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Inhibition of human high affinity copper importer Ctr1 orthologous in the nervous system of *Drosophila* ameliorates Aβ42-induced alzheimer's disease-like symptoms

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Abstract

Disruption of copper homeostasis has been implicated in Alzheimer's disease (AD) during the last two decades; however, whether copper is a friend or a foe is controversial. Within a genetically tractable *Drosophila* AD model, we manipulated the expression of human high affinity copper importer orthologous in *Drosophila* to explore the *in vivo* roles of copper ions in the development of AD. We found that inhibition of Ctr1C expression by RNAi in Aβ-expressing flies significantly reduced copper accumulation in the brains of the flies as well as ameliorating neurodegeneration, enhancing climbing ability and prolonging lifespan. Interestingly, Ctr1C inhibition led to a significant increase in higher molecular weight Aβ42 forms in brain lysates, while it was accompanied by a trend of decreased expression of amyloid-β degradation proteases (including *NEP1-3* and *IDE*) with age and reduced Cu-Aβ interaction-induced oxidative stress in *Ctr1C* RNAi flies. Similar results were obtained from inhibiting another copper importer Ctr1B and overexpressing a copper exporter DmATP7 in the nervous system of AD flies. These results imply that copper may play a causative role in developing AD, as either Aβ oligomers or aggregates were less toxic in a reduced copper environment or one with less copper binding. Early manipulation of brain copper uptake can have a great effect on Aβ pathology.

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Keywords

Alzheimer's disease; *Drosophila*; Copper; Amyloid-β; Neurodegeneration; High affinity copper importer; *Ctr1*; *DmATP7*

1. Introduction

Copper serves as a catalytic center in a broad range of proteins and enzymes, e.g. cytochrome c oxidase, superoxide dismutase, laccase, and ceruloplasmin that are essential for the survival of all living organisms. However, copper is highly toxic when free Cu^{2+} ions are present, due to its capacity to mediate the formation of reactive oxygen species via Fenton reactions (Halliwell and Gutteridge, 1990). Therefore, copper homeostasis is strictly controlled within an organism's cells. Copper dys-homeostasis has been found to induce many human disorders, such as Menkes and Wilson's diseases (Lutsenko and Petris, 2003), Prion disease (Jones et al., 2004), Parkinson's disease (Bharathi et al., 2007) and Alzheimer's disease (Adlard and Bush, 2006; Strozyk et al., 2009).

Alzheimer's disease (AD) is characterized pathologically by cerebral deposition of extracellular amyloid-β (Aβ) plaques. Considerable evidence indicates that the formation of senile plaques is mediated by endogenous biometals, especially Cu, Zn and Fe (Bush et al., 1994; Cherny et al., 1999; Dong et al., 2003). In healthy brains, most Aβ peptides are present as soluble forms; however, Aβ plaques occur, particularly in AD patients. Aβ peptides are produced from proteolytic cleavage of the amyloid precursor protein (APP), which usually constitutes two forms, Aβ40 and Aβ42 (White et al., 1999). Aβ42 forms protofibrils and fibrils much more readily than Aβ40 and is the predominant form of the peptide found in plaques in AD brains. It is known that copper plays critical roles in Aβ plaque formation (Barnham and Bush, 2008; Lovell et al., 1998; Miller et al., 2006); however, the precise function of copper in Aβ amyloid genesis is controversial.

Overexpression of the human Aβ42 peptide in *Drosophila* nervous system tissues results in structural as well as behavioral phenotypes resembling several AD-like symptoms, in a doseand age-dependent manner (Finelli et al., 2004; Iijima et al., 2004). Using Aβ42-expressing flies (Iijima et al., 2008), we can screen modifiers of Aβ42-induced phenotypes, which may lead to the dissection of mechanisms of Aβ toxicity and potentially novel AD therapies.

Given the importance of copper and its close relationship with AD pathology, we explored the effect of genetically controlling the level of a copper importer on AD-like pathology within Aβ42-expressing flies. The *Ctr1* family proteins are high affinity copper importers, and are conserved from yeast to humans. In the *Drosophila* genome, there are three *Ctr1* family copper transporters, Ctr1A, Ctr1B and Ctr1C. *Ctr1A* is constitutively and ubiquitously expressed, and loss of Ctr1A will result in developmental arrest at an early larval stage and a general failure of copper-dependent processes (Turski and Thiele, 2007). *Ctr1B* is expressed exclusively during the late embryonic and larval stages of development and is responsible for copper uptake from the intestine, but is only essential for development and viability under conditions of either extremely limiting or abnormally abundant copper (Zhou et al., 2003). Zhou et al. made an initial characterization of *Ctr1C* in which it was shown able to complement copper import-deficient yeast and to be expressed in *Drosophila* third instar larvae and adult males (Zhou et al., 2003). After that no further studies were made until recently, when Steiger et al. characterized Ctr1C as a copper importer in male fertility (Steiger et al., 2010). Here, we show that RNA interference (RNAi)-induced silencing of Ctr1C in the nervous system of AD flies ameliorates the Aβ42-induced AD-like

symptoms, Similar effects were also produced by silencing another copper importer Ctr1B through RNAi or over-expressing a copper exporter DmATP7 in the nervous system of AD flies. Given the simpler and time-saving of genetic studies in *Drosophila* than in mice, should facilitate our understanding of the role of copper and its homeostasis controlling genes in the development of human AD.

2. Materials and methods

2.1. DNA constructs and transgenic flies

Ctr1C were amplified from a *Drosophila* cDNA pool and cloned into the GAL4-responsive pUAST expression vector. To make the *Ctr1C* RNAi construct, a cDNA fragment corresponding to base pairs 1–700 of *Ctr1C* was PCR-amplified (forward primer, 5'-GGGTCTAGAATGGACCACCATGG-3'; reverse primer, 5'-GGGTCTAGAGCATTAGC AAAAAGGA-3'). Two copies of the PCR fragments were cloned in opposite orientations into the *Xba* I restriction site of the pWIZ expression vector. Transgenic strains were created by embryo injection through P-element-mediated germline transformation. Approximately 3 μg of pUAST-Ctr1C or pWIZ-Ctr1C-RNAi transgenic construct was mixed with 1 μg of helper plasmid in 10 μl of injection buffer. All transgenic flies were generated in the w^{1118} background following standard protocols. All general fly stocks and *Gal4* lines were obtained from the Bloomington *Drosophila* Stock Center, which includes *Actin-Gal4, elav-Gal4, da-GAL4, GMR-GAL4, A9-Gal4*, and *Timan-Gal4*. The *UAS-Aβ42* transgenic *Drosophila* strain was reported previously (Iijima et al, 2008). *DmATP7* overexpression (Bloomington #16866) transgenic strains were acquired from the Bloomington *Drosophila* Stock Center. *Ctr1B* RNAi was obtained from the National Institute of Genetics (NIG#7459R-2)

2.2. Complementation assay

Using a pan-neuronal *elav-Gal4* driver to specifically over-express *Ctr1C* (located on chromosome 1) in the fly nervous system, the flies will be developmentally arrested and die at the second or third instar larval stage when the larvae are fed a normal diet (NF) at 25°C. For the complementation assay, the food was supplemented with a gradient concentration of either bathocuproine disulfonate (BCS; Sigma-Aldrich number 14,662-5) or ethylenedinitrilotetraacetic acid (EDTA). BCS is a specific copper chelator used to deplete copper in the food. The number of rescued flies was scored. Survival rates were calculated relative to fully viable male elav-Gal4 flies.

