Short Article

Increasing glucose uptake into neurons antagonizes brain aging and promotes health and life span under dietary restriction in *Drosophila*

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Highlights

- *In vivo* imaging of *Drosophila* brain reveals aged neurons suffer from energy deficits.
- Increased neuronal glucose uptake is sufficient to ameliorate age-dependent declines in ATP.
- Beneficial effects of increased glucose uptake surpass age-dependent mitochondrial damage.
- Increased neuronal glucose uptake suppresses age-related locomotor decline and extends lifespan under dietary restriction.

Summary

Brain neurons play a central role in organismal aging, but underlying mechanisms are still unclear. Dietary restriction extends lifespan, suggesting that bioenergetic challenges may be beneficial. By contrast, cerebral blood flow and glycolysis decrease during aging, suggesting that brain neurons in aged animals experience an energy deficit. In this study, we analyzed age-associated changes in metabolic states in the brain neurons of *Drosophila* and how such changes affect aging. Using a genetically encoded fluorescent ATP biosensor, we show that ATP concentration decreases in the neuronal somata of the brain during aging. A decrease in glucose content, expression of glucose transporter and glycolytic enzymes, and mitochondrial quality correlated with the reduction in ATP concentration. The age-associated reduction in ATP concentration did not occur in brain neurons in which glycolysis was suppressed or glucose transporter was overexpressed to enhance glucose uptake, suggesting that lower glucose uptake and glycolysis underlies the reduction in ATP in aged neurons. In contrast, increasing the glucose uptake did not rescue age-associated mitochondrial damage and surpassed mitochondrial defects to maintain ATP levels. Furthermore, increasing neuronal glucose uptake suppressed age-dependent locomotor deficits and extended the life span, and life span extension was more prominent on the low-calorie diet. These results suggest that strategies aimed at increasing the neuronal uptake of glucose may further promote the beneficial effects of bioenergetic challenges on aging.

Keywords aging, brain, ATP, glucose transporter, glycolysis, mitochondria, dietary restriction, *Drosophila*

Introduction

Aging is associated with progressive declines in physiological integrity and functions, and increases in vulnerability and the risk of developing a number of diseases (Kirkwood and Austad, 2000). The nervous system regulates motor and sensory functions and orchestrates metabolic control and stress responses in peripheral tissues *via* neuroendocrine signals (Alcedo and Kenyon, 2004; Burkewitz et al., 2015; Cohen et al., 2009; Libert et al., 2007; Satoh and Imai, 2014; Ulgherait et al., 2014; Zhang et al., 2018). Thus, age-associated changes in neurons are likely to play a critical role in organismal aging.

The brain requires a large amount of energy: it consumes about 20% of the oxygen and 25% of the glucose in the human body (Belanger et al., 2011; Mink et al., 1981; Raichle et al., 1970). Most of the ATP required to support neuronal function is supplied by the metabolism of glucose through glycolysis and mitochondrial oxidative phosphorylation (Belanger et al., 2011). However, aging induces changes in both glucose availability and energy production, including declines in glucose uptake, electron transport chain activity, and aerobic glycolysis in the brain (Goyal et al., 2017; Hoyer, 2000). This implies that strategies aimed at increasing glucose metabolism in neurons may protect against organismal aging.

By contrast, dietary restriction (DR) and a reduction in insulin signaling have been demonstrated to have anti-aging effects (Guarente, 2008). DR causes circulating glucose concentrations to fall (Guarente, 2008). Thus, the pro-aging effects of reductions in brain glucose metabolism and the anti-aging effects of reducing insulin-stimulated glucose uptake are apparently contradictory. To resolve this discrepancy, it is necessary to elucidate how aging affects glucose metabolism in brain neurons and how such age-related changes in neurons interact with the anti-aging effects of DR.

In the present study, we show that aged brain neurons suffer from ATP deficits. Using a genetically encoded fluorescent ATP biosensor, we show that ATP concentrations decrease in the somas of brain neurons during aging due to a reduction in glycolysis or glucose availability. Increased neuronal glucose uptake by overexpression of glucose transporter is sufficient to ameliorate age-dependent declines in ATP via glycolysis and surpass mitochondrial damages. Of particular interest, we demonstrate that increasing glucose uptake into neurons significantly extends the organismal lifespan under DR conditions, suggesting that increasing neuronal glucose metabolism optimizes the anti-aging effects of energetic challenges.

Results

An ATP biosensor (ATeam) identifies local changes in ATP levels in Drosophila brain neurons

Neurons are highly polarized structurally and functionally, and require tight local management of ATP production (Rangaraju et al., 2014). To determine the local ATP concentration in fly brain neurons, we used the fluorescence resonance energy transfer (FRET)-based ATP biosensor, ATeam (Tsuyama et al., 2013). ATeam detects changes in ATP concentrations in the range of 0.5 to 4 mM at 25°C (Tsuyama et al., 2013; Tsuyama et al., 2017), which is appropriate for the physiological ATP concentrations in neurons (Erecinska and Silver, 1989; Pathak et al., 2015; Rangaraju et al., 2014). The concentrations of intracellular ATP are much higher than the expression levels of ATeam; therefore, the FRET signal derived from ATeam is not likely to be affected by its intracellular distribution (Tsuyama et al., 2013; Tsuyama et al., 2017).

It has been reported that the ATeam signal decreases in response to the inhibition of cellular respiration in the neurons of *Drosophila* larvae (Tsuyama et al., 2013; Tsuyama et al., 2017). To confirm that ATeam is capable of detecting changes in ATP concentration in adult fly brains, we used the Gal4-UAS system to express ATeam in neurons under the control of a pan-neuronal driver Elav-Gal4 and inhibited respiratory complex III by treatment with antimycin (AM). We focused on the mushroom body, where axons, dendrites, and cell bodies are easily identifiable as lobes, calyxes, and Kenyon cells, respectively. AM treatment significantly reduced the FRET signal from ATeam in both the cell bodies and axons (Figure 1A), which indicates that ATeam can identify reductions in ATP concentrations in fly brain neurons.

Next, we determined whether ATeam could identify local changes in ATP concentration. To reduce ATP concentration only in the axons, axonal mitochondria were genetically depleted by knocking down milton, an adaptor protein for the kinesin motor that is essential for mitochondrial anterograde transport (Figure 1B) (Stowers et al., 2002). The RNAi-mediated neuronal knockdown of milton has been reported to effectively deplete mitochondria in axons (Iijima-Ando et al., 2012), and the FRET signal from ATeam decreased in axons, but not in the cell bodies, of milton-deficient fly brain neurons (Figure 1C). These results indicate that ATeam is capable of identifying local changes in ATP concentration in adult brain neurons.

