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Supplemental Information

Increasing neuronal glucose uptake attenuates brain aging and promotes life span under dietary restriction in *Drosophila*

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Supplemental figure 1

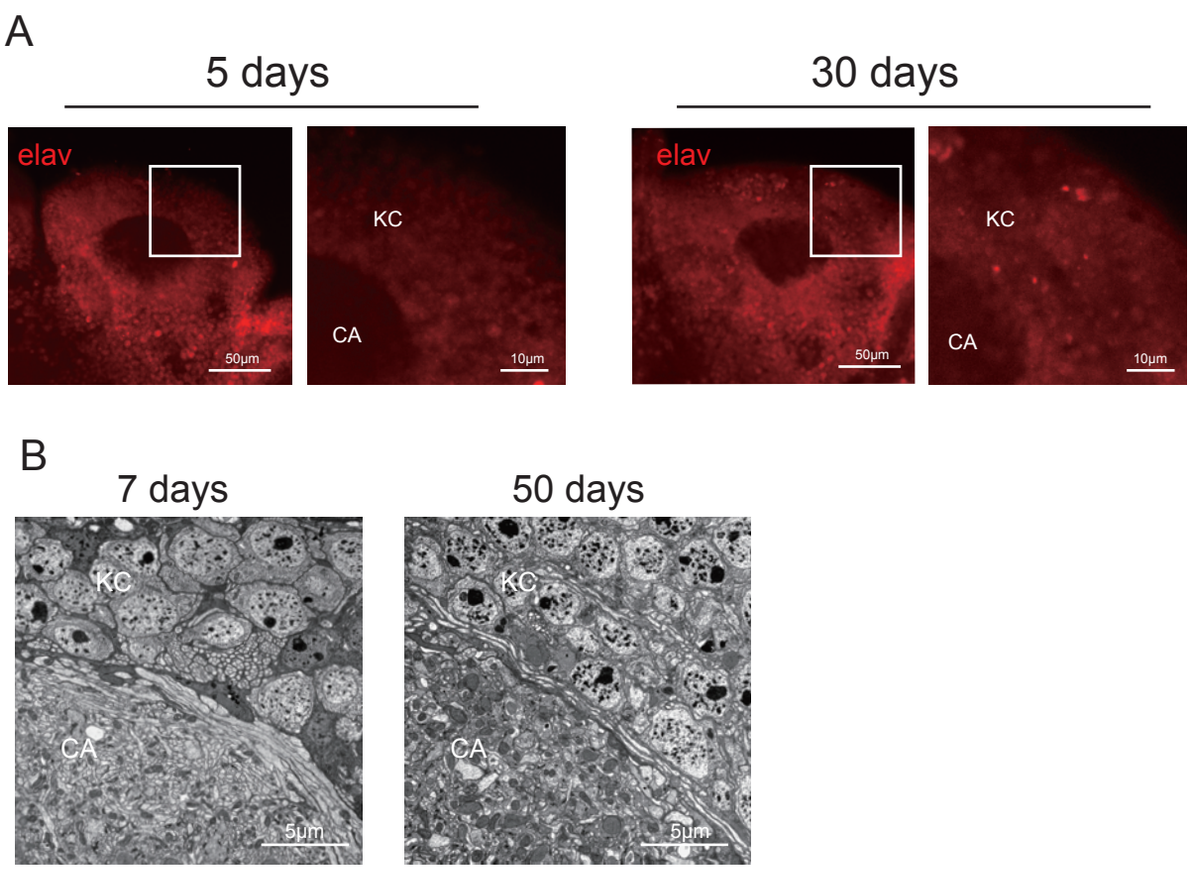

Figure S1. Kenyon cells in aged fly brains are structurally intact, Related to Figure 1

(A) Fly brains expressing an ATP biosensor in neurons were immunostained with an antibody for the neuronal marker elav. Low and high magnification of the maximum intensity projection of Calyx (CA) and Kenyon Cells (KC) are shown. More than six brains were analyzed, and no degeneration was observed. (B) Ultrastructural analyzes of Kenyon cells show no sign of cell death in the aged brains. More than four brain hemispheres from young (7-day-old) and aged (50-day-old) flies were analyzed using transmission electron microscopy (TEM).