2.3. Quantification of neurodegeneration

Adult fly heads were fixed in Carnoy solution (ethanol:chloroform:acetic acid, 6:3:1) overnight at 4 C°, dehydrated in serial ethanol solutions, and then embedded in paraffin and sectioned at 6 μm thickness. H&E staining was performed following standard protocols. Neurodegeneration was assessed by quantification of vacuoles with diameter greater than 3 μm in the fly brains. At least five fly brains were analyzed for each genotype.

2.4. Western blot analysis

Protein samples including SDS-soluble or SDS-insoluble but formic acid-soluble Aβ42 were prepared as previously reported (Iijima et al., 2004). Lysates from an equal number of fly heads were diluted in SDS sample buffer, separated on 10–20% Tris-Tricine gels (Invitrogen), and transferred to nitrocellulose membranes (Invitrogen). Membranes were boiled in PBS for 3 min. Membranes were blocked with 3% BSA and blotted with primary antibody. Primary antibodies used in this study were mouse anti-Aβ42 (6E10, Covance Research Products) and rabbit anti-Actin (Sigma). After washing with TBST 3 times for 5

minutes each, membranes were incubated with secondary antibodies for 1 hr at RT. Then after same 3 washes in TBST, the membranes were incubated with ECL working solution (GE healthcare) and developed on film (Kodak). Data were analyzed with ImageJ software (NIH).

2.5. Pavlovian olfactory associative memory recording

The training and testing procedures were as previously described (Tully and Quinn, 1985; Tully et al., 1994; Yin et al., 1994). During one training session, a group of 100 flies was sequentially exposed for 60 s to two odors, octanol (OCT) or methylcyclohexanol (MCH), with 45 s of fresh air in between. Flies were subjected to foot-shock (1.5 s pulses with 3.5 s intervals, 60 V) during exposure to the first odor (CS+) but not to the second (CS−). To measure "immediate memory (also referred to as "learning")", flies were transferred immediately after training to the choice point of a T-maze and forced to choose between the two odors for 2 min, at which time they were trapped in their respective T-maze arms, anesthetized, and counted. A performance index (PI) was calculated from the distribution of flies in the T-maze. A reciprocal group of flies was trained and tested using OCT as the CS+ and MCH as the CS+, respectively. PIs from these two groups were finally averaged for an $n = 1$ and multiplied by 100. A PI of 0 represented a 50:50 distribution, whereas a PI of 100 represented 100% avoidance of the shock-paired odor.

2.6. Quantitative analysis of gene expression

Total RNA was extracted from the brains of 20 adults for each sample using TRIzol® Reagent (Invitrogen) according to the manufacturers' instructions and subjected to DNA digestion using DNAse I (Ambion) immediately. The concentration and quality of the DNAse-treated total RNA were then tested, and 1 μg total RNA from each sample was used to synthesize cDNA using the Superscript™ II Reverse Transcriptase Kit (Invitrogen) with oligo(dT) primers. For quantitative RT-PCR (qRT-PCR), real-time PCR reactions were monitored on an iCycler (Bio-Rad) by means of SYBR Green (Bio-Rad) dye. Messenger RNA expression levels were determined relative to *rp49* expression by relative quantification. Primers for amplifying *Ctr1C, NEP1, NEP2, NEP3* and Aβ42 are listed in Table S1. Statistical analysis was performed using the Student's *t*-test.

2.7. Metal content and oxidative stress assay

For the metal content analysis, flies were reared on normal food and fly heads were collected and weighed on day 30 after eclosion. Fly heads were dissolved in 1 ml 65% HNO_3, boiled in a 100 °C water bath for 10 min and diluted to 10 ml for quantitative elemental analysis with inductively coupled plasma–mass spectrometry (ICP-MS) XII (Thermo Electron Corp., Waltham, MA, USA) at the Analysis Center of Tsinghua University.

For the oxidative stress assay, flies were collected 2 days after eclosion. After being reared on normal food for 10 days, flies were transferred to filter paper supplemented with 5% sucrose and 20 mM paraquat. Mortality was recorded about every 8 hours. Each vial contained 20–25 flies, and the experiments were repeated at least three times.

2.8. Climbing assay

The climbing assay was referenced to Iijima et al. (2004). Briefly, twenty flies were placed in a plastic vial and gently tapped to the bottom. The number of flies at the top of the vial was then counted after 18 s of climbing under red light (Kodak, GBX-2, Safelight Filter). The data shown represent results from a cohort of flies with four repeats tested serially for 5–50 days. The experiment was repeated more than three times.

2.9. Longevity assay

Twenty to 23 1–2 day newly eclosed flies were placed in a food vial. Each vial was kept at 25 or 29°C, 70% humidity, under a 12-h light–dark cycle. Food vials were changed every 2–3 days, and dead flies were counted at that time. At least 150 flies were prepared from each genotype, and the experiments were carried out more than three times. Percent increases in life span were based on comparing the median survivals. Prism (GraphPad) was used for statistical analysis of lifespan data. Mantel-Cox log-rank statistical analysis was used for testing the statistical significance of the differences between the survivorship curves.

2.10. Statistical analysis

All data were analyzed by Student's *t*-test or two-way ANOVA (otherwise indicated). Statistical results were presented as means ± SEM. Asterisks indicate critical levels of significance (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).

3. Results

3.1. Ctr1C can function as a copper importer in the Drosophila brain and nervous system

To determine whether *Ctr1C* levels could influence *Drosophila* brain copper homeostasis, we first specifically over-expressed *Ctr1C* in the fly nervous system using a pan-neuronal *elav-Gal4* driver. We found that the *elav-Gal4>UAS-Ctr1C* progeny were developmentally arrested and died at the second or third instar larval stage when they were fed on normal food at 25°C. To determine whether the lethality was due to copper toxicity in the flies' nervous systems, we raised fly larvae on normal food supplemented with different concentrations of the copper chelator BCS. Fig. 1A shows that the lethality of *Ctr1C* over-expression in flies was rescued by supplementing with BCS, and such rescue was dose dependent and reached nearly 100% with 500 μM BCS. However, we failed to rescue the lethality by adding EDTA, which is not a copper-specific chelator. When raised at a lower temperature of 18°C, the *elav-Gal4>UAS-Ctr1C* flies could survive to adulthood, but had an abnormal wing phenotype (Figs. 1A) and rough eyes (Fig. 1B). Fig. S1B shows sqRT-PCR results confirming Ctr1C was overexpressed in the brains of the transgenic flies. Flies developed normally when *Ctr1C* was over-expressed with wing (*A9-Gal4*, Figs. 1A), eye (*GMR-Gal4*), or heart (*Timan-Gal4*) specific drivers. These data provide evidence that *Ctr1C* can function as a copper importer in fly brains, that the lethality of pan-neuronal over-expression of *Ctr1C* is due to copper-overloading-induced toxicity to the nervous system, and copper overloading is more toxic to the nervous system than to other tested tissues.

3.2. Knocking down Ctr1C increases the climbing ability and lifespan of Aβ42 flies

As copper has been implicated as a critical factor in the amyloid pathology of AD, we next wished to determine how the inhibition of a copper importer *Ctr1C* would influence Aβ42-induced pathology in a *Drosophila* model. Aβ42 expressing flies have been documented to display locomotor dysfunction after three weeks of age and their lifespan is significantly reduced (Finelli et al., 2004; Iijima et al., 2004; Lang et al., 2012). Fig. S2 shows that in adult fly brains either with or without Aβ42 expression, *Ctr1C* RNAi could lead to about a threefold reduction in *Ctr1C* mRNA levels compared with controls. Assays of climbing ability demonstrated that Aβ42 flies started to have a locomotor defect at 15 days of age compared to *elav-Gal4* flies without expressed Aβ42 (Fig. 2A), while Aβ42 flies with *Ctr1C* RNAi started to have locomotor defects at 30 days of age compared to *elav-Gal4* flies (Fig. 2A). In all the tested time courses, Aβ42 flies with decreased *Ctr1C* expression through RNAi had a delayed climbing deficit compared with age-matched Aβ42 flies (Fig. 2A). As a further control, we tested the climbing ability of transgenic flies without Aβ42 expression

(Fig. 2B). No significant differences were found between age-matched *elav-Gal4* and *Ctr1C -RNAi* flies.

