Supplementary file

High salt intake induces Alzheimer's disease like pathology by promoting tau phosphorylation and mitochondrial reverse electron transport

Jacob Kasanin[1, #], Benjamin Lu[1, #], Xiwei Zheng [1], Kodi Khau[1], Suman Rimal[1, *]

[1] Department of Pathology, Stanford University School of Medicine, R270 Edwards Building, 300 Pasteur Drive, Stanford, CA 94305, USA

# These authors contributed equally

* Correspondence to sumanr45@stanford.edu

Material and Methods

Experimental Model and Subject Details

Fly lifespan analysis

Lifespan assays were performed as described earlier[2]. Briefly, flies were anesthetized using CO_2 and collected in a cornmeal media containing vial 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 transferred into fresh vials every 3 days and the number of dead animals was recorded. Each set of experiment was carried out ≥ 4 times.

Aversive taste memory assay in flies

We performed the taste memory assay as previously described [1]. Briefly, flies were starved for 12–18 hours in empty vials containing moist Kimwipe paper. Following starvation, flies were anesthetized on ice and mounted onto glass slides by securing their wings with nail polish. Each experimental group consisted of 10–15 flies. The mounted flies were then placed in a humid chamber for 2 hours to recover from the handling procedure.

During the pretest phase, a 500 mM sucrose solution, serving as an attractive tastant, was applied to the legs using a Kimwipe wick. Only flies displaying a positive proboscis extension response (PER) were selected for subsequent experiments. In the training phase, flies were simultaneously presented with 500 mM sucrose on the legs and punished with 50 mM caffeine, an aversive tastant, to the extended proboscis. This conditioning protocol was repeated 15 times per fly.

For the final test phase, flies were exposed to 500 mM sucrose at different time points (0, 5, 15, 30, 45, and 60 minutes), and PER responses were recorded. Each experiment was independently repeated at least four times.

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 in the provided lysis buffer and incubated at 37°C for 15 minutes. Lysates were then collected following centrifugation at 12,000 × g for 15 minutes

To measure total NAD^+ amount, NAD^+ extraction solution was added to the lysates and incubated at 37°C for 15 minutes, followed by addition of neutralization solution. Subsequently, NAD^+/NADH working reagent was added, and samples were incubated for 1 hour at room temperature protected from light before measuring absorbance at 460 nm. 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.

We used a genetically encoded NAD^+/NADH sensor SoNar to measure NAD^+/NADH by live imaging. Briefly, the Gal4-UAS system was employed to drive neuronal expression of the *UAS-SoNar* transgene using the pan-neuronal *nSyb-Gal4* driver. Flies, with or without drug treatment, were dissected at the indicated ages, and isolated brains were maintained in Schneider's medium prior to imaging. Samples were immediately imaged using a Leica SP8 confocal microscope with 405 nm and 488 nm laser excitation to detect NADH and NAD^+ associated fluorescence signals, respectively.

Because the SoNar NADH signal is highly susceptible to photobleaching, imaging with the 405 nm laser was performed first. Fluorescence intensity was subsequently quantified using NIH ImageJ software.

ROS measurement

The Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit was used to measure ROS levels according to the manufacturer's instructions. Briefly, tissue samples were homogenized using a loose-fitting homogenizer followed by enzymatic treatment. Cells were collected by centrifugation at 2000 rpm for 5 minutes and incubated for 30 minutes at room temperature with a working solution containing 100 μM Amplex Red reagent and 0.2 U/ml horseradish peroxidase (HRP). Absorbance was measured at 560 nm.

For H_2O_2 measurement by CM-H2DCFDA staining, CM-H2DCFDA (5 uM) was added to freshly dissected brain tissues of flies and allowed to incubate for 30 min. Tissue samples were immediately imaged on a Leica SP8 confocal microscope with an excitation laser of 492–495 nm and detection set for 517–527 nm using 20x or 40x oil-objective lens.

