Supplemental Material to:

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ER stress inhibits neuronal death by promoting autophagy

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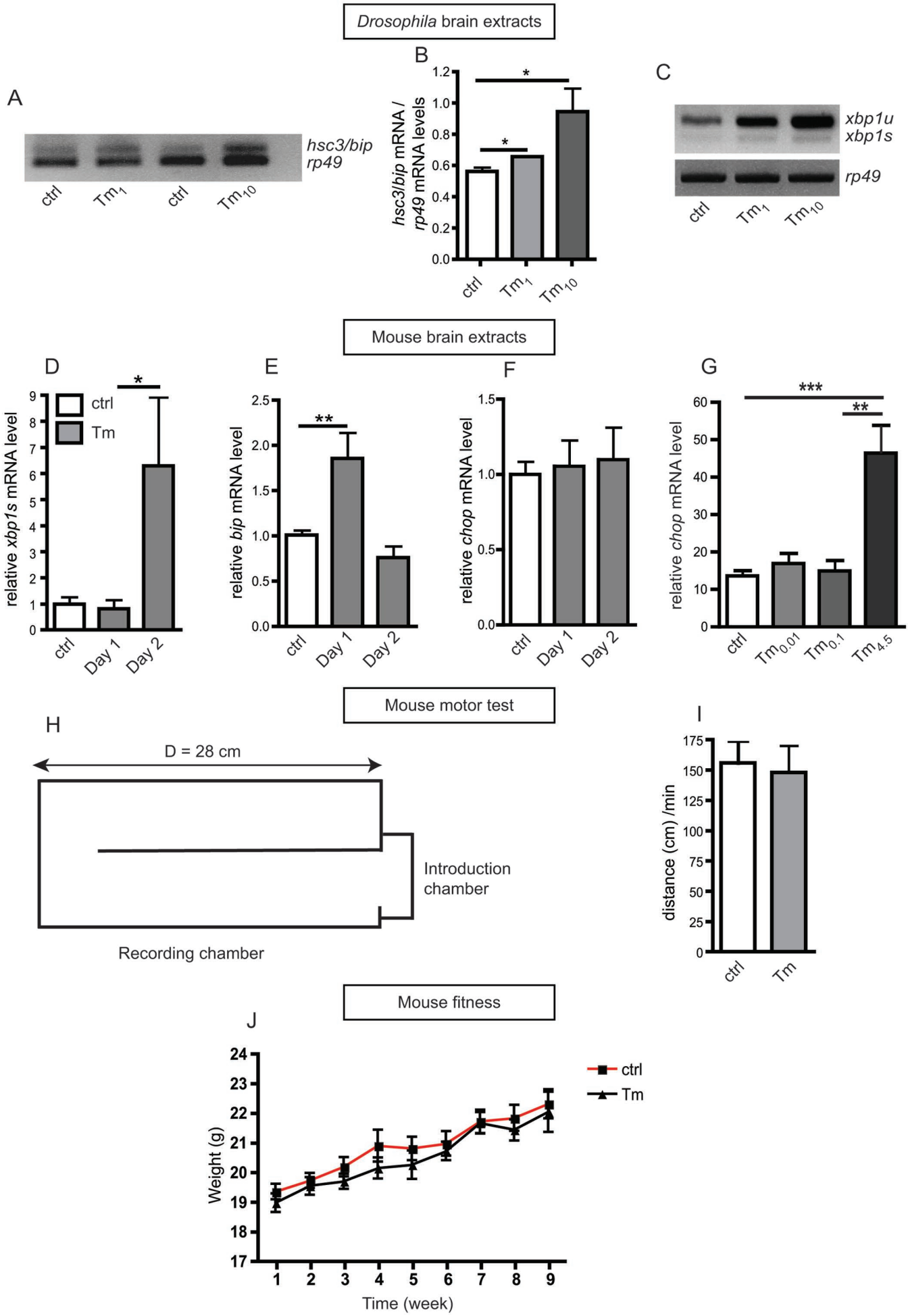
Drosophila brain extracts
A
hsc3/bip
rp49
ctrl
Tm1
ctrl
Tm10
B
hsc3/bip mRNA / rp49 mRNA levels
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
ctrl
Tm1
Tm10
C
xbp1u
xbp1s
rp49
ctrl
Tm1
Tm10
Mouse brain extracts
D
relative xbp1s mRNA level
ctrl
Tm
*
ctrl
Day 1
Day 2
E
relative bip mRNA level
**
ctrl
Day 1
Day 2
F
relative chop mRNA level
ctrl
Day 1
Day 2
G
relative chop mRNA level
***
**
ctrl
Tm0.01
Tm0.1
Tm4.5
Mouse motor test
H
D = 28 cm
Introduction chamber
Recording chamber
I
distance (cm) /min
ctrl
Tm
Mouse fitness
J
Weight (g)
ctrl
Tm
Time (week)