Consistent with the results obtained in the climbing assay, the lifespan of Aβ42 flies was prolonged by RNAi-based knockdown of *Ctr1C* expression (Fig. 2C and D). The *Ctr1C RNAi* transgenic line exhibited an increase of 32.4% in the median lifespan of Aβ42 flies reared at 25 °C. The result indicated that a reduction of *Ctr1C* expression in Aβ42 flies leads to an improved locomotor ability and a longer lifespan. The Aβ42 gene was directly under the control of elav-Gal4, and indeed we did not see changes in Aβ42 mRNA expression when the control and experimental flies had different number of UAS transgenes (Lang et al., 2012). Therefore, the results implied that the locomotion and life span defects resulting from Aβ42 toxicity were closely related to Cu status, and could be modulated through changes in *Ctr1C* expression level.

3.3. Knocking down Ctr1C ameliorates Aβ42-induced neurodegeneration accompanied by a reduction in copper levels

Vacuolization of brain tissues is a major hallmark of neurodegeneration in Aβ42 flies (Muqit and Feany, 2002). Next we wanted to know whether the improved locomotor ability and longer lifespan of Aβ42 flies produced by *Ctr1C* RNAi was associated with ameliorated brain neurodegeneration. Fig. 3A shows that in 30 day old Aβ42-expressing fly brains, there were more and larger bubbles in both the cortex and neuropil regions compared to the brains of Aβ42 flies with *Ctr1C* RNAi. Counting the numbers of vacuoles in the cortex and neuropil revealed that *Ctr1C* knockdown dramatically decreased brain vacuolization in Aβ42 flies (Fig. 3B). These results give evidence that inhibiting *Ctr1C* expression can ameliorate Aβ42-induced neurodegeneration in fly brains.

Using ICP-MS, we determined the copper levels in 30-day-old fly brains when *Ctr1C* was knocked down by RNAi. The results showed that knocking down Ctr1C significantly reduced brain copper compared with controls (Fig. 3C, $p<0.05$); however, other metals, like Zn, Fe and Mn were not significantly disturbed by *Ctr1C* RNAi (Fig. 3D). These results indicate that *Ctr1C* RNAi could specifically reduce copper levels in fly brains without changing the status of other metal ions such as Zn, Fe and Mn, which have also been implicated in the pathogenesis of AD.

3.4. Knocking down Ctr1C does not rescue Aβ42-induced early memory loss

Progressive memory loss is another hallmark of Alzheimer's disease. In Aβ42-expressing adult flies, an obvious memory defect was found at around 10 days old (Chiang et al., 2010; Iijima et al., 2004). To investigate the effect of *Ctr1C* knockdown on Aβ42-induced early memory loss, we used the same extensively characterized Pavlovian olfactory aversive conditioning. *Ctr1C* knockdown did not rescue memory loss at this stage (Fig. 4). As a control, we examined how reduction in *Ctr1C* expression alone might impact memory scores in the absence of Aβ42. Knocking down *Ctr1C* did not significantly influence the memory of normal 10-day-old male flies (Fig. 4). Therefore, reducing Cu levels through knocking down *Ctr1C* did not ameliorate Aβ42 toxicity on fly memory at early stages.

3.5. Knocking down Ctr1C changes fly brain Aβ composition

Because Aβ42 can oligomerize and aggregate into different states (Bitan et al., 2003; Sgourakis et al., 2007), we then asked how *Ctr1C* RNAi affects various forms of Aβ42. Fly brain lysates were used for Western blotting analysis. The result showed that the SDS-soluble Aβ42 (low level aggregate forms) in male brains was at similar levels between Aβ42 flies with and without *Ctr1C* RNAi. However, dramatic increases in SDS-insoluble but

formic acid-soluble Aβ42 were detected in male Aβ42 flies with *Ctr1C* knock down (Fig. 5A and B).

Because of the significant increase in formic acid-soluble Aβ42 fractions brought about by *Ctr1C* RNAi, we then asked whether this was due to an inhibition of Aβ42 clearance. Several proteases (NEP1-3, IDE) have been proposed to act in Aβ degradation (Farris et al., 2003; Iwata et al., 2000); we thus explored whether they were affected by *Ctr1C* RNAi in brains of Aβ flies. We did not find any significant changes in IDE and NEP1-3 mRNA levels in young (5 days old) Aβ42 fly brains induced by *Ctr1C* RNAi (Fig. 5C–F). However, with ageing, IDE, NEP1 and NEP3 mRNA levels were dramatically reduced in 25-day-old Aβ42 fly brains by *Ctr1C* RNAi (Fig. 5C, E and F). Therefore, the increase in SDS-insoluble but formic acidsoluble Aβ42 fractions by *Ctr1C* RNAi possibly resulted from these reductions in degrading proteases.

3.6. Knocking down Ctr1C greatly enhances flies' tolerance to oxidative stress

Reactive oxidative species are associated with many pathological processes, including that in Alzheimer's disease. As copper is a redox-active agent producing ROS *in vivo*, to determine whether the reduction in *Ctr1C* expression levels in brain could alter the flies' susceptibility to oxidative injury, we treated *Ctr1C* RNAi and control Aβ42 flies with paraquat, a widely used free radical generator that can increase ROS levels *in vivo* (Arking et al., 1991; Dias-Santagata et al., 2007). Targeted knock down of *Ctr1C* in the CNS of Aβ42 flies rendered them more resistant to paraquat treatment than control Aβ42 flies (Fig. 6), with a 45.5% increase in mean life span from 16.5 h to 24 h. The two survival curves were significantly different ($p<0.0001$).

3.7. Knocking down Ctr1B and over-expressing DmATP7 increase the climbing ability and lifespan of Aβ42 flies

To further confirm the ameliorating effects through lowering brain Cu accumulation by *Ct1C* RNAi in AD flies, we tested the longevity and climbing ability of AD flies through knocking down another copper importer *Ctr1B* or over-expressing a copper exporter *DmATP7*. Over-expressing *Ctr1B* has been reported to aggravate rough eye phenotype due to its resulted copper overloading (Saini et al., 2010), and over-expressing *DmATP7* could lower copper accumulation in fly brains (Southon et al., 2010). Fig. 7A showed that the longevity of AD flies was significantly increased when *Ctr1B* was knocked down ($p<0.0001$), or *DmATP7* was over-expressed ($p<0.0001$), with a 17.9% and a 33.3% increase in median life-span compared with control Aβ42 flies, respectively. In consistent with the longevity test, the climbing ability of AD flies was also improved significantly through knocking down *Ctr1B* or over-expressing *DmATP7* in the nervous system (Fig. 7B). Taken together, these results corroborated that specifically lowering copper accumulation in brains of AD flies could ameliorate the AD-like symptoms in *Drosophila*.