In vitro RET and FET assays

Isolated mitochondria were incubated in assay buffer containing 125 mM KCl, 20 mM HEPES, 2 mM K_2HPO4, 1 mM $MgCl_2$, 0.1 mM EGTA, and 0.025% BSA (pH 7.0). Forward electron transport (FET) was initiated by the addition of 2.5 mM malate and 2.5 mM glutamate as respiratory substrates. Reverse electron transport (RET) was induced by supplementing mitochondrial preparations with 5 mM succinate and 1 μg/mL oligomycin. To better approximate physiological conditions, in which mitochondria predominantly operate under state 3 or near-state

3 respiration in the presence of ADP, FET or RET derived ROS generation and NAD^+/NADH ratio change were also examined in the presence of ADP (phosphorylating condition). For ROS detection, 1 μM Amplex Red and 5 U/mL horseradish peroxidase were added to the reaction mixture. Fluorescence was subsequently measured at excitation and emission wavelengths of 560 nm and 590 nm, respectively.

Immunofluorescence staining

Immunostaining of adult fly muscle was performed as previously described [2]. Briefly, fly thoraxes were dissected and fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Cat#15710) prepared in phosphate-buffered saline containing 0.3% Triton X-100 (PBS-T). Following fixation, tissues were washed three times with PBS-T and incubated for 30 minutes at room temperature in blocking solution consisting of 0.5% goat serum in PBS-T.

Samples were then incubated overnight at 4°C with the following primary antibodies: anti-ubiquitin (Abcam, ab140601, 1:1000) and anti-p62 (Abcam, ab178440, 1:1000). After primary antibody incubation, tissues were washed three times with PBS-T and incubated for 4 hours at 4°C with Alexa Fluor-conjugated secondary antibodies: Alexa Fluor 488 (Invitrogen, A32723, 1:200) and Alexa Fluor 594 (Invitrogen, A11036, 1:200). Finally, samples were washed three additional times with PBS-T and mounted using Slow Fade Gold mounting medium (Invitrogen) prior to imaging.

Immunostaining of adult brains was performed as previously described[2] . 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-TH, Pel-Freez P40101-150, 1:1000; 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), 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.

Western blotting

Around 5 fly thoraxes or 15 heads 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 and incubated on ice for 30 mins before centrifuging at 15000 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 4-12% Bis-Tris gel (Invitrogen #NP0321) with 1x MOPS (Invitrogen #NP0001) as running buffer as previously described [85]. The proteins were transferred to a PVDF membrane (Sigma, IPVH00010), the membrane was blocked with 5% milk in TBS with 0.05% Tween (TBST) for 1 h at room temperature. The following primary antibodies were used: mouse anti-Tau antibody 1:500 (DSHB: 5A6); rabbit phospho-tau (S262) 1:1000 (Thermofisher scientific, 44-750G); rabbit antii ATG8a 1:1000 (Millipore sigma ABC974). 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.

Figures and Figure Legends

Figure S1. Characterization of high salt induced toxicity in *Drosophila*

(A) Immunostaining of TH^+ dopaminergic (DA) neuron in the PPL1 cluster of control and high salt fed fly brains. (B) Quantification of p62 and ATG8II/ATG8I in control and high salt fedflies.. All data are means ± SEM; statistical significance was decided by two-tailed unpaired Student's *t* test. **p*< 0.05; ***p*< 0.01; ****p*< 0.001. For each set of experiments at least n=4 flies are used. Western blots are representative of at least three repeats.

Figure S2. High salt induced toxicity is related to RET

A) Survival curve showing the effect of AOX and NDI1 overexpression in salt-fed flies. (B) Live imaging with the ROS reporter showing the effect of AOX overexpression on ROS in salt-fed fly brain. (C) Measurement of mitochondrial FET related ROS and NAD^+/NADH changes in mitochondria purified from salt fed flies. All data are means ± SEM; statistical significance was decided by one-way ANOVA or two-tailed unpaired Student's *t* test. Survival curve was quantified using Kaplan-Meier survival analysis. **p*< 0.05; ***p*< 0.01; ****p*< 0.001; *****p*< 0.0001. For each set of experiments at least n=4 flies are used.

Figure S3. RET inhibition alleviates high salt induced tau hyperphosphorylation.