Figure S1

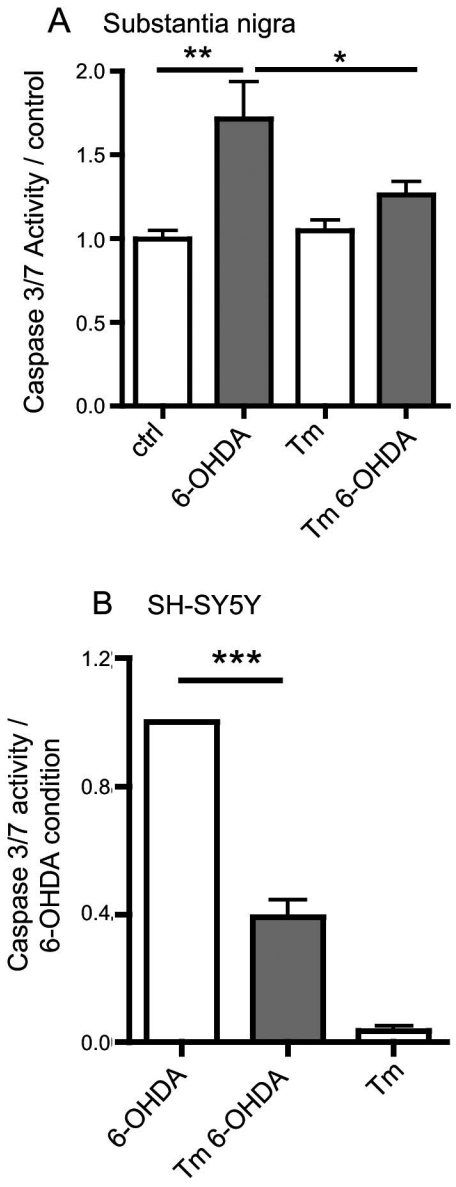
A
Substantia nigra
**
*
Caspase 3/7 Activity / control
2.0
1.5
1.0
0.5
0.0
ctrl
6-OHDA
Tm
Tm 6-OHDA
B
SH-SY5Y
***
Caspase 3/7 activity / 6-OHDA condition
1.2
0.8
0.4
0.0
6-OHDA
Tm 6-OHDA
Tm