4. Discussion

As we know, in healthy and/or young tissue, efficient homeostatic mechanisms are sufficient to maintain normal compartmentalization and release of metals. This compensatory capability of the brain is gradually lost with age, which would lead to an imbalance in brain metal levels and distribution (Bleackley and MacGillivray, 2011). In the last two decades, increasing evidences have demonstrated that disturbances in metal (especially Zn, Cu and Fe) homeostasis play critical roles in the development of AD (Adlard and Bush, 2006; Duce et al., 2010; James et al., 2012; Maynard et al., 2002; Strozyk et al., 2009). However, the exact role of metal ions in AD is still in debate; more research is urgently needed to understand how this disturbance is initiated and how it can be addressed to ameliorate the

disease. Copper is an essential but very active redox agent in living cells. It has been reported that increased brain copper levels are associated with normal ageing and AD (Maynard et al, 2002). In this study, we showed that genetic inhibition of human high affinity copper importer Ctr1 orthologous, Ctr1C, in whole life-course of AD model flies ameliorated brain neurodegeneration and prolonged the flies' life-span with enhanced climbing ability. Our results favors the foe role for increased copper accumulation in promoting brain neurodegeneration, which is also supported by data delivered from a recent review (Brewer, 2012).

Besides the oxidative toxicity of Cu itself in AD process, the level of Cu in tissues also influences the Aβ toxicity. *In vivo* studies have shown that the soluble oligomeric species are more neurotoxic than the insoluble amyloid fibrils, and are likely responsible for neurodegeneration/memory loss (Golde et al., 2006; Haass et al., 2007). Also some reports indicate that a significant increase in SDS insoluble but formic acid-soluble Aβ42 usually signals worsening neurodegeneration (Bieler and Soto, 2004; Horikoshi et al., 2004; Xing and Higuchi, 2002). However, the locomotor ability and longevity of *Ctr1C* RNAi Aβ42 flies were much improved even when the SDS insoluble but formic acid-soluble Aβ42 fractions are markedly increased in our results. These paradoxical results suggest that the Aβ toxicity may not be determined solely by the aggregation form. One possibility is that the toxicity of either Aβ42 oligomers or aggregates is mostly induced by bound Cu ions through the production of ROS radical species by redox reactions. To test this hypothesis *in vivo*, we treated adult flies with an *in vivo* ROS generator, paraquat. Surprisingly, the flies' tolerance to paraquat challenge was much enhanced through pan-neuronal knock down of *Ctr1C*. These results suggested that with less copper or in a copper reduced environment, the Aβ42 soluble fractions or the increased Aβ42 aggregates (here referring only to the SDS insoluble but formic acid-soluble Aβ42 fractions) may have less toxicity. Some recent experiments using synthesized bifunctional compounds (BFCs) to treat copper-mediated Aβ aggregation or preformed Aβ fibrils also supported our observations (Geng et al., 2012; Sharma et al., 2012). BFCs could inhibit the copper mediated Aβ42 aggregation and promote disaggregation of amyloid fibrils accompanying with a dramatic reduction of H_2O_2 production. However, the formation of soluble Aβ42 oligomers in presence of copper ions and the compounds still lead to an increased cellular toxicity (Sharma et al., 2012), which also emphasized the toxicity of soluble Aβ42 oligomers when they are present with copper ions.

As neprilysin (NEP) and the insulin-degrading enzyme (IDE) are well known proteases involved in amyloid degradation (Sudoh et al., 2002; Carty et al., 2013; Yamamoto et al., 2013), we thus measured their mRNA level in brains of young (5-day old) and older (25-day old) flies to explore why *Ctr1C* RNAi led to a significant increase in SDS insoluble but 70% FA soluble Aβ42 fractions. It's interesting that we found the expression levels of potential amyloid-β degradation proteases (including *NEP1-3* and *IDE*) exhibited a trend of decreasing with age when *Ctr1C* was knocked down. We hypothesized that the decreased expression of NEP or IDE may partially explain the increased level of SDS-insoluble but FA soluble fraction of Aβ42 because less monomers are degradated. However, one previous published paper (Sudoh et al., 2002) have clear *in vivo* evidences that the detergent-insoluble fraction extracted with 70% formic acid decreased markedly in IDE overexpression cells, and inhibit NEP expression, the cell will accumulate less insoluble Aβ. Their results showed that both IDE and NEP could degrade SDS soluble and insoluble fractions. One would generally expect that the levels of these proteases should be increased in order to achieve greater clearance of Aβ aggregates and in turn ameliorate AD defects, but we found decreased levels of these proteases accompanied with improved longevity and ameliorated neurodegeneration. Therefore, copper-Aβ interaction-initiated oxidative stress may play a causative role in AD development.

A considerable number of reports indicate that oxidative stress arises early, before the appearance of AD symptoms, and is also a feature of mild cognitive impairment (Butterfield et al., 1999; Keller, et al., 2005; Nunomura, et al., 2001; Reddy, 2006; Yatin, et al., 1999). It has been proven that binding of Aβ42 with Cu^{2+} not only induces the precipitation of Aβ42 (Atwood, et al., 1998; Bush et al., 1994; Lovell et al., 1998) but also induces oxidative stress through the generation of H_2O_2 (Huang et al., 1999). Increased copper binding to Aβ is evident in AD (Bush et al., 1994; Lovell et al., 1998; Syme et al., 2004; Tougu et al., 2011), and overexpression of Aβ42 in *Drosophila* brains may accumulate oxidative stress and cause damage in neuronal nuclei and mitochondrial DNA (Rival et al., 2009). Therefore, controlling copper status from youth may be an effective way to slow or inhibit the progress of AD. Recently, a Phase 2 clinical trial of Cu supplementation based on the "copper deficiency theory" was unsuccessful (Kessler et al., 2008) and the chelation method, such as by using clioquiniol, has effects at the organismal level and is generally harmful. Using a genetic method to manipulate copper specific transporters may be a potentially valuable strategy for treating AD. Through knocking down *Ctr1C* in the nervous system of *Drosophila*, we found oxidative stress was significantly reduced, and brain neurodegeneration was much improved and lifespan was prolonged.