(A) Western blot showing the level of p-S262 tau in brain tissues of male flies. (B) Western blot showing the level of p-S262 tau in the gut of female flies. (C) Western blot and data quantification showing the level of p-tau in high salt fed tau-R406W transgenic flies. (D) Measurement of ROS and NAD^+/NADH in mitochondrial samples respiring under FET condition. (E) Learning and memory assay showing the effect of tau deletion in high salt fed flies. (F, G) Measurement of ROS and NAD^+/NADH in mitochondrial samples from salt-fed tau RNAi flies respiring under RET (F)

or FET (G) conditions. (H) Measurements of ROS and NAD^+/NADH through genetic sensors in high salt fed tau-KD flies. All data are means ± SEM; statistical significance was decided by one-way ANOVA or two-tailed unpaired Student's *t* test. **p*< 0.05; ***p*< 0.01; ****p*< 0.001; *****p*< 0.0001. For each set of experiments at least n=4 flies are used. Western blots are representative of at least three repeats.

Figure S4. Functional significance of RET inhibition under high salt stress.

(A-C) Survival assays showing the effect of Rotenone (A), NDUFS3-RNAi (B), and DES (C) on high salt fed flies. (D) Learning and memory assay showing the effect of DES with high salt feeding. (E) TH immunostaining showing the effect of CPT on high salt feeding induced neuronal loss. (F) Measurement of p62 and ubiquitin aggregates in tau-RNAi and CPT treated flies. (G) Measurement of mitochondrial FET-ROS and FET-NAD^+/NADH in salt fed flies with CPT treatment. (H) Measurement of ROS after CPT and rotenone treatment of high salt fed flies. (I) Measurement of NAD^+/NADH after CPT treatment in high salt fed flies. All data are means ± SEM; statistical significance was decided by one-way ANOVA or two-tailed unpaired Student's *t* test. Survival curve was quantified using Kaplan-Meier survival analysis. **p*< 0.05; ***p*< 0.01; ****p*< 0.001; *****p*< 0.0001. For each set of experiments at least n=4 flies are used. Western blots are representative of at least three repeats.

Figure S5. The protective effect of CPT under high salt stress is dependent on mitochondrial Sirtuin induced autophagy.

(A) Survival curve showing the effect of autophagy inhibition in high salt fed flies. (B) Survival curve of dSirtuin-4 overexpressing flies after autophagy blockade with 3MA. All data are means

± SEM, Survival curve was quantified using Kaplan-Meier survival analysis *p< 0.05; **p< 0.01; ***p< 0.001; ****p< 0.0001. For each set of experiments at least n=4 flies are used.