Figure S2

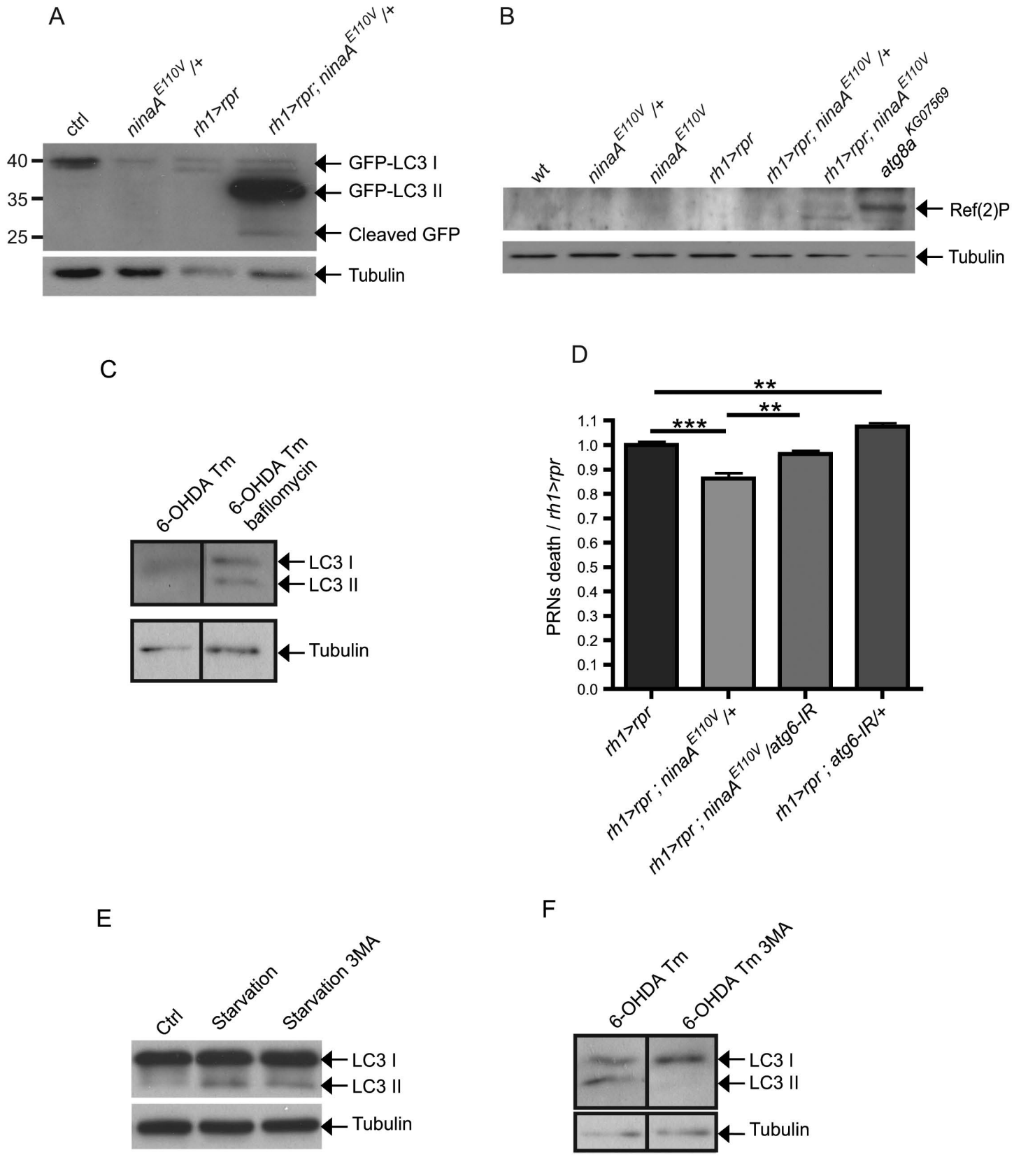
A
ctrl
ninaA E110V /+
rh1>rpr
rh1>rpr; ninaA E110V /+
40
35
25
GFP-LC3 I
GFP-LC3 II
Cleaved GFP
Tubulin
B
wt
ninaA E110V /+
ninaA E110V
rh1>rpr
rh1>rpr; ninaA E110V /+
rh1>rpr; ninaA E110V
atg8a KG07569
Ref(2)P
Tubulin
C
6-OHDA Tm
6-OHDA Tm bafilomycin
LC3 I
LC3 II
Tubulin
D
PRNs death / rh1>rpr
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
**
**
rh1>rpr
rh1>rpr ; ninaA E110V /+
rh1>rpr ; ninaA E110V /atg6-IR
rh1>rpr ; atg6-IR/+
E
Ctrl
Starvation
Starvation 3MA
LC3 I
LC3 II
Tubulin
F
6-OHDA Tm
6-OHDA Tm 3MA
LC3 I
LC3 II
Tubulin

Figure S3

Table S1: RT-PCR primers for *pale*, *vmat*, *rp49*, *xbp-1*, *bip* (*Drosophila* and mice) and *TBP*

Primers	Sequence
pale-F	AGTACGAGCCCGATTTGGACATGA
pale-R	TCCGTACTTGTAGGCGAATGCGAT
vmat-F	TTCACCGTAAACGCAAACACCACC
vmat-R	GTTGATTGCTGTCCAACTGCTGCT
hsc3/bip-F	CAGTGATCGGCATTGATTTG
hsc3/bip -R	ATGTCGTGTTGCACATTGGT
rp49-F	CGGATCGATATGCTAAGCTGT
rp49-R	GCGCTTGTTCGATCCGTA
xbp1tot-F	GTGCAGGCCCAGTTGTCACC
xbp1tot-R	GTGCAGGCCCAGTTGTCACC
xbp1US-F	CAGACTATGTGCACCTCTGC
xbp1US-R	TCTGGGTAGACCTCTGGGAG
bip-F (mouse)	ACGATCAGGGCAACCGCATCA
bip-R (mouse)	CCACCTCCAATATCAACTTG
TBP-F	TGGTGTGCACAGGAGCCAAG
TBP-R	TTCACATCACAGCTCCCCAC

Supplemental material and methods

Climbing Assay

To assess the effect of mild ER stress in *hu-α-syn* expressing flies, climbing performances were evaluated on flies expressing *hu-α-syn* (*elav>UAS-hu-α-syn*) compared to control flies (*elav>UAS-GFP*). Cohorts of 20 flies from each condition were treated once a week with Tm (1µg/ml, Covalab, 11089-65-9) or vehicle solution, and studied for their climbing ability up to 35 days after hatching weekly. The flies were placed in an empty *Drosophila* vial (9,3 cm in height, 5,7 cm^2 in area) and were gently tapped to the bottom of the vial. They were given 8 seconds to climb, and the number of flies remained below 4 cm, was counted. Ten trials were performed for each time point for the same set of flies.