In summary, our study suggests that brain copper level is vital to AD, genetically manipulating copper importer or exporter to lower copper accumulation early in life may markedly ameliorate the Aβ42-induced neurodegeneration. Because metal ions function differently in AD progress, we may need to combine diverse strategies in order to develop a better type of therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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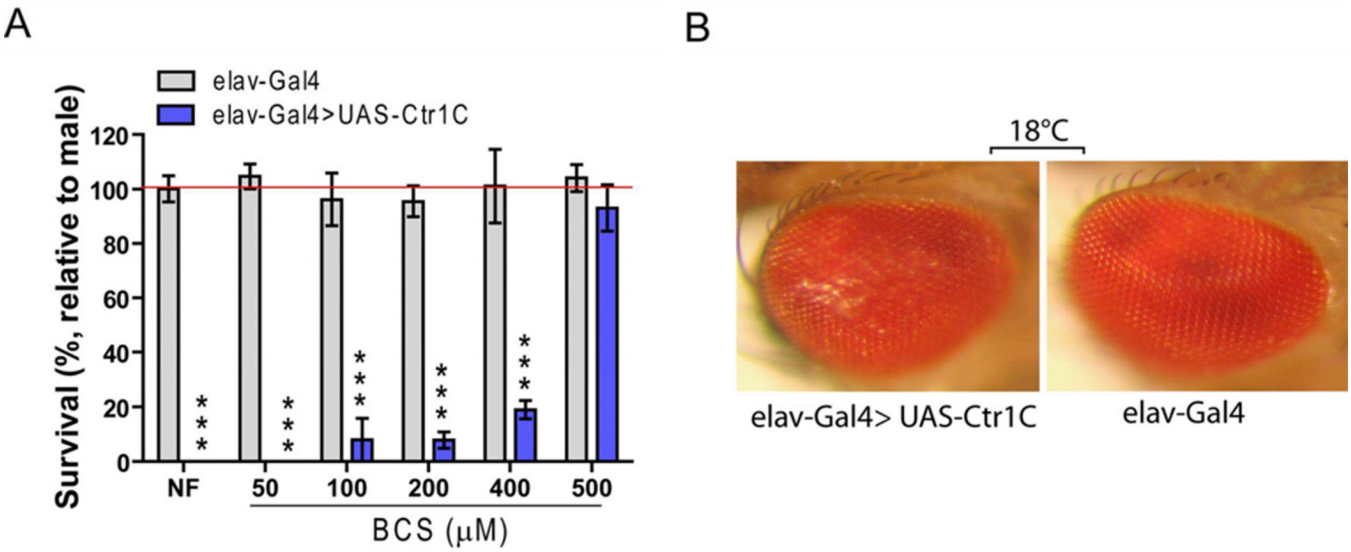
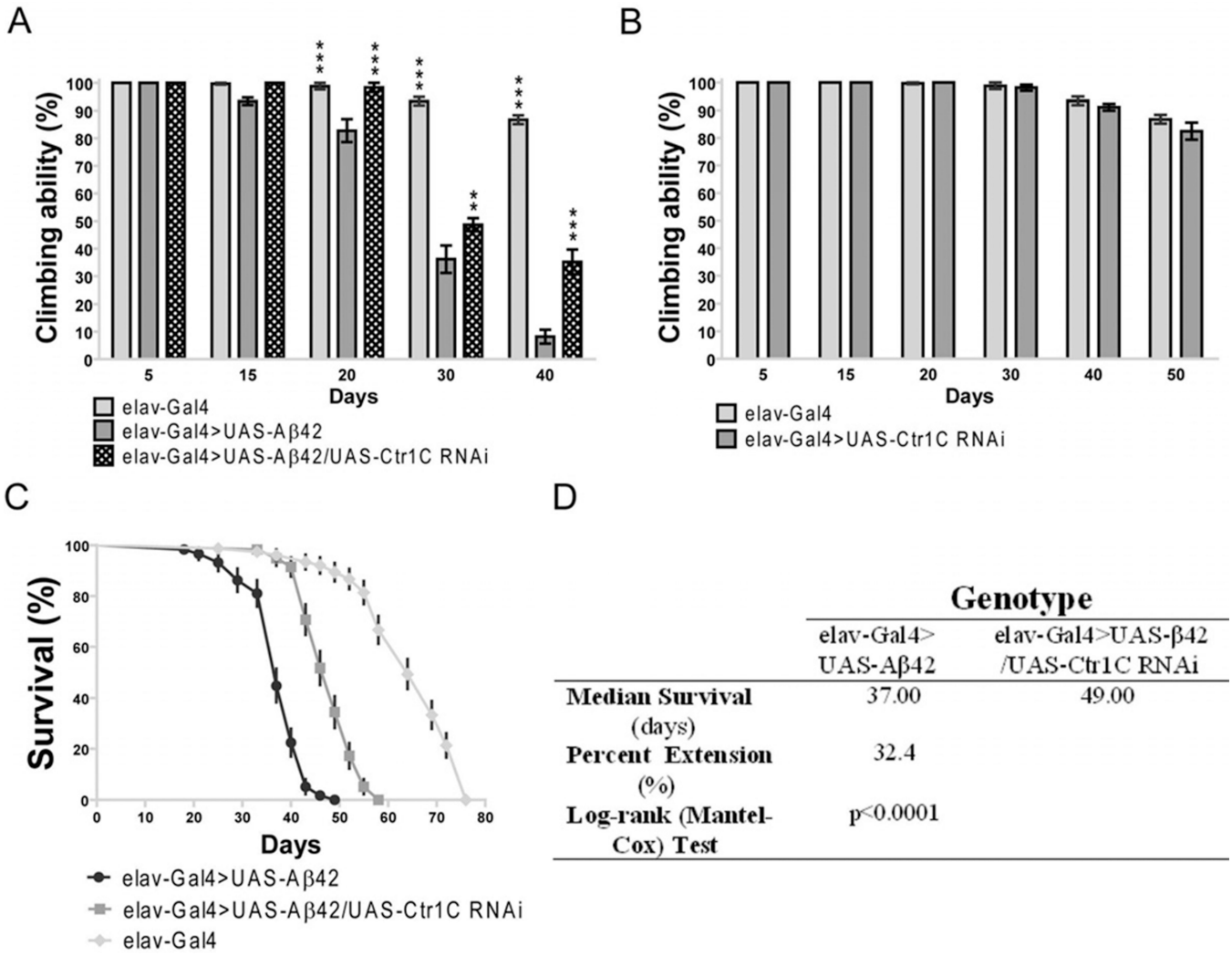


Fig. 1.

Ctr1C can function as a copper importer in fly brain nervous system. Specifically, over-expression of *Ctr1C* in the fly nervous system using a pan-neuronal *elav-Gal4* driver caused copper-dependent lethality when fly larvae were raised on normal food at 25°C, but could be rescued by supplying a concentration gradient of BCS (A). Such flies could survive to adulthood when they were raised at a lower temperature (18 °C), but showed rough eyes (B). The "relative to male" is the calculation of the percentage of the number of eclosed female flies to male flies in each group. Gray column (control) represents progeny of ♂w^{1118}×♀ *elav-Gal4* crosses. Blue column represents progeny of ♂*UAS-Ctr1C*×♀ *elav-Gal4* crosses.



	Genotype	
	elav-Gal4> UAS-Aβ42	elav-Gal4>UAS-β42 /UAS-Ctr1C RNAi
Median Survival (days)	37.00	49.00
Percent Extension (%)	32.4	
Log-rank (Mantel-Cox) Test	p<0.0001	

Fig. 2.

Reduced *Ctr1C* expression can ameliorate the climbing and longevity defects of Aβ42 flies. (A) Aβ42 expression in fly brains (*elav-Gal4>UAS-Aβ42*) induced a climbing deficit as compared with control *elav-Gal* flies. Knocking down *Ctr1C* by RNAi (*elav-Gal4>UAS-Aβ42/UAS-Ctr1C-RNAi*) could significantly increase the climbing ability of Aβ42 flies. Two-way ANOVA, **p<0.01, ***p<0.001 (in comparison with *elav-Gal4>UAS-Aβ42* flies). n=4 independent experiments. (B) In the absence of Aβ42 expression, no significant locomotor deficits were found among *elav-Gal4* and *elav-Gal4>UAS-Ctr1C-RNAi* flies. n=4 independent experiments. (C–D) *Ctr1C* knockdown significantly lengthened the lifespan of Aβ42 flies. The percentage survivorship was plotted against age (C). *Ctr1C* RNAi significantly prolonged the life span of *Aβ42* flies, with a 32.4% extension in median lifespan over that of Aβ42 flies at 25 °C (p<0.0001, C and D). Reported P values are from Mantel-Cox log-rank statistical analysis.