Figure S1

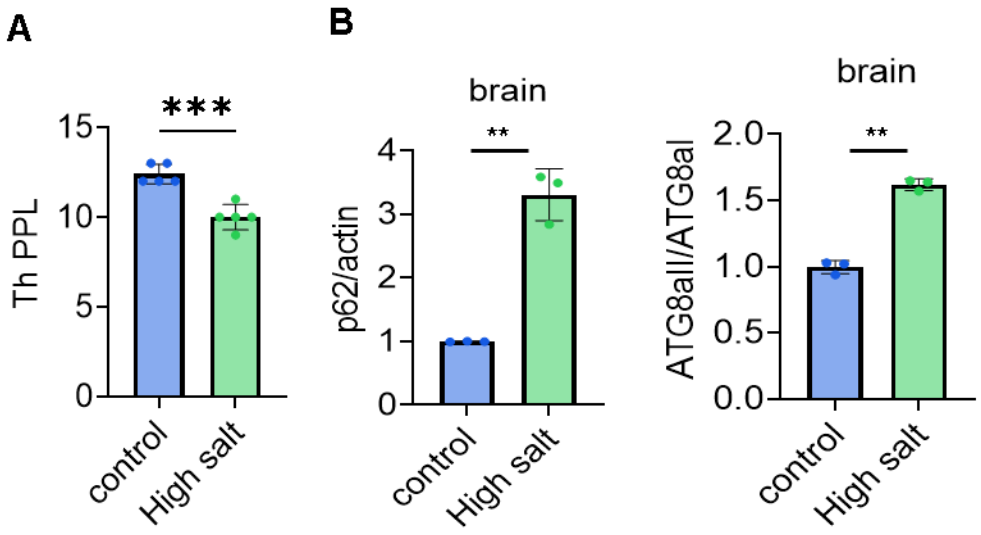


Figure S2

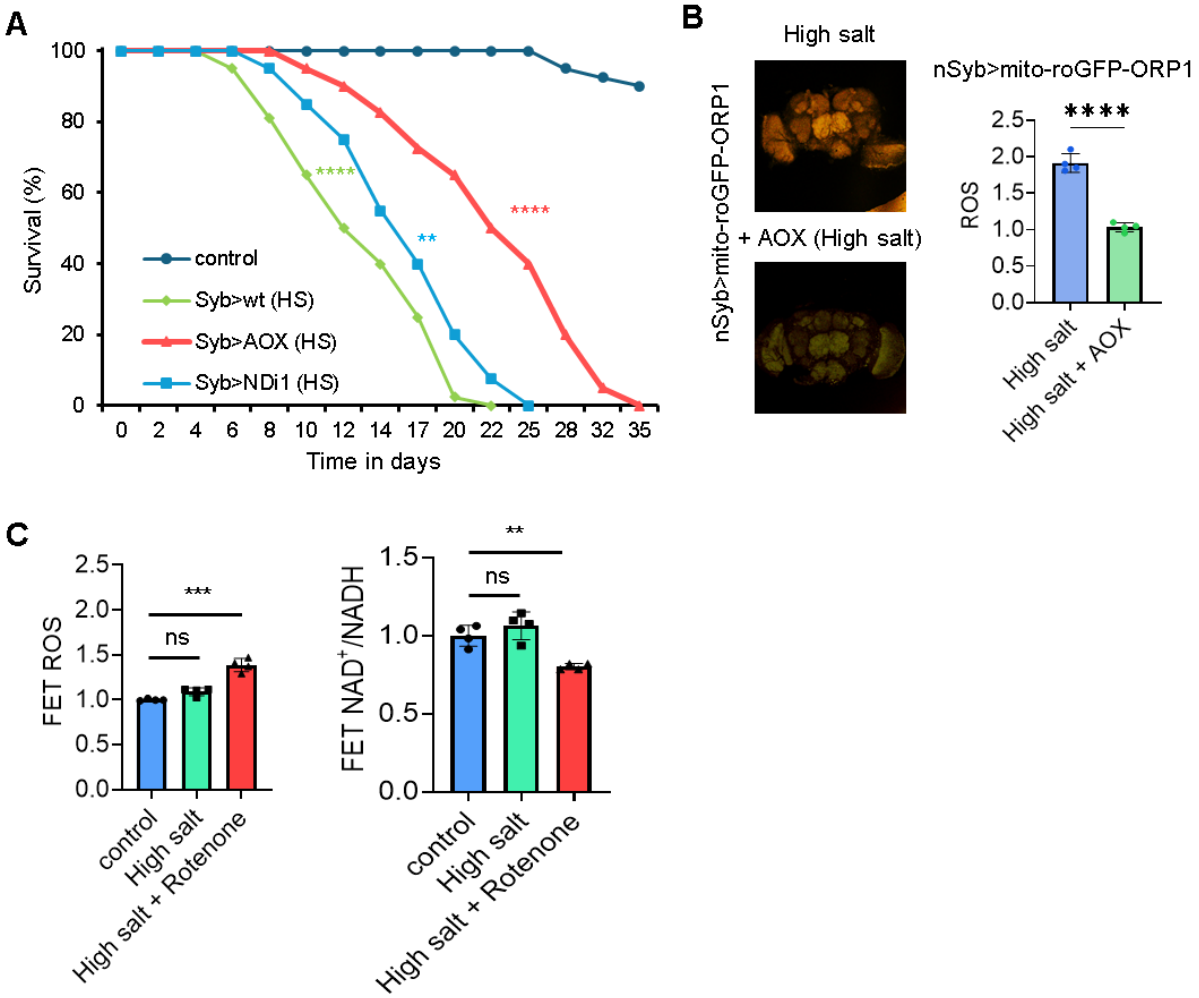

A
Survival (%)
Time in days
control
Syb>wt (HS)
Syb>AOX (HS)
Syb>NDi1 (HS)
****
**
****
B
High salt
nSyb>mito-roGFP-ORP1
+ AOX (High salt)
nSyb>mito-roGFP-ORP1
ROS
****
High salt
High salt + AOX
C
FET ROS
***
ns
control
High salt
High salt + Rotenone
FET NAD+/NADH
**
ns
control
High salt
High salt + Rotenone