Supplemental figure 2

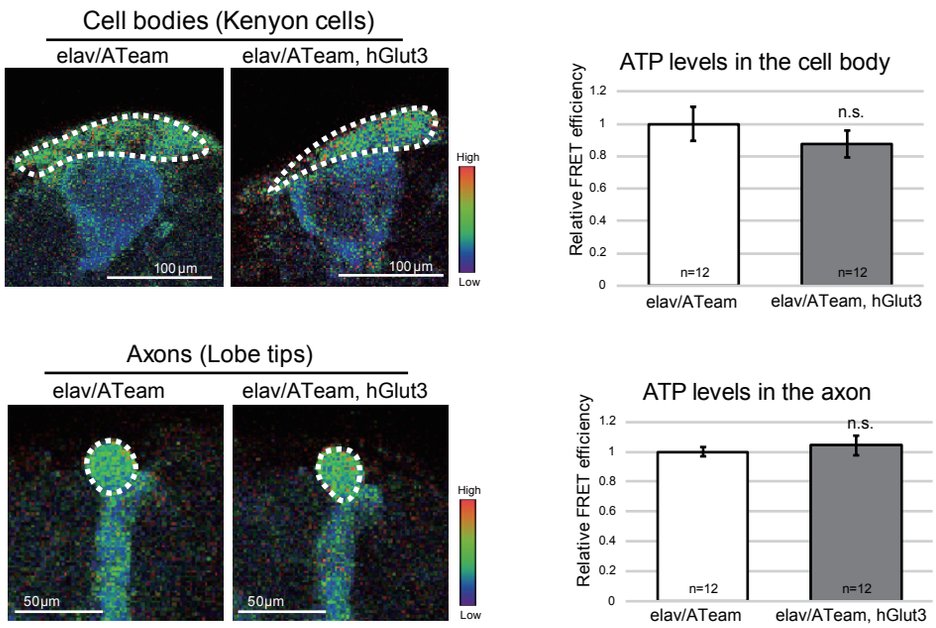

Figure S2. The overexpression of hGlut3 does not increase ATP concentration in the mushroom body neurons of young flies, Related to Figure 2

The FRET signals from ATeam were quantified and are shown as mean ± SE (n=12, n.s.; p>0.05, Student's t-test). The flies were 5 days old.

Supplemental figure 3

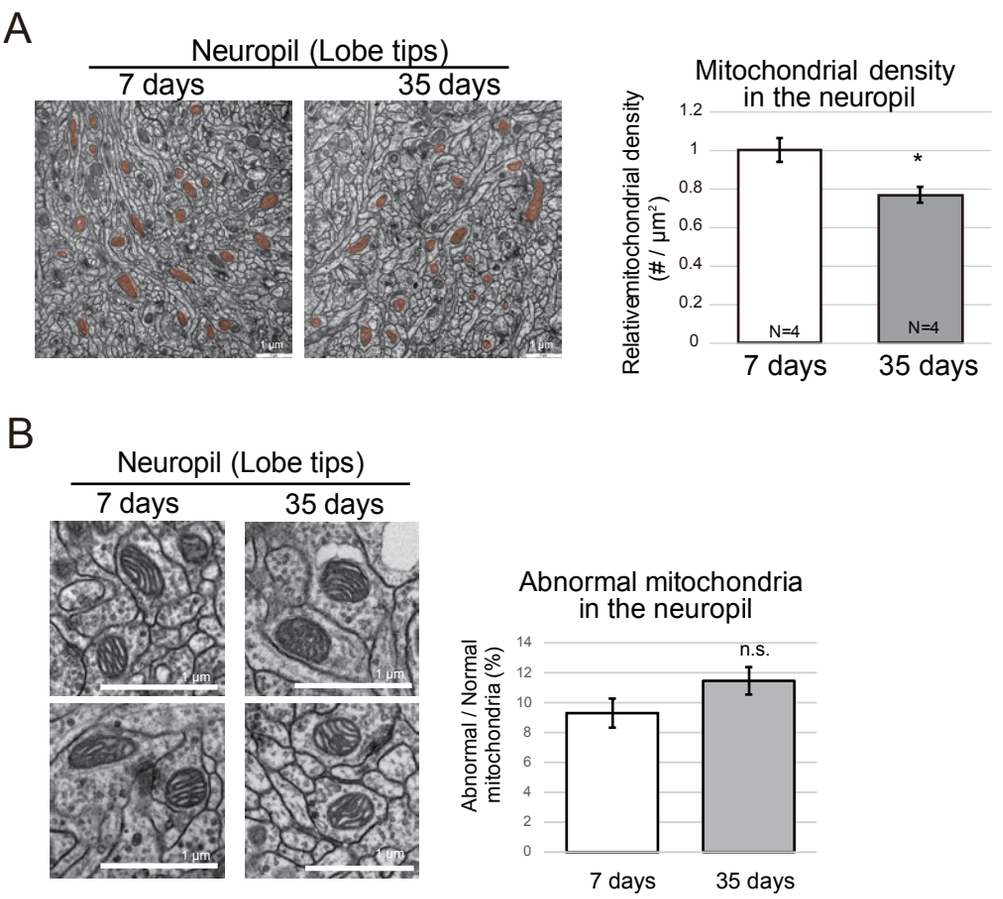

Figure S3. Age-related changes in the number and quality of mitochondria in the axon, Related to Figure 3

