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ER stress inhibits neuronal death by promoting autophagy

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Endoplasmic reticulum (ER) stress has been implicated in neurodegenerative diseases but its relationship and role in disease progression remain unclear. Using genetic and pharmacological approaches, we showed that mild ER stress ("preconditioning") is neuroprotective in Drosophila and mouse models of Parkinson disease. In addition, we found that the combination of mild ER stress and apoptotic signals triggers an autophagic response both in vivo and in vitro. We showed that when autophagy is impaired, ER-mediated protection is lost. We further demonstrated that autophagy inhibits caspase activation and apoptosis. Based on our findings, we conclude that autophagy is required for the neuroprotection mediated by mild ER stress, and therefore ER preconditioning has potential therapeutic value for the treatment of neurodegenerative diseases.

Introduction

The unfolded protein response (UPR) is an evolutionarily conserved adaptive response to perturbations of normal endoplasmic reticulum (ER) physiology,[1-3] and is characteristic of several neurodegenerative diseases. Whether ER stress plays a causative role in certain disease conditions is still being debated.[4,5] To cope with the aberrant accumulation of unfolded proteins, cells trigger the UPR causing the activation of the ERN1/IRE1, ATF6 and EIF2AK3/PERK pathways.[6] Depending on the level of UPR activation and which components of the pathway are activated, ER stress can lead to either cellular death or survival. Specifically, sustained and full-fledged UPR that involves GADD153/CHOP and CASP12/caspase 12 activations is detrimental to the cell.[7,8] By contrast, mild ER stress (ER preconditioning) induces selective activation of X box binding protein (XBP1) accompanied by cellular protection.[9,10] ER preconditioning induces a cytoprotective response, named ER-hormesis, that protects the cell against a stronger insult.[10,11] ER preconditioning has been shown to induce cytoprotection in ischemia/hypoxia models.[12] However, in other models of neurodegenerative diseases, the cellular mechanism that elicits ER-mediated cytoprotection remains to be explored. A candidate mechanism for the ER-mediated protection is autophagy. It has been proposed in yeast that UPR activation stimulates autophagy, which in turn acts as a protective mechanism limiting ER expansion.[13] Mutations in Drosophila or mouse *atg* genes lead to spontaneous neurodegeneration, suggesting that basal autophagy is neuroprotective.[14-16] In addition, defective autophagic responses are observed in several neurodegenerative diseases including both Alzheimer and Parkinson diseases.[17,18]

Because the UPR and autophagic responses are evolutionarily conserved,[2,3,19,20] we studied the protective mechanisms mediated by mild ER stress in Drosophila and mouse model of Parkinson disease (PD). First, we found that mild ER stress is protective in Drosophila and mouse models of Parkinson disease. In addition, we show that combination of mild ER stress and apoptotic signal induces autophagy, which in turn mediates neuroprotection. We discuss the implications of our findings in the light of the antagonistic relationship between autophagy and apoptosis, as well as the physiological relevance of ER stress and autophagy in neurodegenerative diseases.

Results

Mild ER stress is neuroprotective in Drosophila and mouse models of PD. We have previously shown that mild ER stress inhibits cell death both in vitro and in vivo in Drosophila. Drosophila S2 cells pretreated with a mild dose of tunicamycin (Tm), a chemical inducer of the UPR, exhibited increased resistance to cell death. Similar resistance to cell death was observed in adult Drosophila photoreceptor neurons (PRN) where the UPR was genetically induced by mutations in *neither inactivation nor afterpotential A* (*ninaA*), a gene encoding a

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chaperone specific for the folding of Rhodopsin-1 (Rh1).[10] In these experimental conditions, the activation of the Ire1-Xbp1 pathway is well tolerated, does not induce cell lethality but instead increases resistance to exogenous apoptotic insults.

To determine if mild ER stress-mediated protection is effective in Drosophila PD model, we first confirmed that Tm feeding could activate UPR in the Drosophila brain tissues. *hsc3/bip* expression, a hallmark of UPR activation, was detected by RT-PCR in the heads of flies fed on two doses of Tm (1 μg/ml and 10 μg/ml) (**Fig. S1A and S1B**). Furthermore, we observed an increase of spliced and unspliced forms of *xbp1* (**Fig. S1C**). In our assays, we chose the weakest dose of Tm (1 μg/ml) to induce a mild ER stress in Drosophila.

To establish a previously reported Drosophila PD model, we expressed the gene encoding SNCA/human α-synuclein (*hu-α-syn*) in all neurons with *elav-gal4* or selectively in dopaminergic (DA) neurons with *th-gal4*.[21,22] Next, we examined if Tm feeding could improve locomotor functions and dopaminergic neurons viability in *hu-α-syn* expressing flies. Flies expressing *hu-α-syn* using the pan-neuronal driver *elav* exhibited progressive decrease of motility compared with control (**Fig. 1A**). However, Tm feeding clearly improved climbing ability in 21, 28 and 35 d old flies (**Fig. 1B**).