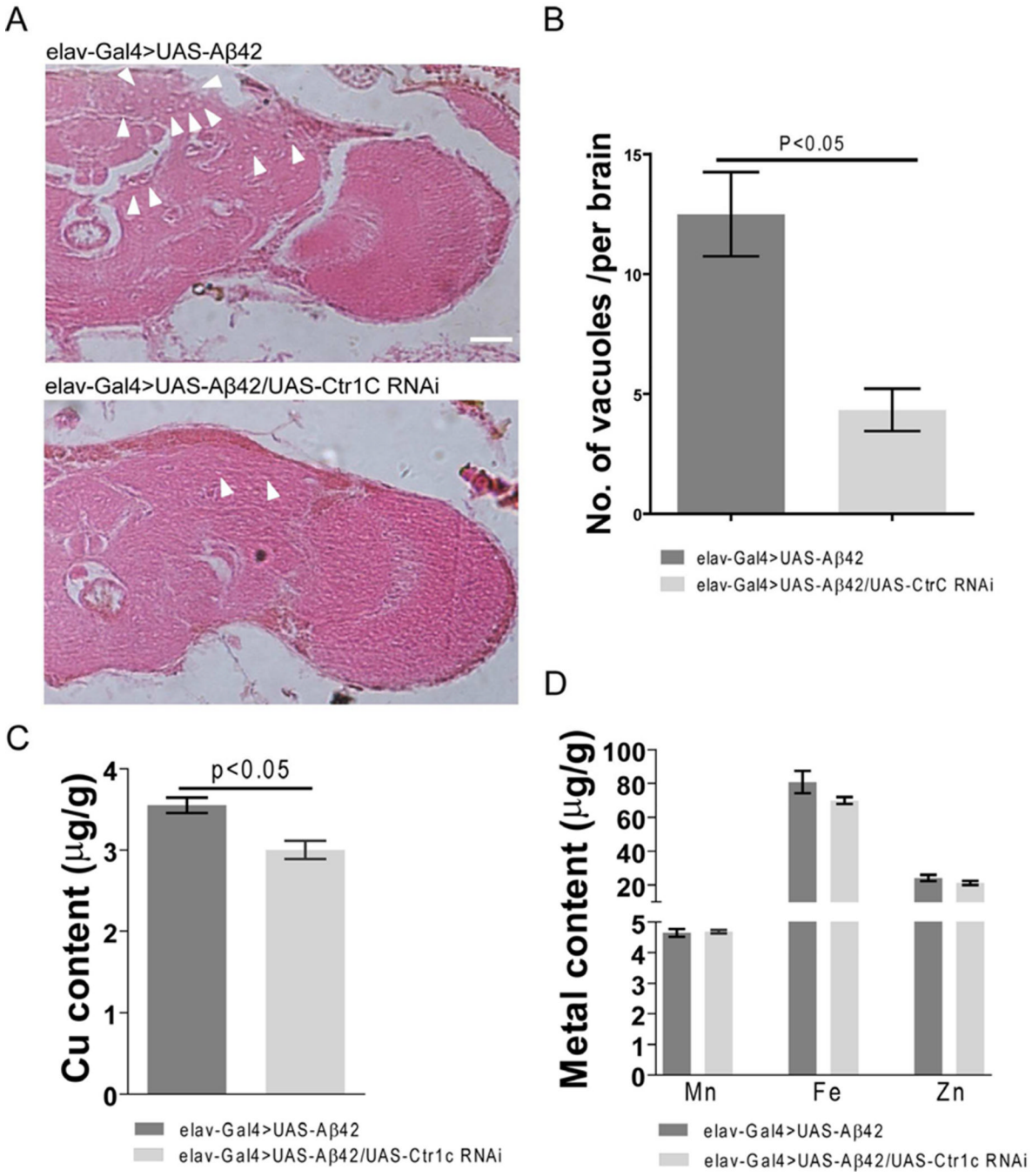


Fig. 3.

Specifically knocking down fly brain *Ctr1C* can ameliorate Aβ42-induced neurodegeneraion with a decrease in copper levels. Paraffin sections of 30-day-old fly brains were stained with H&E (A). Pan-neuronal expression of Aβ42 in fly brains induced neurodegeneration (arrowheads indicate vacuoles) in both the cortex and neuropil regions, while *Ctr1C* RNAi ameliorated neurodegeneration. Scale bar, 50μm. (B) is a statistical analysis of Aβ42-induced neurodegeneration bubbles. Brain sections across the mushroom body somatic region were chosen for comparison. The number of vacuoles (diameter > 3 mm) in each section was counted and summarized. Significant differences were observed between *Ctr1C* RNAi (*elav-Gal4>UAS-Aβ42/UAS-Ctr1C RNAi*) flies and control (*elav-Gal4>UAS-Aβ42*)

flies ($p<0.05$). (C–D) shows metal content in brains of 30-day old flies as measured by ICP-MS. *Ctr1C* RNAi (*elav-Gal4>UAS-Aβ42/UAS-Ctr1C RNAi*) significantly slowed down brain Cu accumulation (C, $p<0.05$), while not significantly disturbing the Zn, Fe and Mn content of *Aβ*42 fly brains (D). Data are expressed as means ± SEM and analyzed by Student's *t*-test. n=3 for each genotype.

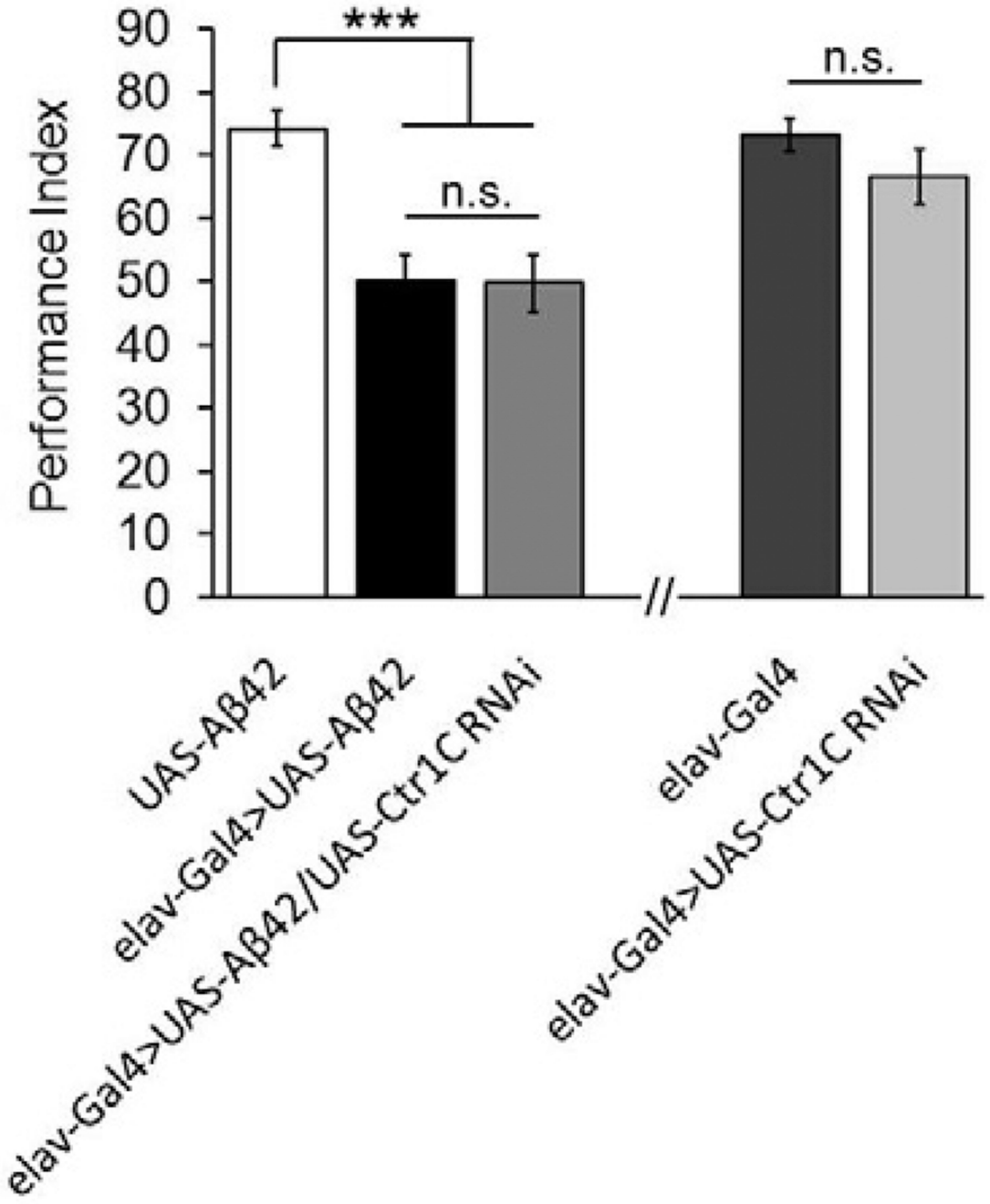


Fig. 4.

