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Extension of Lifespan and Amelioration of Alzheimer's Disease Phenotypes by Genetic Manipulation of Mitochondrial NAD^+/NADH Ratio

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MATERIALS AND METHODS

Resource Availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bingwei Lu (bingwei@stanford.edu).

Materials availability

All unique/stable reagents generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Drosophila

We obtained the following flies from the *Bloomington* Drosophila Stock Center: *elav-GAL4* (B8765), *UAS-APP.C99* (B33783), *UAS-APP; UAS-BACE* (B33798), *UAS-APP* (B6700). The sources of the other fly stocks are as follows: Drs. T. Littleton (*MHC-GAL4*). The indicated *UAS* RNAi and OE fly lines were crossed to *Mhc-Gal4* or *elav-Gal4* driver lines for muscle or pan-neuronal expression, respectively. We used wild type w^{1118} as control. *UAS-mito-LbNox* was generated by cloning the mito-LbNox sequence from a plasmid provided by Dr. Navdeep Chandel into the *pUAST* vector. The transformation vector was injected into w^{1118} embryos (Bestgene Inc.). Fly culture and crosses were performed according to standard procedures. Adult flies were generally raised at 25 °C and with 12/12 h dark/light cycles. Fly food was prepared with a standard receipt (Water, 17 L; Agar, 93 g; Cornmeal, 1,716 g; Brewer's yeast extract, 310 g; Sucrose, 517 g; Dextrose, 1033 g).

Fly lifespan analysis

Flies were reared in vials containing cornmeal medium. Flies were anesthetized using CO_2 and raised at a density of 20 flies/vial. All flies were kept at humidified incubators at 12h on/off light cycle at 25°C. Flies were flipped into fresh vials every 3 days and the number of dead animals was recorded. Each set of experiment was carried out ≥ 4 times.

Climbing activity assay

Climbing assay was performed as described previously [1]. Briefly, around 10-20 flies were transferred to a clean plastic vial. The flies were allowed to get accustomed to the new environment for 3-4 min and subsequently measured for bang-induced vertical climbing distance. The performance was scored as percentage of flies crossing the 8 cm mark within 12 seconds. Each experiment was performed ≥ 4 times.

Aversive taste memory assay in flies

We performed the taste memory assay as previously described [2]. Briefly, flies were starved for 12–18 hours in an empty vial containing wet Kimwipe paper. Flies were anesthetized on ice and fixed onto a glass slide by applying nail polish to their wings. Ten to fifteen flies were used per experimental set. The flies were then incubated in a humid chamber for 2 hours to allow recovery from the procedure. In the pretest phase, flies were presented with a 500 mM sucrose stimulus (attractive tastant) applied to their legs using a Kimwipe wick. Only flies that exhibited a positive proboscis extension response were selected for the subsequent phases. During the training phase, flies were presented with 500 mM sucrose at their legs while simultaneously being punished with a 50 mM caffeine (aversive tastant) solution applied to their extended proboscis. This training was repeated 15 times for each fly. In the

final test phase, flies were presented with 500 mM sucrose at their legs without applying caffeine to their proboscis, at various time intervals (0, 5, 15, 30, 45, and 60 minutes) after training, and proboscis extension responses (PER) were recorded. Each experiment was performed at least four times.

Aversive olfactory learning and memory assay

Groups of 40–60 flies were acclimated in fresh food vials for at least 20 minutes in a training room under dim red light (~70% humidity). Flies were then loaded into a training tube and exposed to fresh air for 30 seconds. Training consisted of 12 electric shocks (2s each, 3s intervals, 60 V) paired with a conditioned odor (CS+, either 0.05% OCT or 0.08% MCH in mineral oil) for 1 minute, followed by 30 seconds of fresh air. Subsequently, flies were exposed to the neutral odor (CS–) without shock for 1 minute, followed by 30 seconds of fresh air. Immediately after training, flies were transferred to a T-maze and allowed to choose between two arms for 2 minutes: one containing the CS+ odor and the other the CS– odor. The performance index (PI) was calculated as: (#flies in the CS- arm) - (# flies in the CS+ arm)) / (# total flies in both arms). Flies that remained in the middle were excluded from the analysis. Each experiment was carried out at least 4 times.