Figure 1

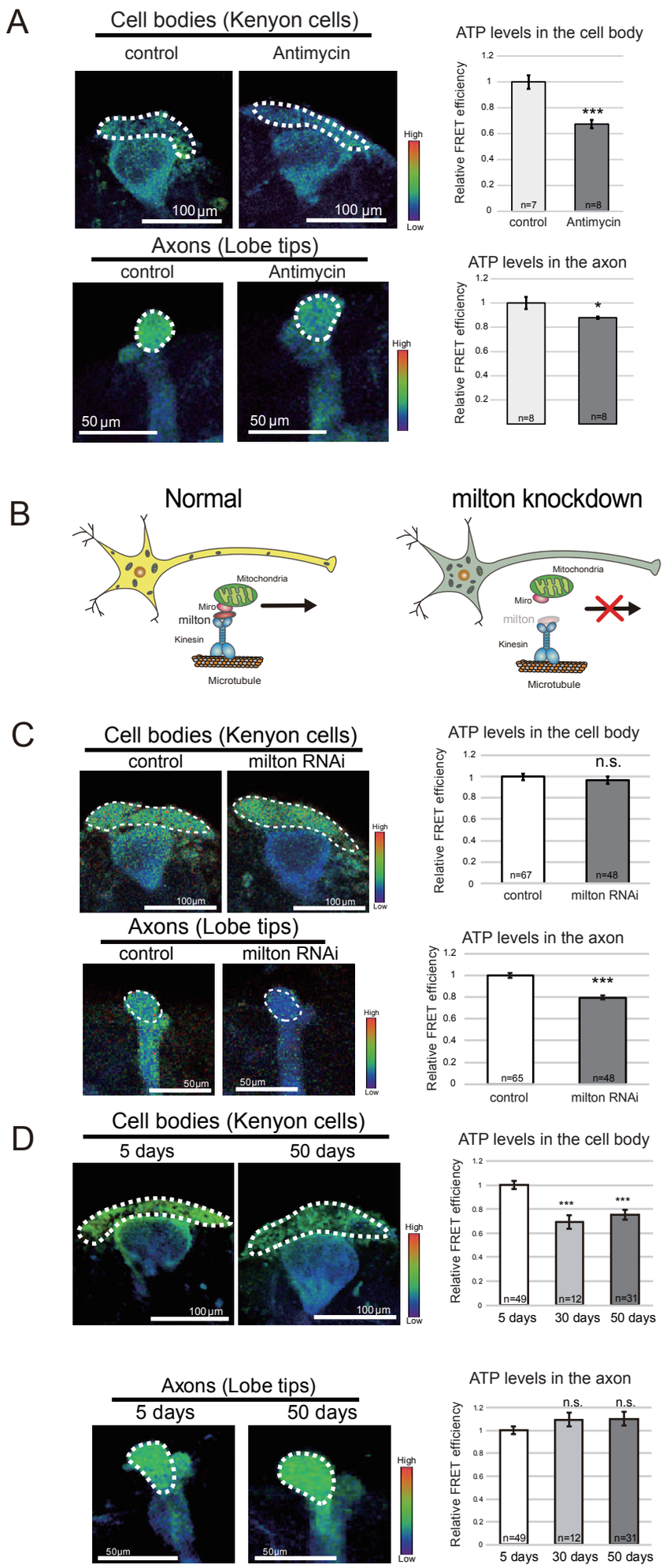

A
Cell bodies (Kenyon cells)
control
Antimycin
100 µm
High
Low
ATP levels in the cell body
Relative FRET efficiency
***
n=7
n=8
control
Antimycin
Axons (Lobe tips)
50 µm
ATP levels in the axon
*
B
Normal
milton knockdown
Mitochondria
Miro
milton
Kinesin
Microtubule
C
control
milton RNAi
100µm
50µm
n.s.
n=67
n=48
n=65
D
5 days
50 days
n=49
n=12
n=31
30 days

Figure 1. ATP concentration decreases in the cell body but not in the axon during aging.
(A) A reduction in ATP concentration, caused by the inhibition of mitochondrial respiration by antimycin in adult fly brain neurons, was identified using ATeam. ATeam expression was driven by the pan-neuronal driver Elav-GAL4. Quantification of the FRET signal (right panels) is shown (mean ± SE, n=7–8; *p<0.05, ***p<0.001; Student's t-test). (B) Schematic representation of mitochondrial transport. The knockdown of milton, an adaptor protein for mitochondrial transport, depleted mitochondria in the axon. (C) Neuronal knockdown of milton reduced ATP concentration in Kenyon cells and lobe tips. Flies carrying the driver alone (Elav-GAL4/+) were used as controls. Data are mean ± SE, n=48–65; n.s., p>0.05, ***p<0.001; Student's t-test). (D) The FRET efficiency of ATeam in Kenyon cells (A) and lobe tips (B) of the mushroom body of 5-, 30-, and 50-day-old flies (n=12–49; n.s., p>0.05, ***p<0.001; one-way ANOVA, followed by Tukey's HSD multiple comparisons test. ATeam expression was driven in neurons by the pan-neuronal driver Elav-GAL4.

ATP concentration decreases in the cell body, but not in the axon, of brain neurons during aging

To determine whether ATP concentration changes in the brain neurons in an age-related manner, we compared the ATP concentration in the mushroom body structure of young (5 days old), middle-aged (30 days old), and old (50 days old) flies. We found that the ATP concentration in the cell bodies in the mushroom body structure of middle-aged (30 days old) and old (50 days old) flies was ~70% of that in young flies (5 days old) (Figure 1D). By contrast, there were no significant changes in the ATP concentrations in the axons during aging (Figure 1D). The brains of 50-day-old flies were structurally intact (Figure 1D and (He and Jasper, 2014; Tonoki and Davis, 2015; White et al., 2010)), which suggests that the reduction in ATeam signal is not due to the loss of neurons. Taken together, these results show that the ATP concentration, specifically in the cell bodies of brain neurons, decreases during aging.

Decreases in glycolytic gene expression contribute to the age-related reduction in ATP

Most of the ATP produced in the brain is supplied by the metabolism of glucose (Belanger et al., 2011). To gain insight into the underlying mechanism, we asked whether glucose availability in the brain decreases during aging in *Drosophila*. We found that the glucose concentration in head extracts was significantly lower in aged flies (30- and 50-day-old) than in young (5-day-old) flies (Figure 2A), which