To assess the toxicity of *hu-α-syn* expression on DA neurons, we measured DA neurons viability and the transcriptional activity of *pale*, a gene responsible for dopamine metabolism and vesicular monoamine transporter (*vmat*), a gene specific for dopamine transport.[23-25] After 42 d of *hu-α-syn* expression in DA neurons, we observed 30% loss of DA neurons in the protocerebral posterior medial clusters (PPM1/2) compared with control flies (**Fig. 1C**). We then detected by RT-qPCR a decrease of *pale* and *vmat* expression in *th* > *hu-α-syn* flies starting from 20 d onward (**Fig. 1D and E**). In *hu-α-syn* expressing flies that were regularly fed on Tm diet, we observed a rescue of DA neuron number in the PPM1/2 cluster (**Fig. 1C**). Similarly, the expression of *pale* and *vmat* was restored following Tm treatment (**Fig. 1D and E**). Together these results show that Tm feeding induces the UPR in Drosophila brain and is neuroprotective in the *hu-α-syn* model of Parkinson disease.

To validate these findings in a mammalian PD model, we tested whether mild ER-stress can induce neuroprotection in the 6-OHDA mouse model and in the human SH-SY5Y neuroblastoma cells.[26-28] The 6-OHDA mouse model recapitulates the common features of PD, including the loss of dopaminergic (DA) neurons and gradual onset of locomotor dysfunction.[29,30] We first assessed if Tm activates the UPR in the mouse brain. We monitored UPR activation by visualizing spliced *xbp1* (*xbp1s*) and *bip* mRNA (**Fig. S1D and S1E**) after intraperitoneal (I/P) injection of Tm. Increases in *xbp1s* and *bip* mRNA were detected at a low dose of Tm (0.1 mg/kg) in the *substantia nigra* (SN), where DA neurons of the nigra-striatal pathway are located (**Fig. S1D and S1E**). No toxic effect or locomotor deficit was observed following chronic injection of Tm (0.1 mg/kg) into mice (**Fig. S1H–J**). Moreover, following Tm injection at 0.1 mg/kg, the expression of *chop*, a transcription factor inducing cell death,[31] was not increased in the SN of mice (**Fig. S1F and S1G**). The *chop* level was only elevated at high doses of Tm (4.5 mg/kg, ED_{50}) whereas *xbp1s* remained at the basal level at this dose (**Fig. S1G** and data not shown). These findings show that low doses of Tm activate a nontoxic, mild UPR in the SN.

Stereotaxic injection of 6-OHDA into the mouse left striatum induces an asymmetrical loss of DA in the nigra-striatal circuit (**Fig. 2A, B, E** and ref. 32). To determine if mild ER stress is protective against the 6-OHDA-induced DA loss, we counted TH-positive DA neurons in the bilateral SN (**Fig. 2A–E**). After 6-OHDA injection, 20% more DA neurons remained in Tm-treated (0.1 mg/kg) than nontreated mice (**Fig. 2E**). Similarly in the human SH-SY5Y neuroblastoma cell line, we observed that Tm treatment reduced cell death induced by 6-OHDA (**Fig. 2G**). In addition, in both SN extracts and in SH-SY5Y cells, caspase activation induced by 6-OHDA was significantly inhibited by Tm treatment (**Fig. S2A and S2B**). These results indicate that Tm treatment-induced ER stress mediates neuron survival by blocking apoptosis both in vitro and in vivo.

Next, we studied the effects of Tm treatment on the rotational behavior in the 6-OHDA mouse model (**Fig. 2F**). The 6-OHDA lesion triggered a progressive increase of unilateral rotational behavior induced by apomorphine treatment. Tm pretreatment markedly reduced the number of turns, indicating that Tm antagonizes 6-OHDA-induced rotational behavior. In summary, our results indicate that Tm treatment protects against the toxic effects of 6-OHDA both in the mouse model and in human neuroblastoma cell line (**Fig. 2**). These findings are pertinent to Parkinson disease progression and treatment.

Autophagy activation is required for the ER-mediated protection. Autophagy and cell death are highly conserved cellular processes during evolution.[19,20,33-35] We therefore chose to study the contribution of autophagy in the ER-mediated protection against neuronal cell death in Drosophila. To achieve this goal, we examined autophagy activation in Drosophila retina submitted to genetically-induced ER stress (*ninaA* mutant) and apoptotic signal (*reaper* overexpression). In *ninaA* mutant PRN where apoptosis was induced by the expression of *reaper* (*rpr*) under the control of *rh1* promoter,[10] we coexpressed the GFP-LC3/Atg8 reporter fusion protein construct (referred to as GFP-LC3) and sought GFP-LC3 puncta in dissected whole-mount retina[36] (**Fig. 3A–D and A'–D'**). We found that PRNs subjected to mild ER stress and apoptosis exhibited a marked increase of GFP-LC3 puncta (**Fig. 3D, D' and E**). In contrast, diffuse GFP-LC3 staining similar to that in wild-type controls was observed in *ninaA* mutant PRN and in PRN expressing *rpr* alone (**Fig. 3A–C and 3A'–C'**). LC3-I was converted into the active form of LC3 (LC3-II) only in *ninaA* mutant PRN subjected to *rpr* expression (**Fig. 3F**). On the western blot, we could also detect in this latter condition the appearance of free GFP, which is more resistant to lysosomal degradation than LC3 (**Fig. S3A**). This result suggests that autophagosomes have fused with lysosomes and that autophagic flux is functional. Next, we examined the expression of Ref(2)P/p62, as an indirect mean to evaluate the flux of autophagy in vivo. Ref(2)P/p62 is a multifunctional scaffold protein that is retained in autophagic vacuoles when the

