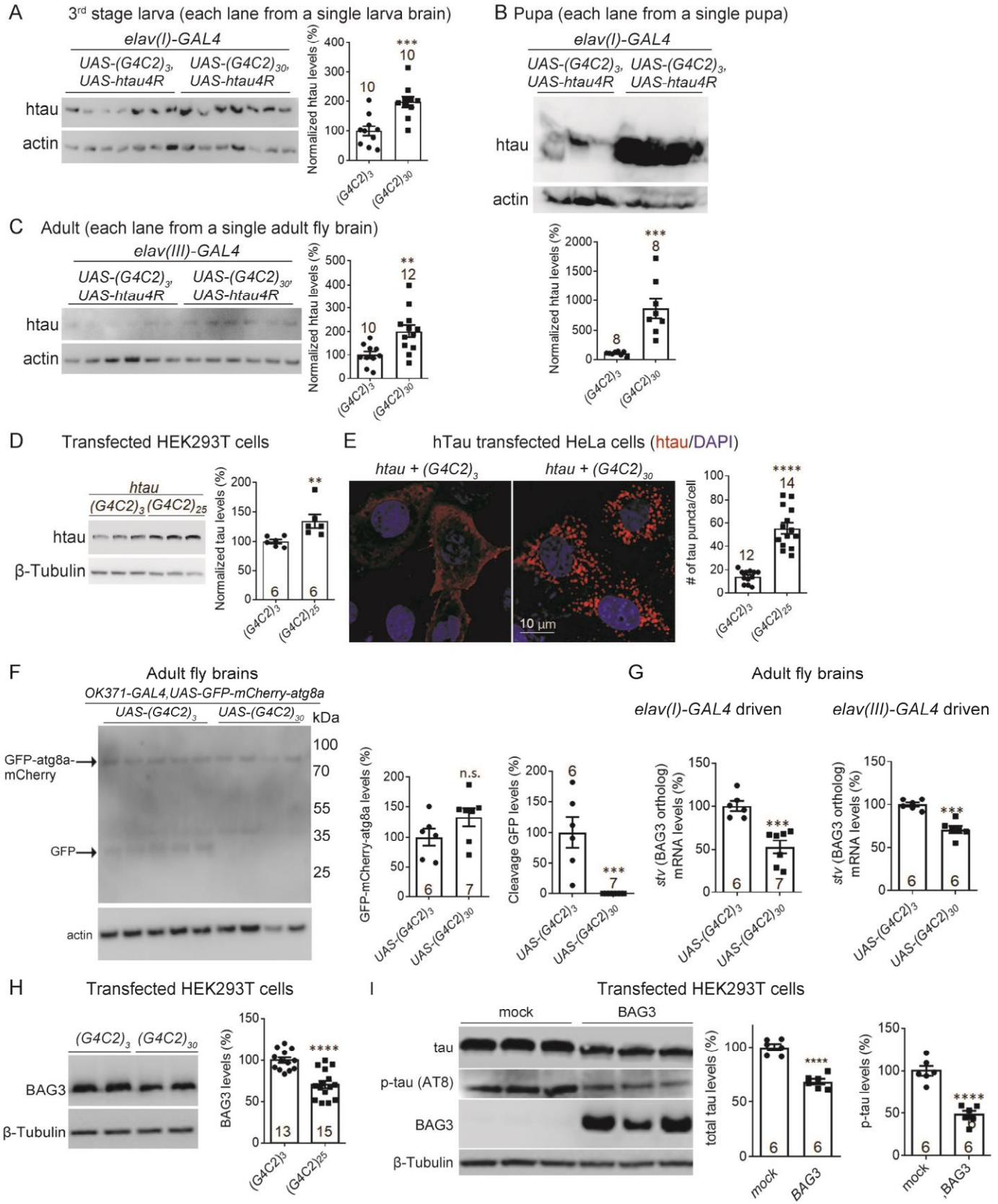
A
3rd stage larva (each lane from a single larva brain)
elav(I)-GAL4
UAS-(G4C2)3, UAS-htau4R
UAS-(G4C2)30, UAS-htau4R
htau
actin
Normalized htau levels (%)
(G4C2)3
(G4C2)30
B Pupa (each lane from a single pupa)
elav(I)-GAL4
UAS-(G4C2)3, UAS-htau4R
UAS-(G4C2)3, UAS-htau4R
htau
actin
Normalized htau levels (%)
C Adult (each lane from a single adult fly brain)
elav(III)-GAL4
UAS-(G4C2)3, UAS-htau4R
UAS-(G4C2)30, UAS-htau4R
htau
actin
Normalized htau levels (%)
D Transfected HEK293T cells
htau
(G4C2)3 (G4C2)25
htau
β-Tubulin
Normalized tau levels (%)
E hTau transfected HeLa cells (htau/DAPI)
htau + (G4C2)3
htau + (G4C2)30
10 µm
# of tau puncta/cell
F Adult fly brains
OK371-GAL4,UAS-GFP-mCherry-atg8a
UAS-(G4C2)3
UAS-(G4C2)30
kDa
GFP-atg8a-mCherry
GFP
actin
GFP-mCherry-atg8a levels (%)
n.s.
Cleavage GFP levels (%)
G Adult fly brains
elav(I)-GAL4 driven
elav(III)-GAL4 driven
stv (BAG3 ortholog) mRNA levels (%)
H Transfected HEK293T cells
(G4C2)3 (G4C2)30
BAG3
β-Tubulin
BAG3 levels (%)
I Transfected HEK293T cells
mock
BAG3
tau
p-tau (AT8)
BAG3
β-Tubulin
total tau levels (%)
p-tau levels (%)