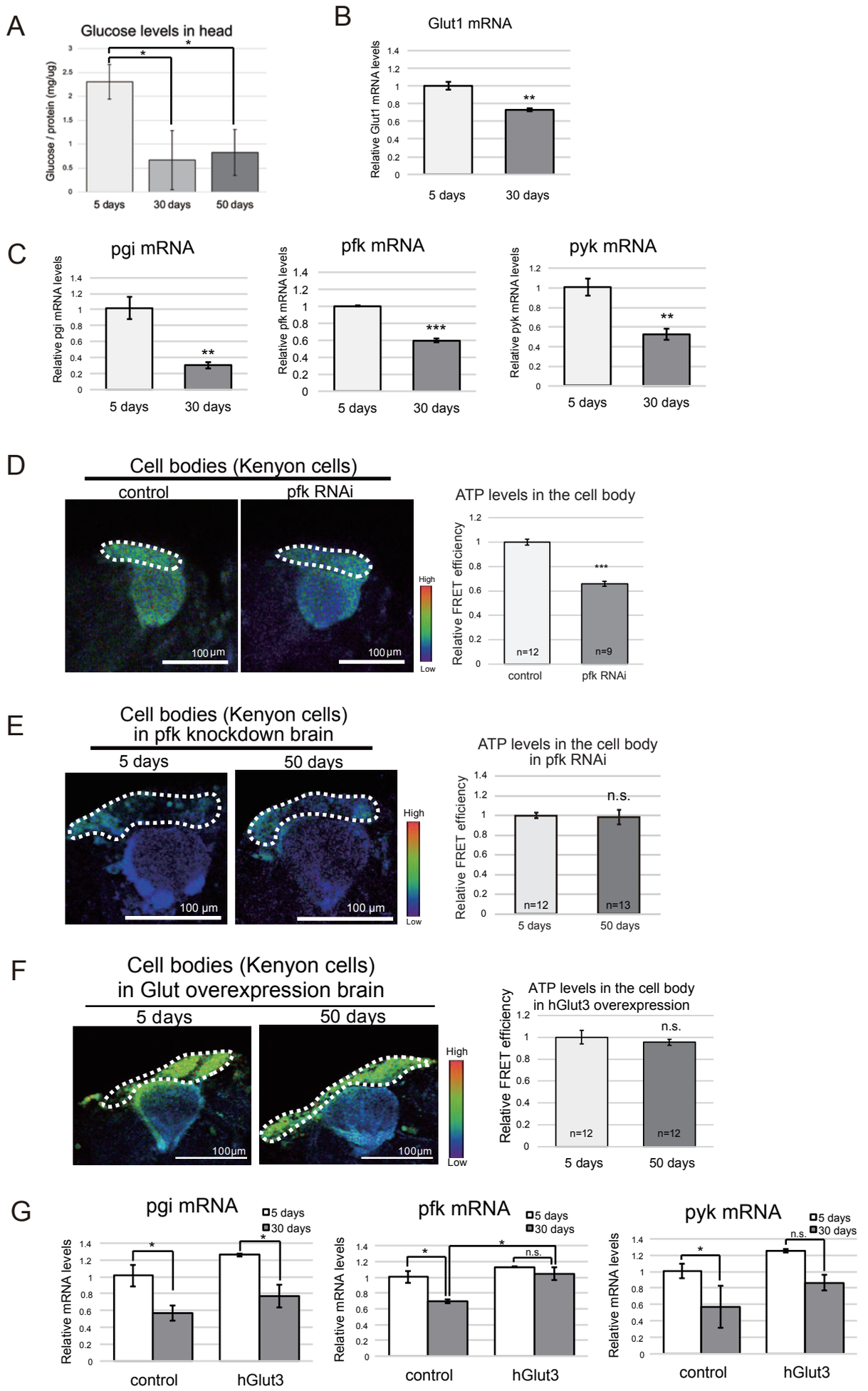
suggests that glucose availability in brain neurons decreases during aging. Glucose uptake by brain neurons is mostly constitutive because the glucose transporter isoforms expressed in CNS neurons, Glut1 and Glut3 in mammals and Glut1 in *Drosophila*, are constitutively localized to the plasma membrane and insensitive to insulin (Chintapalli et al., 2007; Flier et al., 1987). We measured *dGlut1* mRNA expression during aging using qRT-PCR, which revealed that its expression was slightly but significantly lower in the heads of aged than in those of young flies (Figure 2B).

It has been reported that the rate of glycolysis changes during aging in human brains (Goyal et al., 2017). To determine the contribution of changes in glycolytic rate to the age-related reduction in ATP in the cell bodies of brain neurons, we measured the expression of the genes encoding the key glycolytic enzymes glucose-6-phosphate isomerase (*Pgi*), phosphofructokinase (*Pfk*), and pyruvate kinase (*Pyk*) in young and aged fly heads. qRT-PCR analysis revealed that the mRNA expression of these genes was significantly lower in aged than young flies (Figure 2C). To evaluate the contribution of glycolysis to ATP production in brain neurons, we knocked down *Pfk*, which encodes phosphofructokinase, a rate-limiting enzyme in glycolysis. Neuronal knockdown of *Pfk* reduced ATP concentration by up to 65% in 3-day-old flies (Figure 2D), indicating that glycolysis plays a critical role in ATP production in brain neurons. Next, to determine whether the decline in glycolytic activity contributes to the age-related decrease in ATP concentration, we measured the ATeam signal in *Pfk*-deficient young and aged brain neurons. The neurons of 5-day-old flies with *Pfk* knockdown showed a reduced ATP concentration, with no further reduction in 50-day flies (Figure 2E). This result suggests that a lower rate of glycolysis and/or events upstream of glycolysis are responsible for the age-related reduction in ATP in cell bodies of brain neurons.

Neuronal overexpression of glucose transporter ameliorates the age-related reduction in ATP concentration in brain neurons and glycolytic gene expression

We next asked whether increasing glucose uptake could ameliorate the age-related reduction in ATP in the brain neurons. Overexpression of *hGlut3* in *Drosophila* cells has been reported to increase glucose uptake (Besson et al., 2015). We found that neuronal overexpression of *hGlut3* ameliorated the age-related reduction in ATP in the neuronal cell bodies (Figure 2F). By contrast, neuronal overexpression of *hGlut3* did not increase the ATP concentration in young flies (Figure S1). These results suggest that glucose availability is the rate-limiting factor specifically in the brain neurons of old flies, and that overexpression of glucose transporter can increase ATP

Figure 2


A
Glucose levels in head
Glucose / protein (mg/ug)
5 days
30 days
50 days
B
Glut1 mRNA
Relative Glut1 mRNA levels
5 days
30 days
C
pgi mRNA
Relative pgi mRNA levels
pfk mRNA
Relative pfk mRNA levels
pyk mRNA
Relative pyk mRNA levels
D
Cell bodies (Kenyon cells)
control
pfk RNAi
100 µm
High
Low
ATP levels in the cell body
Relative FRET efficiency
n=12
n=9
E
Cell bodies (Kenyon cells)
in pfk knockdown brain
5 days
50 days
ATP levels in the cell body
in pfk RNAi
n.s.
n=13
F
Cell bodies (Kenyon cells)
in Glut overexpression brain
ATP levels in the cell body
in hGlut3 overexpression
G
Relative mRNA levels
control
hGlut3

production in the neurons of aged flies. We also examined whether glucose transporter overexpression ameliorates not only the age-related reduction in ATP but also the age-related changes in glycolytic activity and found that neuronal overexpression of *hGlut3* suppressed the age-related decrease in the mRNA expression of glycolytic genes, *Pgi*, *Pfk*, and *Pyk* (Figure 2G).Taken together, these results suggest that neuronal overexpression of *hGlut3* ameliorates age-related decline in ATP concentration by increasing glucose metabolism in aged fly neurons.