Mitochondrial purification

Intact mitochondria from *Drosophila* tissues were purified and quality controlled for the absence of contamination by other organelles according to established procedures. Briefly, brain or thoracic muscle were dissected and homogenized using a Dounce homogenizer. After two steps of centrifugation (1,500 g for 5 minutes and 13,000 g for 17 minutes), the mitochondria pellet was resuspended and washed twice with HBS buffer (5 mM HEPES, 70 mM Sucrose, 210 mM Mannitol, 1 mM EGTA, 1x protease inhibitor cocktail), then resuspended and loaded onto Percoll gradients. After centrifugation (16,700 rpm, 15 minutes, Beckman SW-40Ti rotor), the fraction between the 22% and 50% Percoll gradients containing intact mitochondria was carefully transferred into a new reaction tube, mixed with 2 volumes of HBS buffer, and pelleted by centrifuging at 20,000g for 20 minutes at 4°C to collect the mitochondrial samples for further analyses.

NAD^+/NADH measurement

NAD^+/NADH was measured essentially as described, using an NAD^+/NADH quantification colorimetric kit according to the manufacturer's instructions (AAT Bioquest #15273). Briefly, cell pellets or tissue samples were lysed using the lysis buffer for 15 min at 37°C and lysates were collected after centrifugation at 12,000g for 15 min. For the measurement of total NAD^+ amount, NAD extraction solution into the lysates were added and incubated at 37°C for 15 minutes, thereafter neutralization solution was added to neutralize the NAD extracts. Absorbance was monitored at 460 nm after adding NAD/NADH working solution and 1hr incubation at room temperature with protection from light. To measure total NAD^+ and NADH amount - NAD(H) (total), control extraction solution was added into the lysates and incubated at 37°C for 15 minutes, thereafter again control extraction solution was added. Absorbance was monitored at 460 nm after adding NAD/NADH working solution and 1hr incubation at room temperature with protection from light. The ratio of NAD^+/NADH was determined by the following equation: ratio = NAD^+/ NAD(H) (total) – NAD^+. Each experiment was performed ≥ 4 times.

ROS measurement

Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermofisher) was used for the measurement of ROS according to the manufacturer's instructions. Briefly, fly muscle tissues were homogenized in

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PBS with a loose-fitting homogenizer, and homogenates were centrifuged at 2,000 rpm for 5 minutes. The supernatant containing intact cells was used for ROS determination with the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit.

***In vitro* RET and FET assays**

Isolated mitochondria were incubated in an assay medium (125 mM KCl, 20 mM HEPES, 2 mM K2HPO4, 1 mM MgCl2, 0.1 mM EGTA, 0.025% BSA, pH7.0). To induce forward electron transport, 2.5 mM malate and 2.5 mM glutamate were supplemented as substrates. To induce reverse electron transport, 5 mM succinate and 1ug/ml oligomycin were supplemented into the mitochondrial samples. After 30 minutes of incubation, the mitochondrial samples were subjected to NAD^+/NADH ratio measurements as described above.

Immunofluorescence staining

Immunostaining of adult fly muscle was performed as previously described [3]. Briefly, fly thoraxes were dissected and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, cat# 15710) in phosphate buffered saline and 0.3% Triton X-100 (PBS-T). The tissues were then washed three times with PBS-T. Samples were incubated for 30 min at room temperature in blocking buffer: 0.5% goat serum in PBS-T. The following primary antibodies were added, and samples were incubated overnight at 4 °C: anti-Ubiquitin (Abcam, ab140601, 1:1000); anti-P62 (Abcam, ab178440, 1:1000). The samples were washed three times with PBS-T and subsequently incubated with the following secondary antibodies for 4h at 4°C: Alexa Flour 488 (A32723, Invitrogen), Alexa flour 594 (A11036, Invitrogen), both at 1:200. The tissues were washed three times with PBS-T and mounted in slow fade gold buffer (S36936, ThermoFisher).