Figure 2. *G4C2r* expression increased tau protein levels *in vivo* and in cells *via* reducing BAG3-mediated autophagy.

A-C) Representative Western-blots and quantifications showing that the total tau levels were increased by $(G4C2)_{30}$ expression in the 3rd instar larva, pupa and adult stage flies, compared to the $(G4C2)_3$ expression controls. n indicates the number of individual flies. The statistical analysis was performed by two-tailed unpaired t tests.

D) Representative immunofluorescence images of whole-mount fly brains showing increased transgenic htau levels in $(G4C2)_{30}$ flies. All the flies was dissected and stained at day 16.

E) Representative Western-blots and quantifications showing that total tau levels were increased by $(G4C2)_{25}$ compared to $(G4C2)_3$ controls in transfected HEK293T cells. The statistical analysis was performed by two-tailed unpaired t tests.

F) Representative immunofluorescence images and quantifications (measured by the number of tau puncta *per* cell) showing that $(G4C2)_{25}$ expression increased tau aggregates in HeLa cells, compared to the $(G4C2)_3$ expressing controls. The statistical analysis was performed by two-tailed unpaired t tests. The aggregates were tested in HeLa cells, because tau aggregates were hardly visible in HEK293T cells.

G) Representative Western-blots and quantifications showing that the cleaved product (free GFP cleaved from GFP-mCherry-atg8) band is largely missing in motor neurons expressing $(G4C2)_{30}$, suggesting an inhibition of autophagy activity in these neurons. n indicates the number of individual flies. The statistical analysis was performed by two-tailed unpaired t tests.

H) qPCR showing a reduction of *starvin (stv)* mRNA levels by $(G4C2)_{30}$ expression compared to the $(G4C2)_3$ expressing controls. The results were consistent in both *elav(I)-GAL4* or *elav(III)-GAL4* driven flies.

I) Representative Western-blots and quantifications of lysates from transfected HEK293T cells showing that BAG3 protein levels were reduced by $(G4C2)_{25}$ expression compared to the $(G4C2)_3$ expressing controls.

For all plots, bars indicate mean ± SEM. Asterisks denote statistically significant differences (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$，****$p \leq 0.0001$).