Figure 2. Neuronal overexpression of glucose transporter ameliorates the age-related reduction in ATP concentration in brain neuron and glycolytic gene expression. (A) Glucose concentration in head lysates decreased during aging. The glucose concentrations in 5-, 30- and 50-day-old fly heads, normalized to protein concentration, are shown (mean ± SE, n=6; **p*<0.05; one-way ANOVA, followed by Tukey's HSD multiple comparisons test). (B) dGlut1 mRNA expression decreased in fly heads during aging. qRT-PCR was performed using RNA extracted from the heads of 5- and 30-day-old flies (mean ± SE, n=3; ***p*<0.01; Student's *t*-test). (C) The expression of glycolytic genes decreased in the fly brain during aging. The mRNA expression of genes encoding glycolytic enzymes in the heads of 5- and 30-day-old flies was quantified using qRT-PCR (mean ± SE, n=3; ***p*<0.01, ****p*<0.001; Student's *t*-test). (D) Neuronal knockdown of *Pfk*, which encodes a rate-limiting glycolytic enzyme, reduced ATP concentration in 3-day-old fly neurons (mean ± SE, n=9–12; ****p*<0.001; Student's *t*-test). (E) The age-related reduction in ATP concentration in neuronal cell bodies was not identified in *Pfk*-deficient neurons. The expression of ATeam and *Pfk* RNAi was driven by the pan-neuronal driver Elav-GAL4. Data are mean ± SE, n=12–13; n.s., *p*>0.05, **p*<0.05; Student's *t*-test. (F-H) Neuronal overexpression of hGlut3 ameliorates the age-related reduction in ATP concentration and glycolytic gene expression and the decline in physical function. (F) ATP concentration did not decrease with aging in neurons overexpressing hGlut3. Expression of ATeam and hGlut3 was driven by the pan-neuronal driver Elav-GAL4 (mean ± SE, n=12; n.s., *p*>0.05; Student's *t*-test). (G) Neuronal overexpression of hGlut3 ameliorated the age-associated decline in glycolytic gene expression. The mRNA expression of genes encoding key glycolytic enzymes was quantified using qRT-PCR in the heads of 5- and 30-day-old flies. Flies carrying the driver alone were used as controls. Data are mean ± SE, n=3; n.s., *p*>0.05, **p*<0.05, ***p*<0.01; one-way ANOVA, followed by Tukey's HSD multiple comparisons test.

The age-related reduction in mitochondrial quality is dispensable for maintaining ATP levels when glucose transporter was overexpressed

Since mitochondrial dysfunction is implicated in aged brains, we examined whether the reduced number and increased damage of mitochondrial is associated with an age-related reduction in ATP levels in brain neurons. We first measured the mitochondrial density in the brain neurons of young and aged flies by transmission electron microscopy (TEM). We found that in the cell body, where ATP concentration decreases (Figure 1C), the number of mitochondria did not change significantly during aging (Figure 3A). Thus, the age-related reduction in ATP in the cell bodies of neurons is not the result of a decrease in the number of mitochondria.

Next, we analyzed the quality of mitochondria in the brain neurons of young and aged flies. Dysfunctional mitochondria are recognized by the abnormal structure of their cristae (Brandt et al., 2017; Daum et al., 2013). Ultrastructural analyses of the mitochondria revealed that the number of abnormal mitochondria in cell bodies slightly but significantly increased during aging (7% in 7-day-old flies and 13% in 35-day-old flies, Figure 3B). These results indicate that a slight but significant increase in the number of damaged mitochondria occurs alongside a decrease in ATP concentration in the cell bodies of neurons during aging.

Interestingly, however, while neuronal overexpression of *hGlut3* ameliorated the age-related reduction in ATP in the neuronal cell bodies (Figure 2F), it did not recover either the number of or quality of mitochondria (Figure 3C). These results suggest that, although the mitochondrial quality is reduced, increasing glucose uptake is sufficient to ameliorate energetic deficits in aged brain neurons.

Increased glucose uptake in neurons ameliorates physical performance in aged flies and extends lifespan further on a low-calorie diet

Finally, we examined whether the increase in glucose uptake into neurons promotes the health and life span of flies. We first analyzed the effects of neuronal overexpression of hGlut3 on the age-related decline in physical function using a climbing assay, a robust behavioral assay taking advantage of the innate negative geotaxis behavior. In wild-type flies, climbing ability declined by 45 days of age, whereas flies that neuronally expressed hGlut3 performed significantly better at this age (Figure 4A), indicating that the increase in glucose uptake into neurons promotes healthspan in flies. We also found that flies with neuronal expression of hGlut3 lived slightly but significantly longer than control flies (Figure 4B).