Inhibiting Ctr1C does not affect Aβ42-induced early memory loss. Performance indices represent the memory ability of different genotypes in 10-day-old flies. Pan-neuronal knock down of *Ctr1C* did not influence memory performance as well as affecting the memory defect caused by excessive Aβ42. All behavioral data are normalized to control flies. Data are expressed as means ± SEM. n=8 PIs for each genotype. One-way ANOVA, ***p<0.001. n.s., not significant.

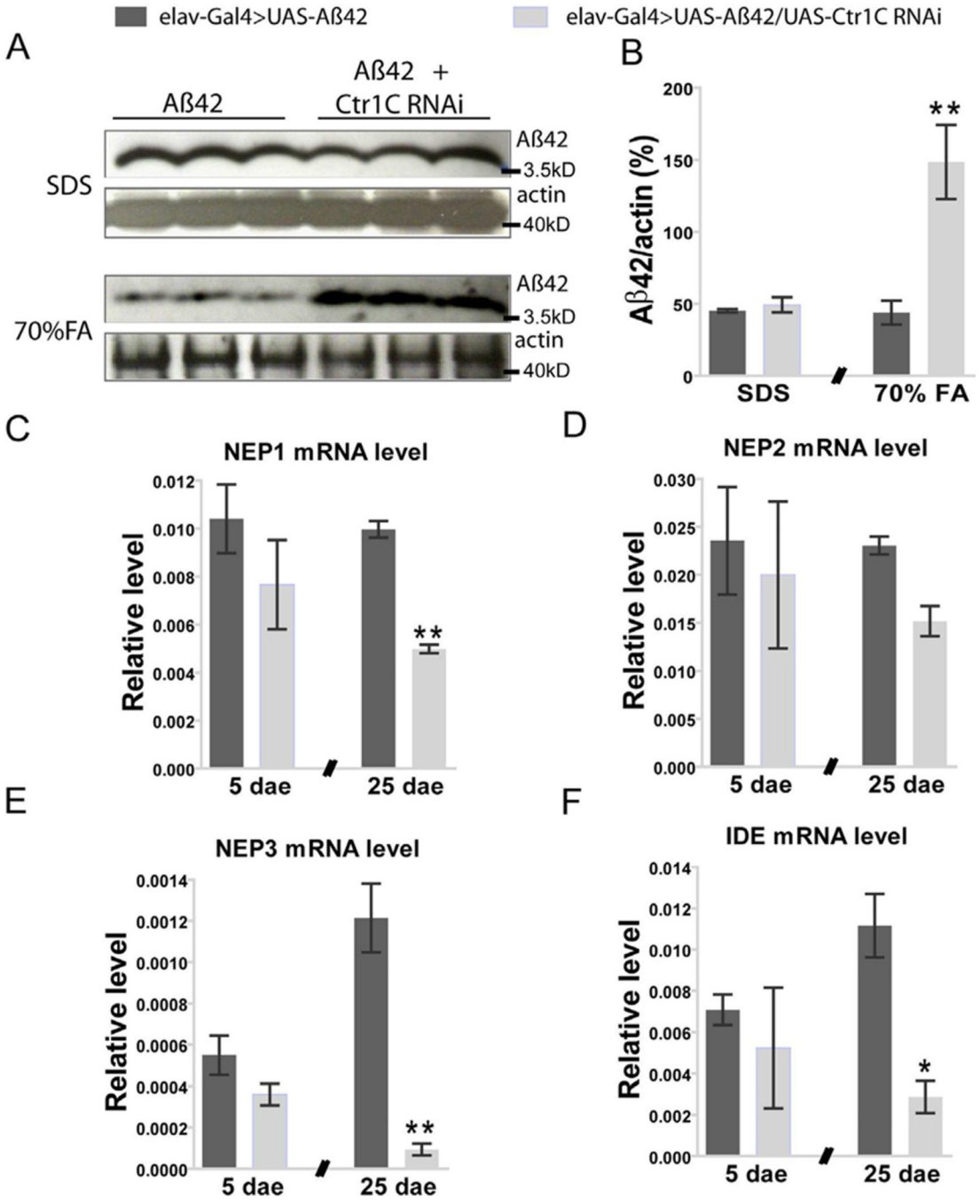


Fig. 5.

Knocking down *Ctr1C* promotes aggregation of higher molecular weight Aβ42 forms with a trend for decreased *NEP* and *IDE* expression levels in brains of Aβ42 flies. Representative western blotting data (A) and statistical results (B). The amount of SDS-soluble Aβ42 was equivalent in Aβ42-expressing flies with *Ctr1C* RNAi and those without *Ctr1C* RNAi. However, SDS-insoluble but formic acid (FA, 70%) soluble Aβ42 forms were dramatically increased after knocking down *Ctr1C*. The amount of Aβ42 and actin proteins in each well were quantified by using ImageJ software, and the data showed the relative percentage of amount of Aβ42 to actin protein. Student's *t*-test, **p<0.01 (in comparison with *elav-Gal4>UAS-Aβ42* flies). n=3 independent experiments. The impact of *Ctr1C* RNAi on

NEP1(C), NEP2 (D), NEP3 (E) and IDE (F) mRNA expression levels in brains of 5 and 25-day-old flies were determined by qRT-PCR. Statistical analysis showed no significant differences for the four tested enzymes between *elav-Gal4>UAS-Aβ42* and *elav-Gal4>UAS-Aβ42/UAS-Ctr1C RNAi* flies at day 5 after eclosion. However, at day 25 after eclosion, *NEP1*(C), *NEP3* (E) and *IDE* (F) mRNA expression levels were significantly decreased in brains of *Ctr1C* RNAi flies (*elav-Gal4>UAS-Aβ42/UAS-Ctr1C RNAi*). Student's *t*-test, *p<0.05, **p<0.01 (in comparison with *elav-Gal4>UAS-Aβ42* flies). The relative *NEP1-3* and *IDE* expression levels against *rp49* were from three independent biological replicates and plotted with SEM (error bars).