Immunostaining of adult brains was performed as previously described [1] . Briefly, brain tissues of adult flies were dissected and fixed on ice for 30-45 min in fixing buffer (940 µl of 1% PBS-T and 60 µl of 37% formaldehyde). Tissues were washed three times in 0.1% PBS-T and blocked overnight at 4°C in blocking buffer (1 ml 1x PBS, 0.1% Triton-X, 5 mg/ml BSA). This was followed by incubating for 16 h at 4°C with the following primary antibodies: anti-Ubiquitin (Abcam, ab140601, 1:1000); anti-p62 (Abcam, ab178440, 1:1000). Tissues were washed three times with 0.1% PBS-T and subsequently incubated with the following secondary antibodies for 4h at 4°C: Alexa Flour 488 (A32723, Invitrogen), Alexa flour 594 (A11036, Invitrogen), both at 1:200. Samples were finally mounted in slow fade gold buffer (Invitrogen) and viewed using a Leica SP8 confocal microscope.

Immunoblotting

Around 5 fly thoraxes were homogenized in 75 µl of regular lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X100, protease inhibitors) on ice. Samples were homogenized using a hand-held mechanical homogenizer for 30 secs. The homogenized samples were incubated on ice for 30 mins before centrifuging at 15,000 rpm for 20 mins at 4ºC. 30 µl of supernatant was mixed with 10 µl of 4x Lammaelli buffer (BioRad #161-0747) and boiled for 5 mins at 100ºC. The protein lysate was cooled, centrifuged and loaded onto 16% Tricine gel (Invitrogen #EC66955) with 1x MES (Invitrogen #NP0002) as running buffer as previously described [4]. Protein concentration was measured by Pierce BCA protein assay kit (Thermo Fisher, 23225). Equal amounts of total protein were subjected to SDS-PAGE. After transferring the proteins to a PVDF membrane (Sigma, IPVH00010), the membrane was blocked with 5% milk in TBS with 0.05% Tween (TBST) for 1 hr at room temperature. The following primary antibodies were used: mouse anti-APP (6E10, Biolegends, 803001); mouse anti actin (Santa Cruz Biotechnology, Sc4778). Antibodies were incubated overnight at 4°C. The corresponding HRP

conjugated secondary antibodies were detected by ECL western blot reagents (Revvity, NEL105001EA). Immunoblot bands was analyzed with Fiji software.

Statistical analysis

Images and western blots were analyzed using Fiji. Analysis was performed with GraphPad Prism 10. Data were tested for normality using Shapiro-Wilk test prior to analysis. For normally distributed data, results were presented as mean ± SEM from at least 4 independent experiments. An unpaired two-tailed Student's *t* test was used to test difference between two groups, group analysis using multiple *t* test with Sidak-Bonferroni corrections, or one-way ANOVA or two-way ANOVA followed by Sidak's or Tukey's post hoc test was used for multiple comparisons. Data with n=3 biological repeats where normality test has limited power, we first confirmed low variation or spread in the measurements (coefficient of variance <10%) and used parametric test such as two-way ANOVA followed by Sidak's post hoc test for multiple comparisons. Large data with n≥6, data were first assessed for normality using Shapiro-Wilk test. Following confirmation of normal distribution, data were analyzed using two-way ANOVA followed by Sidak's or Tukey's Post hoc test for multiple comparisons. All data showed coefficient of variance <15% within groups. Lifespan was analyzed by Kaplan Meier survival analysis. All quantitative graphs with error bars are presented as means ± SEM. *p < 0.05, **p <0.01, ***p < 0.001, ****p < 0.0001.

Supplementary References

[1] Rimal S, Tantray I, Li Y, Pal Khaket T, Li Y, Bhurtel S*, et al.* (2023). Reverse electron transfer is activated during aging and contributes to aging and age-related disease. EMBO Rep, 24:e55548.
[2] Masek P, Worden K, Aso Y, Rubin GM, Keene AC (2015). A dopamine-modulated neural circuit regulating aversive taste memory in Drosophila. Current biology, 25:1535-1541.
[3] Li S, Wu Z, Li Y, Tantray I, De Stefani D, Mattarei A*, et al.* (2020). Altered MICOS Morphology and Mitochondrial Ion Homeostasis Contribute to Poly(GR) Toxicity Associated with C9-ALS/FTD. Cell Rep, 32:107989.
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