DR has been reported to extend the life span in most species, including

Figure 3

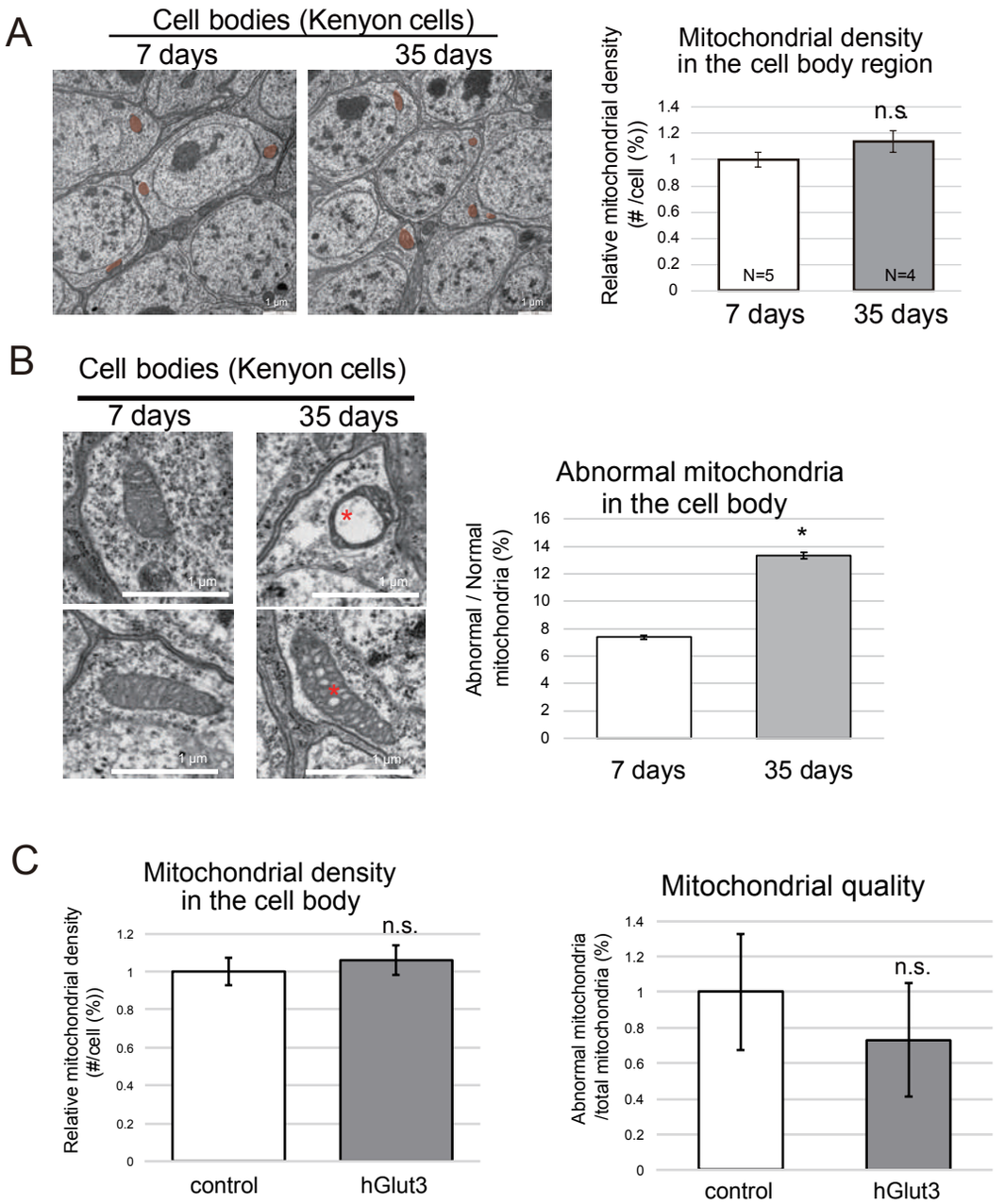


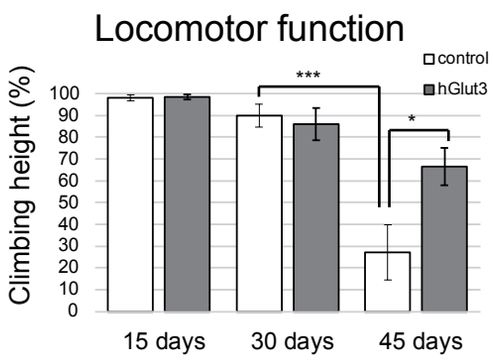
Figure 3. Age-related changes in the number and quality of mitochondria in the brain neurons. (A) The number of mitochondria in the neuronal cell body did not significantly change during aging. More than four brain hemispheres from flies of each age were analyzed using transmission electron microscopy (TEM), and the numbers of mitochondria in more than 30 cells in each hemisphere were counted. Representative images are shown, with the mitochondria highlighted in orange. (B) The ratio of the number of abnormal mitochondria to the total number of mitochondria increased in the cell body during aging. More than four brain hemispheres from flies of each age were examined using TEM, and more than 30 cells were analyzed in each hemisphere. The number of mitochondria containing vacuoles or disorganized cristae (asterisks) was counted. (mean ± SE, n=4–5; n.s., $p>0.05$, $*p<0.05$; Student's t-test). (C) Neuronal overexpression of hGlut3 did not affect the age-associated changes in mitochondria in the brain. The mitochondrial density (Left) and the ratio of the number of abnormal mitochondria to the total number of mitochondria (Right) in neurons expressing hGlut3 in the Kenyon cell region of the brains were analyzed under TEM 50 days after eclosion (mean ± SE, n=4–6; n.s., $p>0.05$; Student's t-test).

Drosophila (Bauer et al., 2005). Since DR causes circulating glucose concentrations to fall (Guarente, 2008), the anti-aging effects of DR and that of the increase in glucose uptake into neurons might be independent and additive. To determine the relationship between neuronal uptake of glucose and DR in aging, we analyzed the effects of neuronal expression of hGlut3 on life span under various dietary conditions. Flies were raised on regular cornmeal food, and after eclosion, they were fed regular food, DR food (10%) (10% yeast extract, 10% sucrose, no cornmeal) or DR food (1%) (1% yeast, 1% sucrose, no cornmeal). Flies on DR food showed reduced levels of glucose in the head and body with more effects with 1 % food than 10% food (Figure S2A). The life span was significantly extended on 1% food (Figure S2B). Of particular interest, life span extension by hGlut3 was more prominent under DR Conditions. While the median lifespans on regular food were 55 days in control and 59 days in hGlut3-overexpressing flies maintained on regular food (Figure 4B), on 1% food, the median lifespans were 59 days in control and 73 days in hGlut3-overexpressing flies (Figure 4D). Maintaining flies on 10% food did not affect the life span of control flies, while it extended the life span of hGlut3-overexpressing flies (the median lifespans; 48 days in control and 56 days in hGlut3-overexpressing flies (Figure 4C)). These results suggest that increased glucose uptake by neurons enhances the anti-aging effects of DR at the organismal level.

Figure 4. An increase in the neuronal uptake of glucose ameliorates physical performance in aged flies and extends lifespan additively further under dietary restriction conditions. (A) Neuronal overexpression of hGlut3 ameliorated the age-associated neuronal dysfunction. Flies were tapped to the bottom of the vial, and the distance that they then climbed in 30 sec was measured (mean ± SE, n=12–39; *p<0.05, ***p<0.001; Student's t-test). Flies carrying the driver alone were used as controls. (B-D) Neuronal overexpression of hGlut3 and DR synergistically extended lifespan. Flies were raised on regular cornmeal food and maintained on regular cornmeal food (B) or two types of DR diet (C and D) (C: food without cornmeal and containing 10% (w/v) of yeast extract and sucrose (10%), D: food without cornmeal and containing 1% (w/v) of yeast extract and sucrose) after eclosion. Error bars represent 95% confidence intervals on observed survivorship. Data are Mean±SE, n=131-601, ***; p<0.001, ****; p<0.0001, Log-rank test.

Figure 4

A



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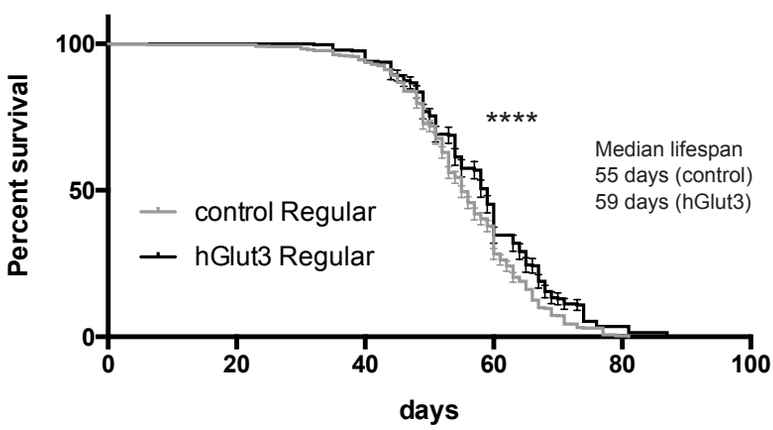

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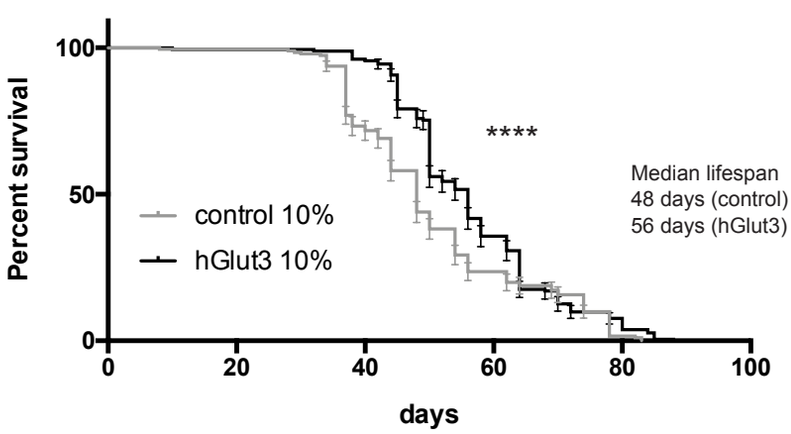

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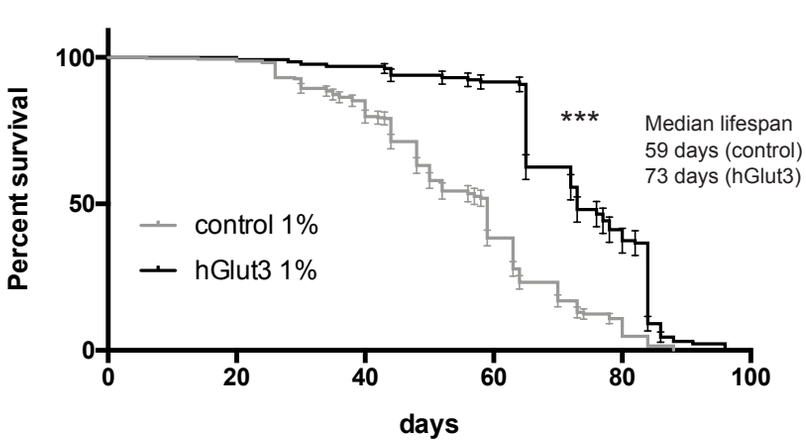