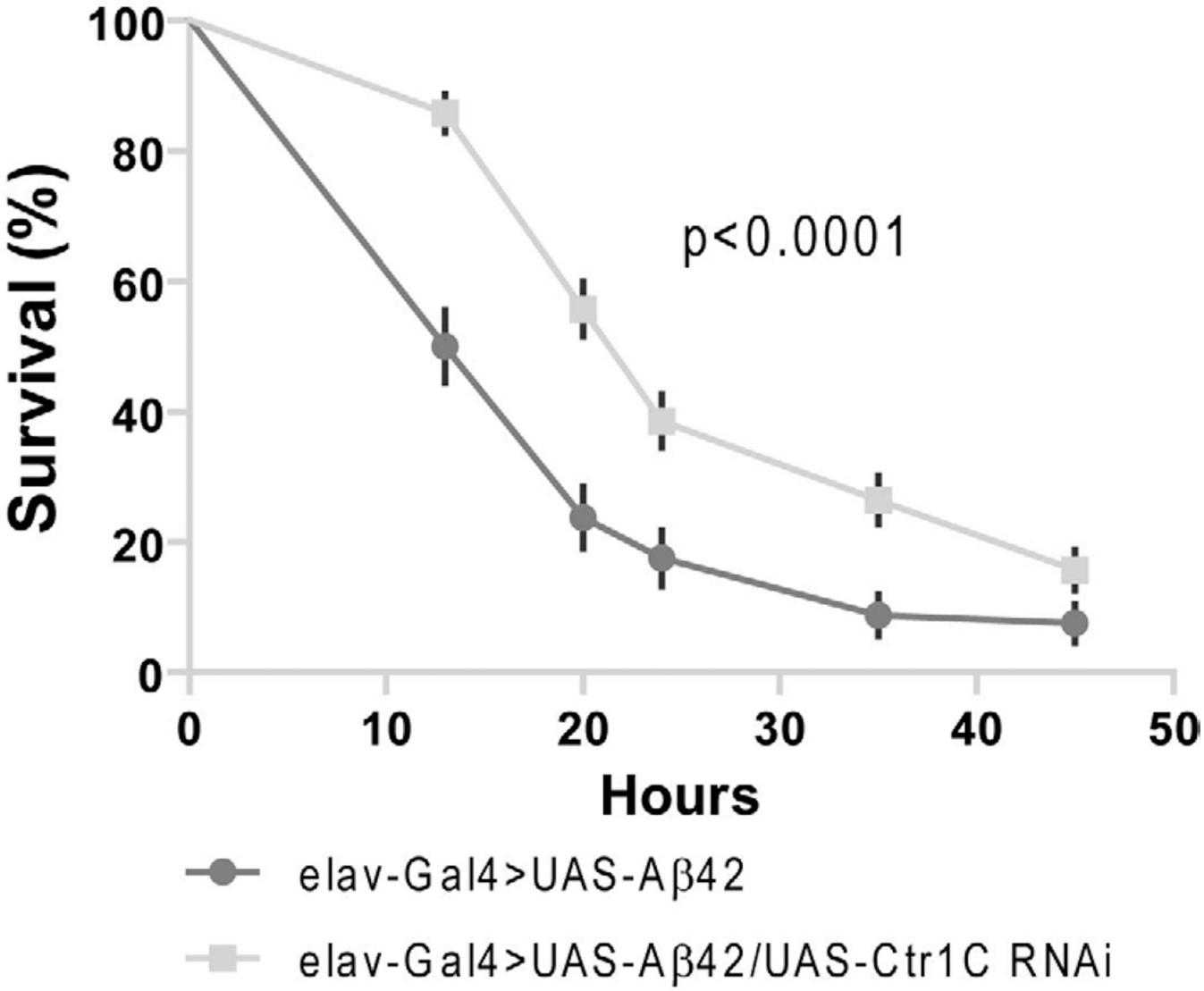


Fig. 6.

Ctr1C RNAi significantly increased Aβ42 flies' tolerance to oxidative stress. About 10-day old normal growing flies were treated in vials with filter paper supplemented with 5% sucrose and 20 mM paraquat. Mortality was recorded about every 8 hours and life span was measured over 45 h. *Ctr1C* RNAi flies (*elav-Gal4>UAS-Aβ42/UAS-Ctr1C RNAi*) survived much better than control Aβ42 flies (*elav-Gal4>UAS-Aβ42*), with a 45.5% increase in mean life span. The two survival curves are significantly different ($p<0.0001$). Reported P values are from Mantel-Cox log-rank statistical analysis. The experiments were repeated at least three times.

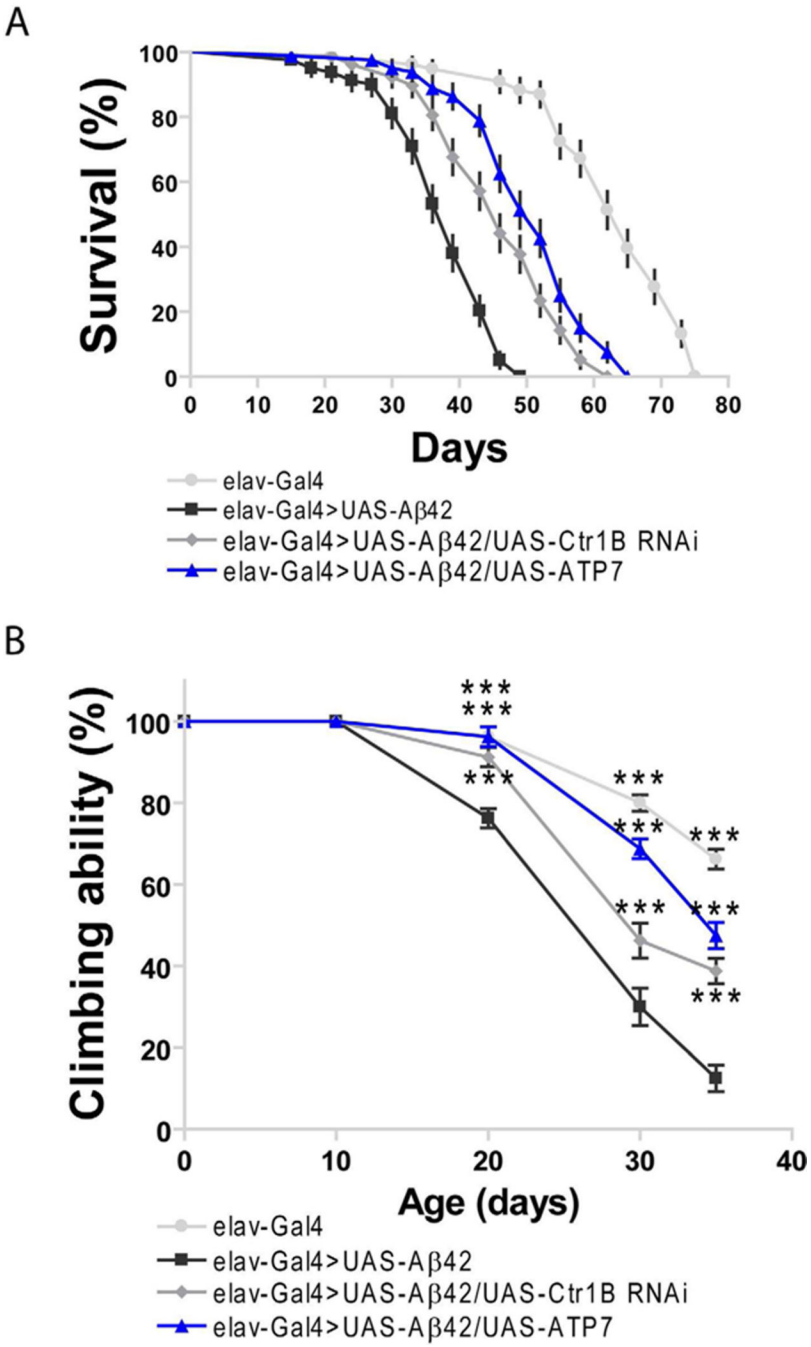


Fig. 7.

Kocking down *Ctr1B* and over-expressing *DmATP7* can ameliorate the climbing and longevity defects of Aβ42 flies. (A) C*tr1B* knockdown and *DmATP7* over-expression significantly lengthened the lifespan of Aβ42 flies, with a 17.9% and a 33.3% extension in median lifespan over that of Aβ42 flies at 25 °C ($p<0.0001$), respectively. Reported P values are from Mantel-Cox log-rank statistical analysis. The percentage survivorship was plotted against age. n=4 independent experiments. (B) The climbing deficit of Aβ42 expression flies was rescued through knocking down *Ctr1B* by RNAi or over-expressing *DmATP7*. Two-way ANOVA, ***$p<0.001$ (in comparison with *elav-Gal4>UAS-Aβ42* flies). n=4 independent experiments.