(A) The number of mitochondria in the axon significantly decreased 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 the lobe tip in at least 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 did not significantly change in the axon during aging. The number of mitochondria containing vacuoles or disorganized cristae was counted. Scale bar: 1 μm. Mean ± SE, n=4–5; n.s., $p>0.05$, *$p<0.05$; Student's t-test.

Supplemental figure 4

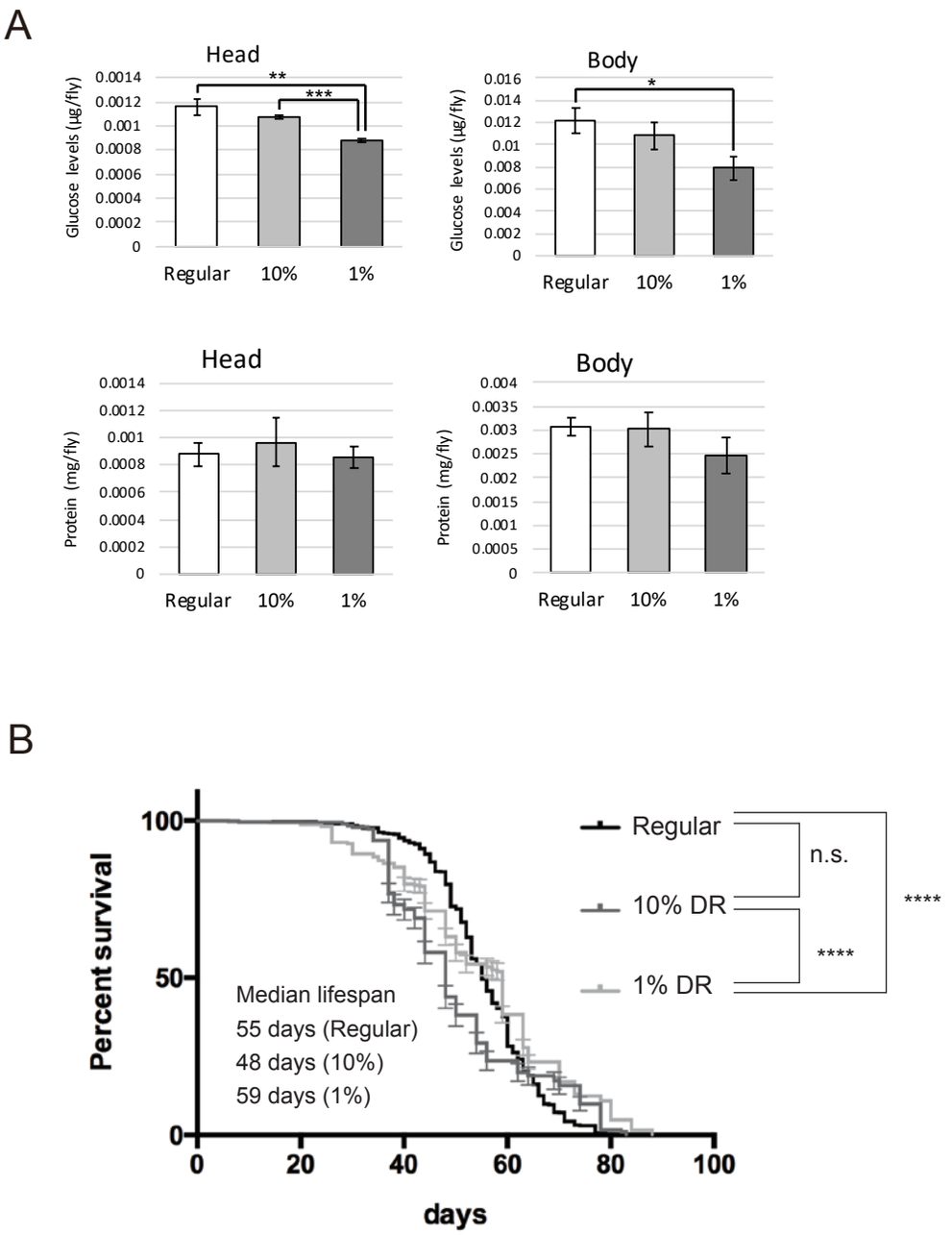

Figure S4. The effects of dietary restriction (DR) on glucose levels and life span, Related to Figure 4