DISCUSSION

In the present study, we showed that aged brain neurons suffer from energy deficits, along with decreases in glucose concentration, the expression levels of glucose transporter and glycolytic enzymes (Figure 1-2), and mitochondrial quality (Figures 3). Increasing glucose uptake by neurons was sufficient to ameliorate energetic deficits (Figure 2) despite persistent mitochondrial damage (Figures 3) and suppressed age-dependent locomotor decline (Figure 4). Interestingly, the effects of increasing glucose uptake on life span extension were more prominent under DR (Figure 4B-D), suggesting that strategies aimed at increasing the neuronal uptake of glucose work in concert to promote beneficial effects of bioenergetic challenges on health and lifespan in flies.

The ATP supply to brain neurons decreases during the lifetime because of global reductions in glucose metabolism, including in oxygen consumption, glycolysis, and mitochondrial respiratory capacity (Conley et al., 2000; Goyal et al., 2017; Jang et al., 2018; Kety, 1956; Martin et al., 1991). Our results suggest that glucose uptake is a limiting factor for ATP supply in aged brain neurons. An increase in glucose uptake not only ameliorated the reduction in neuronal ATP concentration during aging, but also ameliorated the age-related reduction in the expression of glycolytic enzymes (Figure 2), which may be mediated by activation of HIF-1α (Lum et al., 2007) in response to the elevated concentration of intracellular glucose (Guo et al., 2008). In contrast, an increase in glucose uptake did not suppress the age-related reduction in mitochondrial quality (Figure 3), suggesting that enhanced glucose supply can surpass mitochondrial damage to produce sufficient ATP *in vivo*.

DR is known to extend lifespan, but it may reduce glucose supply to the brain and worsen energy deficits in neurons. Our results suggest that the increased glucose uptake by neurons further improves the anti-aging effects of DR, presumably by counteracting neuronal energy deficits under DR conditions (Figure 4). A previous study showed that neuronal expression of hGlut3 did not affect the life span (Niccoli et al., 2016), and this discrepancy is presumably due to different dietary conditions.

The anti-aging effects of DR have been reported to be mediated by a reduction in the intake of protein or amino acids (Mirzaei et al., 2014). Amino-acid restriction affects lifespan through effects on amino-acid sensing pathways, such as mechanistic target of rapamycin complex 1 and the integrated stress response (Mirzaei et al., 2014). The additive effects of DR and enhancement of neuronal glucose uptake on lifespan may be explained by the activation of these anti-aging pathways by DR in the peripheral

tissues and an increase in glucose metabolism by enhanced glucose uptake in neurons. Thus, a strategy aimed at both increasing glucose uptake by neurons and restricting amino-acid intake may be effective at reducing the effects of aging.

In summary, we have shown that glucose uptake by brain neurons is a critical regulator of organismal as well as brain aging. Further elucidation of the cross-talk among nutrient signaling pathways in the central nervous system and peripheral tissues during aging will lead to the identification of novel strategies for the extension of the healthy lifespan of an organism.

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Author Contributions

M.O., K.M.I and K.A. designed the experiments, interpreted the data, and wrote the manuscript; M.O., A.A., T.S. and E.S. conducted the experiments; and M.O., E.S. and K.A. analyzed the data. All authors read and approved the manuscript.

Declaration of Interests

The authors declare no competing interests.

Materials & Methods

Fly strains

The transgenic fly line carrying UAS-ATeam was a kind gift from Dr. Hiromi Imamura (Kyoto

University)(Tsuyama et al., 2013). The transgenic fly line carrying the human Glut3 was reported previously and a kind gift from Dr. Marie Thérèse Besson (Besson et al., 2015). UAS-PFK RNAi (B36782) and UAS-luciferase RNAi (B31603) were obtained from the Bloomington stock center. UAS-PFK RNAi (v 101887) was obtained from Vienna Drosophila Resource Center. Genotypes of the flies used in the experiments are described in Table S1. Flies were reared in a standard medium containing 10% glucose, 0.7% agar, 9% cornmeal, 4% yeast extract, 0.3% propionic acid, 0.1% n-butyl p-hydroxybenzoate per liter. Flies were maintained at 25°C under light-dark cycles of 12:12 hours, and food vials were changed every 2-3 days.

FRET imaging and analysis

FRET-based ATP biosensors, ATeams, consist of mseCFP as a FRET donor, cp173-mVenus as a FRET acceptor, and the epsilon subunit of Bacillus subtilis FoF1 ATP-synthase, which links the donor with the acceptor (Imamura et al., 2009). Binding of ATP to the epsilon subunit induces its large conformational change into a folded form, resulting in an increase in FRET (Imamura et al., 2009). For FRET imaging, *Drosophila* were kept at 25°C, where the probe is optimized to detect changes in physiologically relevant ATP levels (Tsuyama et al., 2013). Flies expressing ATeam were imaged using C2 confocal microscope (Nikon) with a 440-nm solid-state laser for excitation two emission filters (482±17.5 for CFP and 537±13 for YFP-FRET) A series of z-sections was projected if necessary. To subtract background signals, a region adjacent to regions of interest (ROIs) was set as background regions. The mean fluorescent intensity of the background regions was subtracted from that of the ROI. FRET signals (FRET/CFP emission ratio) within ROIs were calculated by dividing the mean intensity of FRET emission by that of the CFP emission using Microsoft Excel (Microsoft). Ratio images were generated using NIS-Elements imaging software (Nikon). For imaging of adult brain preparations, brains were dissected in HL3.1 buffer containing 70mM NaCl, 5mM KCl, 0.2mM $CaCl_2$, 20mM $MgCl_2$, 10mM $NaHCO_3$, 5mM Trehalose, 5mM HEPES. To inhibit mitochondrial respiration, dissected brains were treated with 100 μM of Antimycin A (Santa Cruz Biotechnology) for 15 min.

Electron microscopy

Proboscis was removed from decapitated heads, which were then incubated in primary fixative solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer) at R.T. for 2 hours. After washing heads with 3% sucrose in 0.1 M sodium cacodylate buffer, fly heads were post-fixed for 1 hour in secondary fixation (1% osmium tetroxide in 0.1 M sodium cacodylate buffer) on ice. After washing with H_2O, heads were dehydrated with ethanol and infiltrated with propylene oxide and Epon mixture (TAAB and Nissin EM) for 3 hours. After infiltration, specimens were embedded with Epon mixture at 70°C for 2~3 days. Thin-sections (70 nm) of mushroom body

were collected on copper grids. The sections were stained with 2% uranyl acetate in 70% ethanol and Reynolds' lead citrate solution. Electron micrographs were obtained with a VELETA CCD Camera (Olympus Soft Imaging Solutions GMBH) mounted on a JEM-1010 electron microscope (Jeol Ltd.).