Figure S3

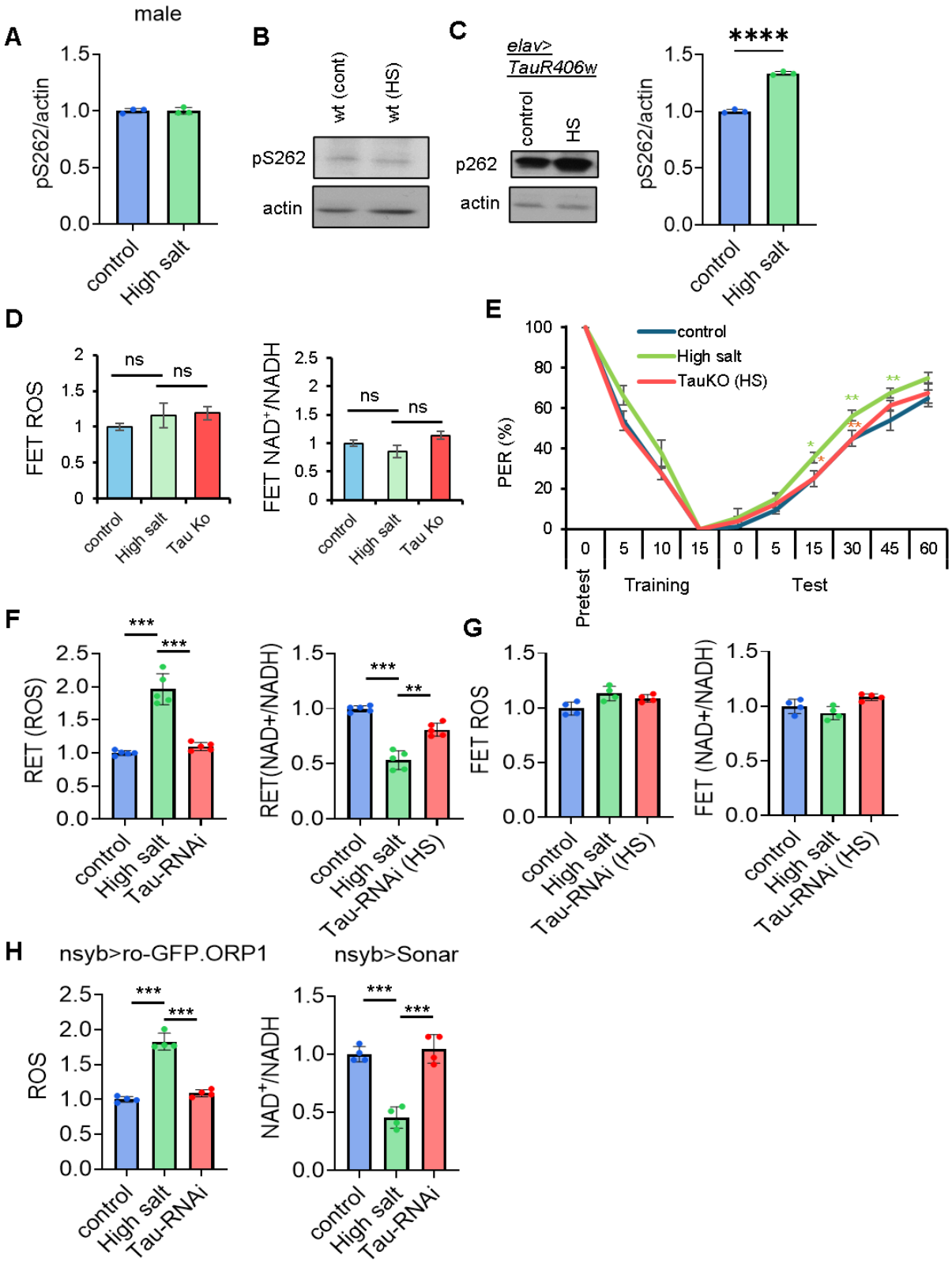

male
A
pS262/actin
control
High salt
B
wt (cont)
wt (HS)
pS262
actin
C
elav>
TauR406w
control
HS
p262
actin
****
pS262/actin
control
High salt
D
FET ROS
ns
ns
control
High salt
Tau Ko
FET NAD+/NADH
ns
ns
control
High salt
Tau Ko
E
control
High salt
TauKO (HS)
PER (%)
Pretest
Training
Test
0
5
10
15
0
5
15
30
45
60
F
RET (ROS)
control
High salt
Tau-RNAi
RET(NAD+/NADH)
control
High salt
Tau-RNAi (HS)
G
FET ROS
control
High salt
Tau-RNAi (HS)
FET (NAD+/NADH)
control
High salt
Tau-RNAi (HS)
H
nsyb>ro-GFP.ORP1
nsyb>Sonar
ROS
control
High salt
Tau-RNAi
NAD+/NADH
control
High salt
Tau-RNAi