(A) Flies undergoing DR have lower levels of glucose in their heads. The glucose and protein concentrations in the heads of flies subjected to DR. Flies were raised on regular cornmeal food and after eclosion, 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 and glucose, 1%: food without cornmeal and containing 1% (w/v) of yeast and glucose). Flies were 30-day-old. 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. Data are mean ± SE, n=131-601, n.s.; p>0.05, ****; p

Supplemental figure 5

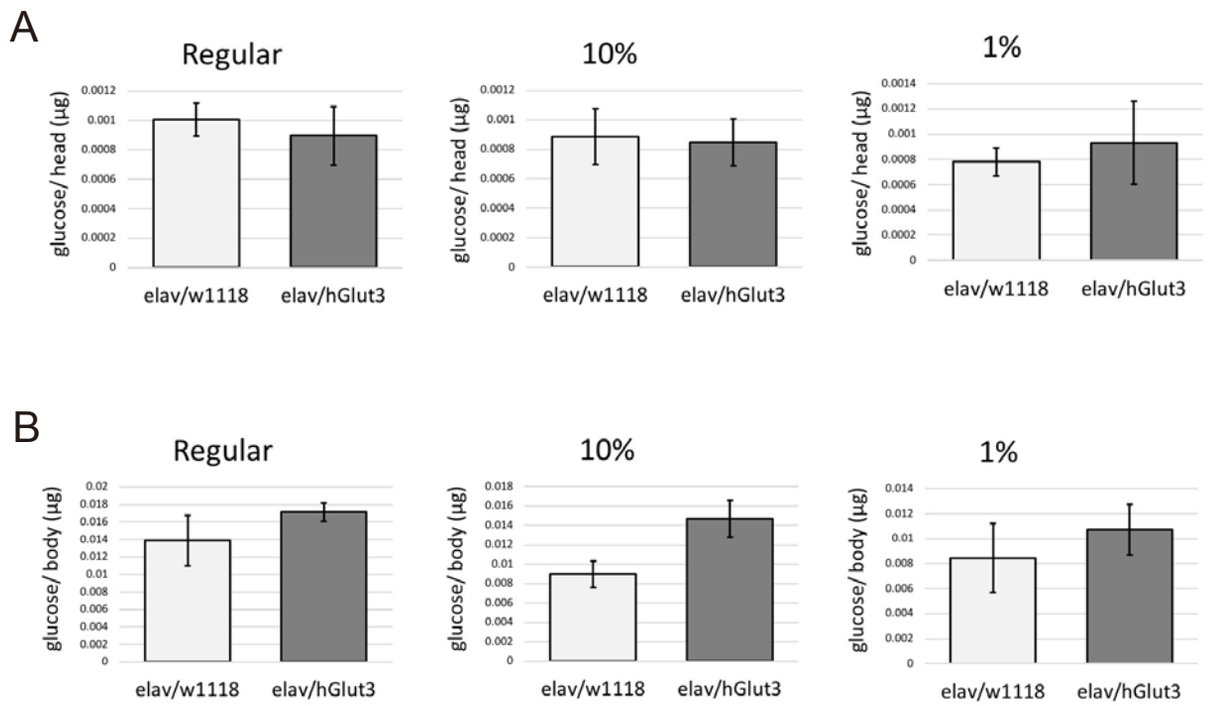

Figure S5. Glucose levels in the head or body were not increased by neuronal expression of hGlut3 under regular or dietary restriction (DR) conditions, Related to Figure 4

Flies were raised on regular cornmeal food and maintained on regular cornmeal food or two types of DR diet (10% and 1%) (10%: food without cornmeal and containing 10% (w/v) of yeast and glucose, 1%: food without cornmeal and containing 1% (w/v) of yeast and glucose) after eclosion. The glucose concentrations in 30-day-old fly heads (A) and bodies (B) are shown (mean ± SE, n=3, Student's t-test, $p>0.05$).