RNA extraction and quantitative real-time PCR analysis

Heads from more than 25 flies were mechanically isolated, and total RNA was extracted using ISOGEN (NipponGene) followed by reverse-transcription using PrimeScript RT reagent kit (Takara). The resulting cDNA was used as a template for PCR with THUNDERBIRD SYBR qPCR mix (TOYOBO) on a Thermal Cycler Dice real-time system TP800 (Takara). Expression of genes of interest was standardized relative to *rp49*. Relative expression values were determined by the ΔΔCT method (Livak and Schmittgen, 2001). Experiments were repeated three times, and a representative result was shown. Primers were designed using DRSC FlyPrimerBank (Harvard Medical School). Primer sequences are shown below;

Glut1 for 5'-TTACCGCGGAGCTCTTCTCC-3'
Glut1 rev 5'-GCCATCCAGTTGACCAGCAC-3'
Pgi for 5'-AGATACTGCTGGACTACTCGAAG-3'
Pgi rev 5'-TCCGTGATGTTAATGTGCTGG-3'
Pfk for5'-GGTCGCTTGAAAGCCGCTA-3'
Pfk rev 5'-CTGACGGAACAGATTGGCG-3'
Pyk for 5'-GCAGGAGCTGATACCCAACTG-3'
Pyk rev 5'-CGTGCGATCCGTGAGAGAA-3'

Climbing Assay

Climbing assay was performed as previously described (Iijima-Ando et al., 2009). Approximately 30 flies were placed in an empty plastic vial (2.5 cm in diameter x 8 cm in length). The vial was gently tapped to knock the flies to the bottom, and the height that the flies climbed in 30 seconds after tapping to the bottom of the vials was measured. Experiments were repeated more than three times, and a representative result was shown. Food vials were changed every 2-3 days.

Lifespan Analysis

Food vials containing approximately 25 flies were placed on their sides at 25°C under conditions of a 12 hour:12 hour light:dark cycle. Food vials were changed every 2-3 days, and the number of dead flies was counted each time. At least three vials for each genotype were prepared.

Calorie restriction

Flies were raised on the regular cornmeal. After eclosion, female flies were maintained on regular cornmeal food, on the food in which cornmeal was removed and the concentration of yeast extract and sucrose were reduced to 10% of the regular food (10%), or the food in which cornmeal was removed and the concentration of yeast extract and sucrose were reduced to 1% of the regular food (1%). Flies were placed at 30 flies/vial, and food vials were changed every 2-3 days.

Glucose assay

Glucose assay (HK) was performed as previously described with mild modifications (Tennessen et al., 2014). Flies were frozen with liquid nitrogen and heads and bodies were isolated. 40 heads and 10 bodies were collected and homogenized in 100 µl PBS. The sample extract was centrifuged at 16,000 g at 4°C. The supernatant was heated at 70 °C for 10 min and centrifuged at 16,000 g for 3 min at 4°C. Glucose levels were measured using by HK reagent (Sigma). Protein levels were measured using by Bradford assay.

Supplemental information

Figure S1-S2

Supplemental Table

Figure 1A	control	elav-Gal4/+
Figure 1C	control	elav-Gal4/+
	milton RNAi	elav-Gal4/milton RNAi
Figure 1D	control	elav-Gal4/+
Figure 2A	control	elav-Gal4/+
Figure 2B	control	elav-Gal4/+
Figure 2C	control	elav-Gal4/+
Figure 2D	control	elav-Gal4/+
	pfk RNAi	elav-Gal4/+; pfk RNAi/+
Figure 2E	pfk RNAi	elav-Gal4/+; pfk RNAi/+
Figure 2F	hGlut3	elav-Gal4/+; hGlut3/+
Figure 2G	control	elav-Gal4/+
	hGlut3	elav-Gal4/+; hGlut3/+
Figure 3A	control	elav-Gal4/+
Figure 3B	control	elav-Gal4/+
Figure 3C	control	elav-Gal4/+
	hGlut3	elav-Gal4/+; hGlut3/+
Figure4A	control	elav-Gal4/+
Figure4B	control	elav-Gal4/+
	hGlut3	elav-Gal4/+; hGlut3/+
Figure 4C	control	elav-Gal4/+
	hGlut3	elav-Gal4/+; hGlut3/+
Figure 4D	control	elav-Gal4/+
	hGlut3	elav-Gal4/+; hGlut3/+
S1	control	elav-Gal4/+
	hGlut3	elav-Gal4/+; hGlut3/+
S2A	control	elav-Gal4/+
S2B	control	elav-Gal4/+

Supplemental Table. The genotypes of flies used in each experiment.

Supplemental figure 1

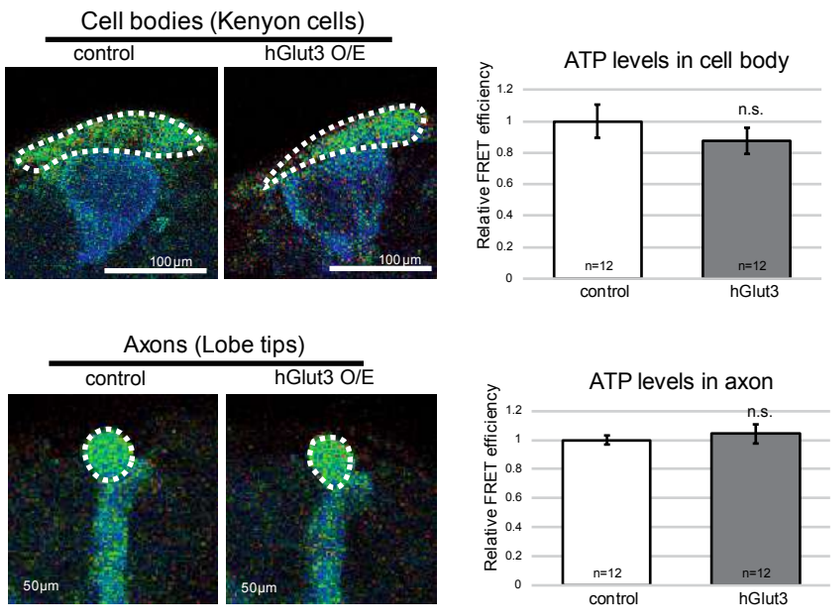


Figure S1. The overexpression of hGlut3 does not increase ATP concentration in the mushroom body neurons of young flies. The FRET signals from ATeam were quantified and are shown as mean ± SE (n=12–13, n.s.; p>0.05, Student's t-test). The flies were 5 days-old.