Cornea neutralization method

Cornea neutralization was used to visualize the photoreceptor cells integrity in living *Drosophila*.[1] Anesthetized *Drosophila* were immobilized on a 40°C agarose bed previously poured in a 50 mm Petri dish. The eyes were covered with water (4°C) and the fluorescence in the retina was observed using a 40X immersion objective with a LSM 510 confocal microscope (Zeiss).

RT PCR analysis

The effect of tunicamycin in *Drosophila* brain on ER stress was monitored by RT–PCR. mRNA was extracted from 25 treated fly heads using Nucleospin RNA II Kit (Macheney

Nagel, 740902.50). 1µg of mRNA was used to synthesize cDNA using the Enhanced Avian RT First Strand Synthesis Kit (Sigma Aldrich, STR1-1KT) following manufacturer's instruction. Briefly, mRNA is incubated at 70°C for 10min with dNTP (0.5mM) and oligodT (3.5µM) and then incubated at 60°C for 1h with 1X buffer, reverse transcriptase enzyme (20 unit) and RNase inhibitor (20 unit). PCR was performed using GoTaq (5 units) in GreenGoTaq Buffer (1X) (Promega, M3178) with 0.1mM dNTP to perform PCR. cDNA was first denatured for 5 min at 94°C and amplified during 27 cycles: 94°C for 30s, 58°C for 30s, 72°C for 30s; followed by an incubation at 72°C for 7min. Amplified cDNA was segregated on 2% agarose gel. Quantification was performed using ImageJ gel analyzer and statistic analyses were done.

Western blotting

Total protein extract was obtained from *Drosophila* retina using a lysis buffer (1% NP40, 20mM Tris HCl, 100mM NaCl, 2mM DTT and protease inhibitor cocktails 1X). Samples were centrifuged for 15 min at 12,000g. Protein quantification was performed using the Bradford assay following the manufacturer's guidelines (Biorad). Samples were boiled for 4 min and 10µg of protein was loaded onto a 12% acrylamide gel and transferred onto nitrocellulose membranes. Anti-GFP (1:400, Roche, 11814460001) and anti-Ref(2)P antibodies [2] were incubated overnight at 4°C and HRP-coupled secondary antibody (1:10 000, GE Healthcare, NA9310) was then incubated for 2h at RT. To compare the level of expression of GFP or Ref(2)P in the different conditions, membranes were reprobed with an anti-tubulin antibody (1/1000, Sigma, T6199) followed by an HRP-coupled antibody (1:10 000, GE Healthcare, NA9310).

Immunoreactivity was detected by chemiluminescence using the ECL reagent Kit Plus, western blotting detection system (GE Healthcare, RPN2132). All the experiments were done in triplicate.

Caspase activity assay

Caspase activity was assessed using the caspase Glo kit (Promega, G8090) following manufacturer's instructions. Briefly, proteins were extracted in a lysis buffer (1% NP40, 20mM Tris HCl, 100mM NaCl, 2mM DTT). 7µg of protein was added to 100µl of caspase glo solution and incubated for 1h at room temperature and in the dark. Caspase activity was observed using Veritas microplate luminometer (Turner Biosystems).

Quantitative real-time PCR

The primers used for the study using quantitative real time RT PCR are presented in table S1. Briefly, cDNA synthesis was performed using the RT Superscript II kit (Invitrogen, 18064014) following the manufacturer's instructions. 1µg of mRNA was used for the cDNA synthesis and samples were treated with 1 µl RNase H (5U, NEB, M0297L) for 20 min at 37°C to degrade all parental mRNA. cDNA diluted 1/60 in RNase free water was amplified using the Absolute qPCR SYBR Green ROX mix (Roche, 4913850) as follows: hold 95ºC 15 min; 40 cycles of 95ºC 15s, 60ºC (*Drosophila pale* and *vmat*, mouse *xbp1s*, *chop*, and *TBP*) or 58°C (mouse *bip*) 15s, 72ºC 30s; 1 cycle 95ºC 30s; 1 cycle 50ºC 30s and the melt ramp from 50ºC to 95ºC at 1ºC/10s to determine the product specificity. All samples were run on the Corbett instrument (Roche). The relative

expression of *Drosophila* DA neurons marker against the HK gene rp49, previously determined to be the most stable HK gene, was performed using the Pfaffl equation.[3]

Mouse Motor test

A mouse was placed in the introduction chamber and the chronometer started. The distance travelled by the mouse during 2 min was measured in centimeter (cm). Mice chronically treated with Tm (0.1mg/kg) for 9 weeks (n=7) and vehicle (n=8) were used in this test.