Table S1. The genotypes of flies used in each experiment, Related to Figure 1-4 and Transparent Methods

Figure 1A	control	elav-Gal4/+;;UAS-ATeam/+
Figure 1C	control RNAi	elav-Gal4/+;;UAS-luciferase/UAS-ATeam
	milton RNAi	elav-Gal4/UAS-milton RNAi^{v42508};;UAS-ATeam
Figure 1D	control	elav-Gal4/+;;UAS-ATeam
Figure 2A	control	elav-Gal4/+
Figure 2B	control	elav-Gal4/+
Figure 2C	control	elav-Gal4/+
Figure 2D	control RNAi	elav-Gal4/+;;UAS-luciferase^{B31603}/UAS-ATeam
	pfk RNAi	elav-Gal4/+; UAS-pfk RNAi^{B36782}/+; UAS-ATeam/+
Figure 2E	pfk RNAi	elav-Gal4/+; UAS-pfk RNAi^{v101887}/+; UAS-ATeam/+
Figure 2F	hGlut3	elav-Gal4/+; UAS-hGlut3/+; UAS-ATeam/+
Figure 2G	control	elav-Gal4/+
	hGlut3	elav-Gal4/+; UAS-hGlut3/+
Figure 3A	control	elav-Gal4/+
Figure 3B	control	elav-Gal4/+
Figure 3C	control	elav-Gal4/+
	hGlut3	elav-Gal4/+; hGlut3/+
Figure4A	control	elav-Gal4/+
	hGlut3	elav-Gal4/+; hGlut3/+
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/+
S1A	control	elav-Gal4/+;;UAS-ATeam/+
S1B	control	elav-Gal4/+
S2	control	elav-Gal4/+;;UAS-ATeam
	hGlut3	elav-Gal4/+; UAS-hGlut3/+; UAS-ATeam/+
S3A	control	elav-Gal4/+
S3B	control	elav-Gal4/+
S4A	control	elav-Gal4/+
S4B	control	elav-Gal4/+
S5	control	elav-Gal4/+
	hGlut3	elav-Gal4/+; hGlut3/+

Transparent 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 (v101887) and RNAi-TK (v60100) as KK RNAi control were obtained from Vienna Drosophila Resource Center (VDRC). UAS-milton RNAi was obtained from VDRC (v41508) and outcrossed to [*w1118*] for five generations in our laboratory. Control flies for Pfk RNAi (Figure 2D) were obtained by crossing elav-GAL4 with UAS-luciferase RNAi (Perkins et al., 2015). Control flies for hGlut3 expression were obtained by crossing elav-GAL4 flies or elav-GAL4;UAS-ATeam flies with the parental strain obtained from the laboratory that generated the UAS-hGlut3 fly line (Besson et al., 2015). 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% Brewer's yeast, 0.3% propionic acid and 0.1% n-butyl p-hydroxybenzoate [w/v]. 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, increasing FRET (Imamura et al., 2009). For FRET imaging, flies 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. The flies were handled in the same incubator and analyzed under the same experimental conditions. The experiments were repeated with timely independent cohorts of flies obtained from different crosses.

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

The 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 described above. After eclosion, female flies were maintained on regular cornmeal food, food without cornmeal and containing 10% (w/v) of yeast and glucose (10%), or food without cornmeal and containing 1% (w/v) of yeast and glucose (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.

Immunohistochemistry and image acquisition

For adult brain dissections, females were collected 1-2 days after eclosion and maintained at 25 °C until the required age. Dissections were performed in PBS, and samples were fixed for 30min in formaldehyde (4% v/v in PBS) at room temperature and washed for 10 min with PBST containing 0.1% Triton X-100 three times. For blocking, samples were incubated for 1h at room temperature in 1% normal goat serum (Wako, Cat# 143-06561) in PBST and incubated overnight with the primary antibody, anti- Elav (1:50; Developmental Studies Hybridoma Bank, Cat# 9F8A9, RRID:AB_528217), diluted in 1% NGS/PBST at 4 °C. After the primary antibody, samples were washed for 10 min with PBST including 0.1% Triton X-100 three times and incubated with the secondary antibody for 3 hours at room temperature. Samples were mounted in Vectashield (Vectorlab Cat#H-1100) and captured images on a Keyence BZ-X700.

Statistics

The number of replicates, what n represents, precision measurements, and the meaning of error bars are indicated in Figure Legends. Data are shown as mean ± SEM. For pairwise comparisons, Student's t-test was performed with Microsoft Excel (Microsoft). For multiple comparisons, data were analyzed using one-way ANOVA with Tukey's HSD multiple-comparisons test in the GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA). For survival analysis, p-value by log-rank test compares the entire curve in the GraphPad Prism 6.0 software. All results were considered statistically significant a p-value of less than 0.05 was considered to be statistically significant.

Supplemental references

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