Supplemental figure 2

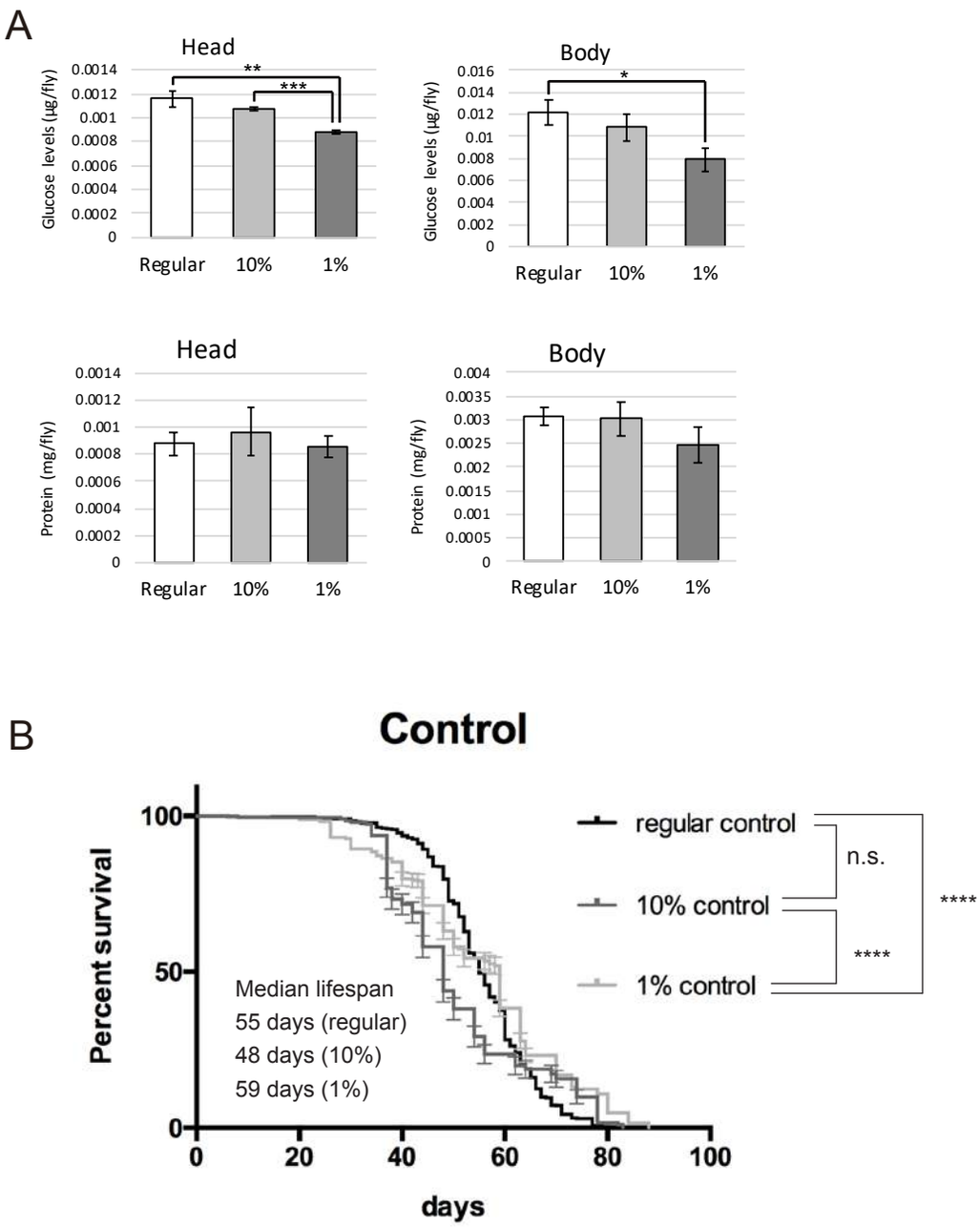
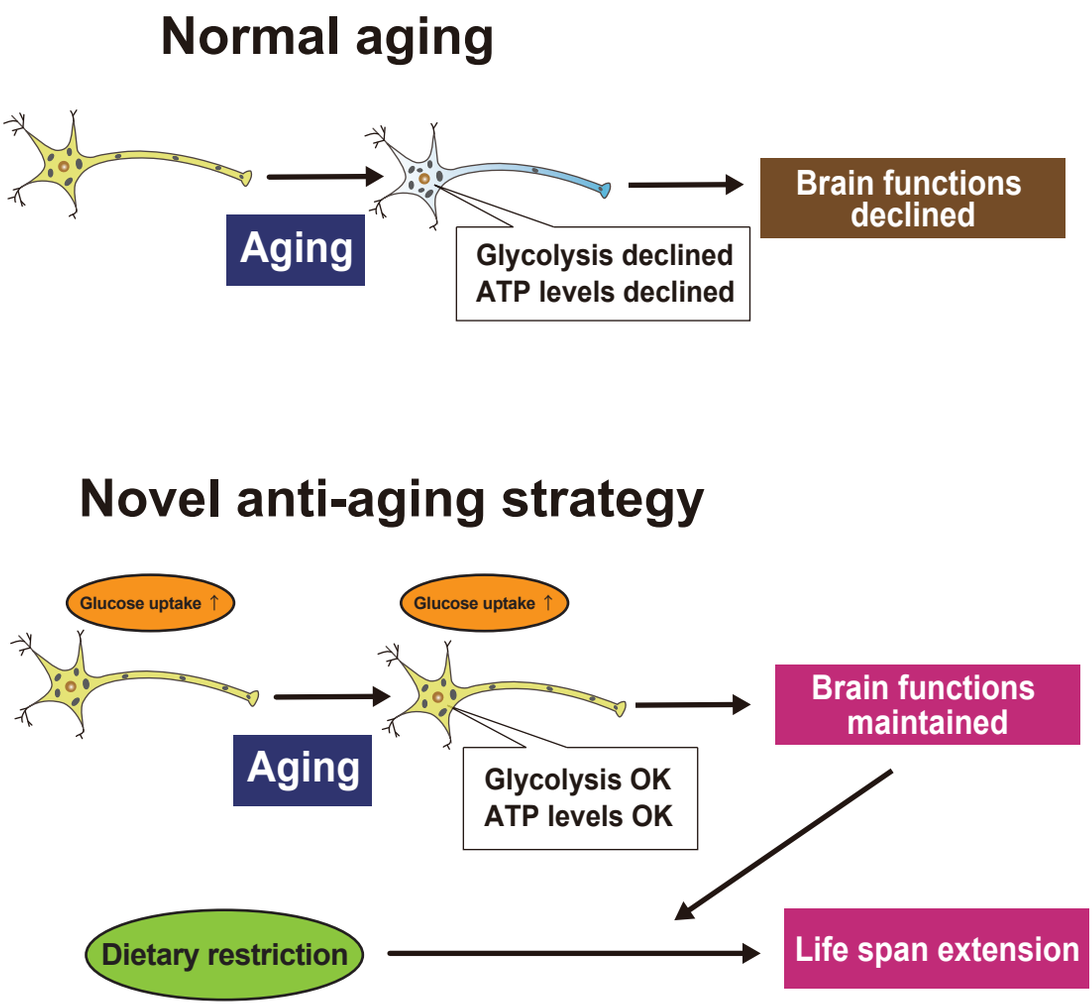


Figure S2. The effects of DR diets on glucose levels and life span. (A) Flies maintained on DR diet contain lower levels of glucose in their heads. The glucose and protein concentrations in the heads of flies subjected to dietary restriction (DR). Flies were raised on regular cornmeal food. After eclosion, they were maintained on regular corn meal food (Regular) or two types of DR diet (10% and 1%) (10%: food without cornmeal and containing 10% (w/v) of yeast extract and sucrose, 1%: food without cornmeal and containing 1% (w/v) of yeast extract and sucrose). Data are mean ± SE, n=3, **; $p<0.01$, ***; $p<0.001$, Student's t-test). (B) Flies maintained on 1% food showed longer lifespan. Error bars represent 95% confidence intervals on observed survivorship. Data are Mean ±SE, n=131-601, n.s.; $p>0.05$, ****; $p<0.0001$, Log-rank test.

Graphical abstract



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