Supplemental references

1. Mollereau B, Wernet MF, Beaufils P, Killian D, Pichaud F, Kuhnlein R, et al. A green fluorescent protein enhancer trap screen in Drosophila photoreceptor cells. Mech Dev 2000; 93:151-60.
2. Nezis IP, Simonsen A, Sagona AP, Finley K, Gaumer S, Contamine D, et al. Ref(2)P, the Drosophila melanogaster homologue of mammalian p62, is required for the formation of protein aggregates in adult brain. J Cell Biol 2008; 180:1065-71.
3. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29:e45.

Supplemental legends

Fig. S1 Tm activates the UPR in *Drosophila* and mouse brain tissues.

(A-C) Effect of Tm feeding on *hsc3/bip* expression and splicing of *xbp1* mRNA was assessed in *Drosophila* brain**.** (A) *hsc3/bip* mRNA level is evaluated by RT-PCR experiments after Tm treatment at 1μg/ml (Tm1) or 10μg/ml (Tm10). (B) Quantification of *hsc3/bip* mRNA relative to *rp49* in 3 independent experiments (*$p \leq 0.05$)**.** (C) Spliced *xbp1* (*xbp1s*) and unspliced *xbp1* (*xbp1u*) levels are evaluated by RT-PCR after Tm treatment at 1μg/ml (Tm1) or 10μg/ml (Tm10). The result is representative of 3 experiments. (D-F) mRNA levels of *xbp1s*, *bip* and *chop* were determined by quantitative RT-PCR 1 and 2 days after I/P injection of Tm at 0.1mg/kg in mouse brain. (n=5-6, t-test * $p \leq 0{,}05$). (G) *chop* mRNA level was determined by quantitative RT-PCR in mouse brain 4 days after Tm injection 0.01mg/kg (Tm0.01), 0.1mg/kg (Tm0.1) or 4.5mg/kg (Tm4.5). (H-J) Mice injected weekly over 9 weeks period with Tm at 0.1mg/kg (n=7). (H and I) Motricity test in mice treated with Tm or vehicle. (H) Schematic representation of the system used to assess mice motricity. (I) Distance travelled by mice treated for 9 weeks with Tm (n=7) and control mice (n=8). Walking distance (cm) was measured over a 2 min period. This test was repeated 3 times. (J) Weight of mice injected weekly over 9 weeks period with Tm at 0.1mg/kg (n=7) or vehicle (n=8).

Fig. S2 Tm treatment inhibits caspase activation in mice SN and in SH-SY5Y cells.

(A, B) Caspase 3/7 activity was measured using Caspase Glo assay. (A) Caspase Glo assay was performed on SN of mice injected with 6-OHDA and/or Tm. Results are expressed as ratio of caspase activity compared to control (n=7-10). (B) Caspase Glo

assay was performed on SH-SY5Y cells treated with 6-OHDA and/or Tm. Results are expressed as ratio of caspase activity compared to 6-OHDA treated cells (n=3). (* $p < 0.05$, ** $p < 0.01$ in Student's t-test)

Fig. S3 Autophagy is functional and protective in *Drosophila* PRNs and in human SH-SY5Y cells submitted to ER stress and cell death signals

(A, B) Autophagic flux was evaluated by following GFP-LC3 maturation and Ref(2)P/p62 expression by western blot from *Drosophila* dissected retina. (A) Western blot with anti-GFP antibody showing the conversion of GFP-LC3-I to GFP-LC3-II and cleaved GFP in retina. (B) Western blot showing the amount of Ref(2)P/p62 in retina mutant for *ninaA* and over-express *rpr (rh1>rpr)*. The western blot shown is representative of three independent experiments. (C) Western blot showing the level of endogenous LC3 protein in SH-SY5Y cells treated with 6-OHDA and Tm in the presence or absence of bafilomycin A1. (D) Quantification of PRN loss in 20 hour-old living flies expressing GFP. PRNs over-express *rpr* (*rh1>rpr*), *atg6-IR* (*rh1>atg6-IR*) and carry $ninaA^{E110V/+}$. The quantification of PRN loss in the various mutants is relative to *rh1>rpr* (n=5-12). (E, F) Western blots showing the conversion of endogenous LC3-I to LC3-II in SH-SY5Y cells. (E) Cells are starved for 16h with or without 3MA. (F) Cells are treated with Tm, 6-OHDA and bafilomycin A1, with or without 3MA. The western blots shown are representative of two independent experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in Student's t-test).