Figure S4

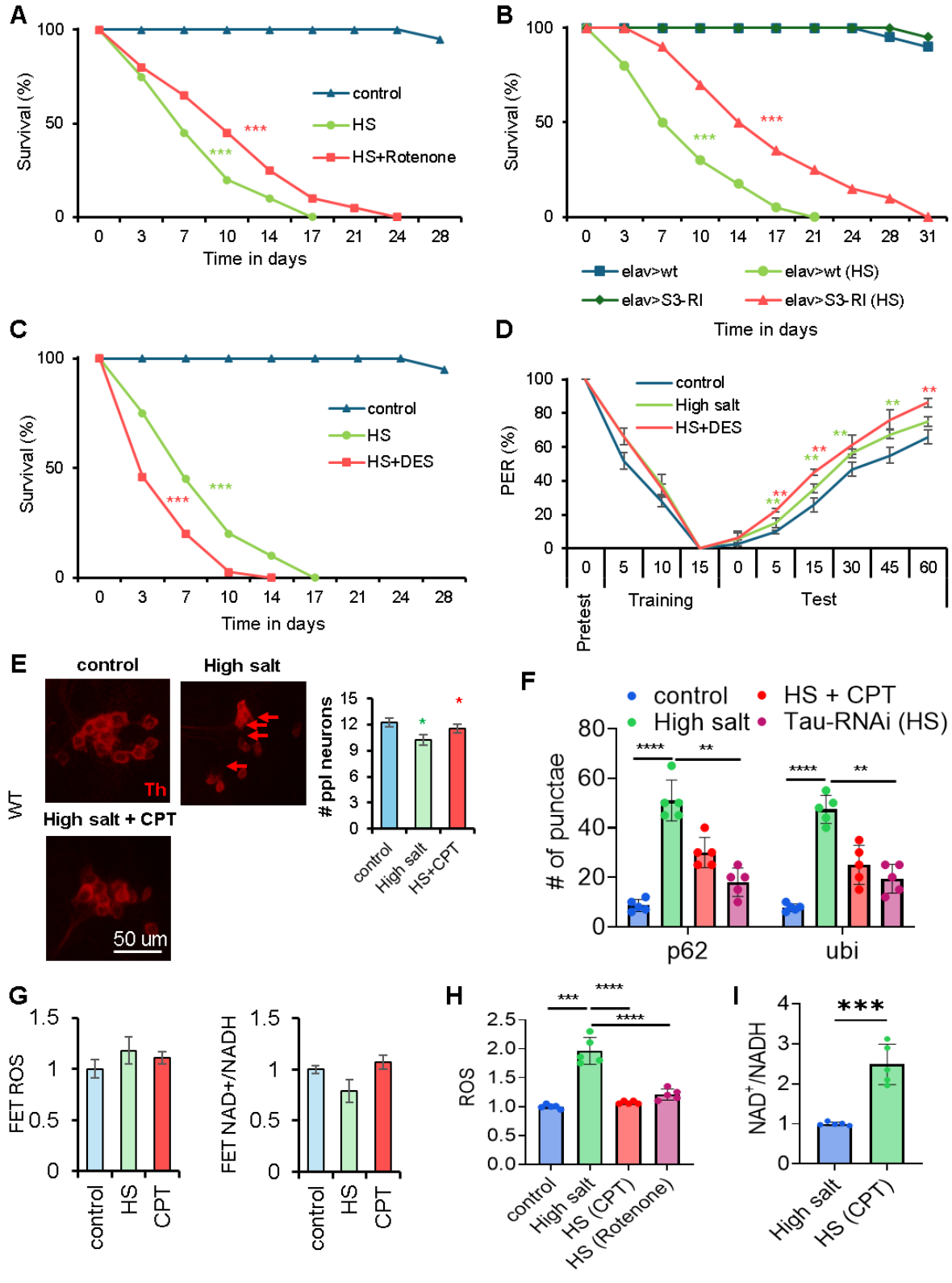
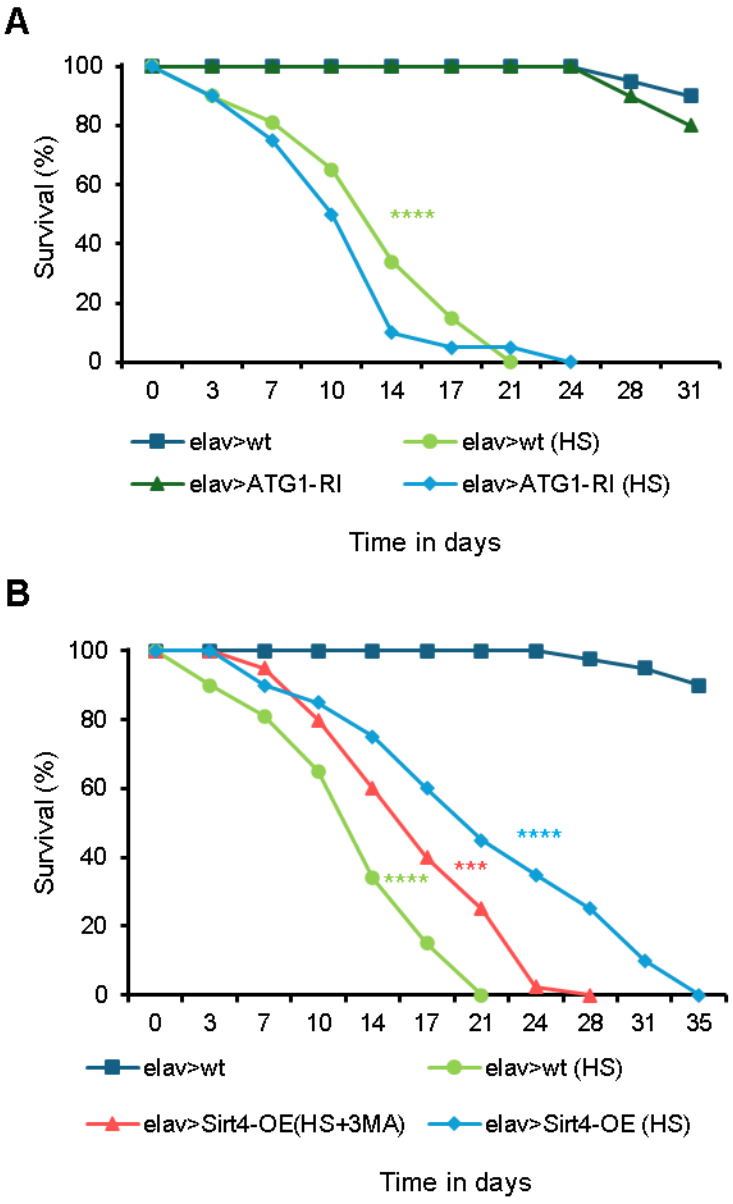

A
Survival (%)
Time in days
control
HS
HS+Rotenone
B
Survival (%)
Time in days
elav>wt
elav>wt (HS)
elav>S3-RI
elav>S3-RI (HS)
C
Survival (%)
Time in days
control
HS
HS+DES
D
PER (%)
control
High salt
HS+DES
Pretest
Training
Test
E
control
High salt
High salt + CPT
WT
Th
50 um
ppl neurons
control
High salt
HS+CPT
F
control
High salt
HS + CPT
Tau-RNAi (HS)
# of punctae
p62
ubi
G
FET ROS
FET NAD+/NADH
control
HS
CPT
H
ROS
control
High salt
HS (CPT)
HS (Rotenone)
I
NAD+/NADH
High salt
HS (CPT)

Figure S5



References

1. Masek, P., Worden, K., Aso, Y., Rubin, G.M. & Keene, A.C. A dopamine-modulated neural circuit regulating aversive taste memory in Drosophila. *Current biology* **25**, 1535-1541 (2015).

2. Rimal, S. et al. Reverse electron transfer is activated during aging and contributes to aging and age-related disease. *EMBO Rep* **24**, e55548 (2023).