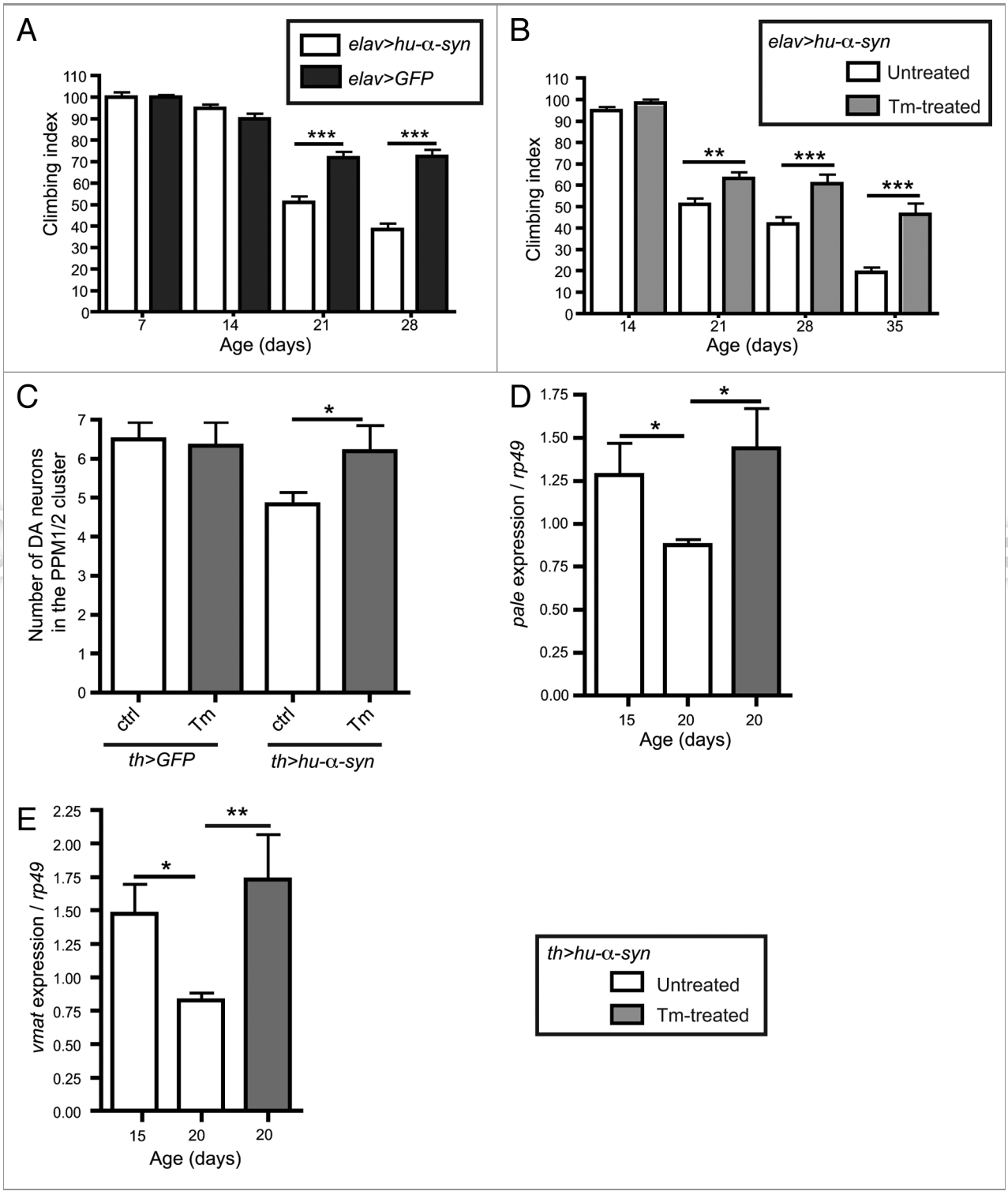


Figure 1. Tm is protective in the *α-syn* Drosophila Parkinson disease model. Flies expressing *hu-α-syn* in all neurons (A and B) with *elav-gal4* driver, or in the dopaminergic neurons (C–E) with the tyrosine hydroxylase driver (*th-gal4*). (A) Climbing ability of aged matched flies expressing *hu-α-syn* and control flies expressing GFP in all neurons (n = 100–120 flies). (B) Climbing ability of flies expressing *hu-α-syn* with or without Tm treatment (n = 100–120 flies). (C) Number of DA neurons in the PPM1/2 brain cluster in *hu-α-syn* or GFP expressing flies with or without Tm treatment. (D and E) Expression of *pale* or *vmat* mRNA normalized to *rp49* in flies expressing *hu-α-syn* with or without Tm (n = 15 flies). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 in Student's t-test.

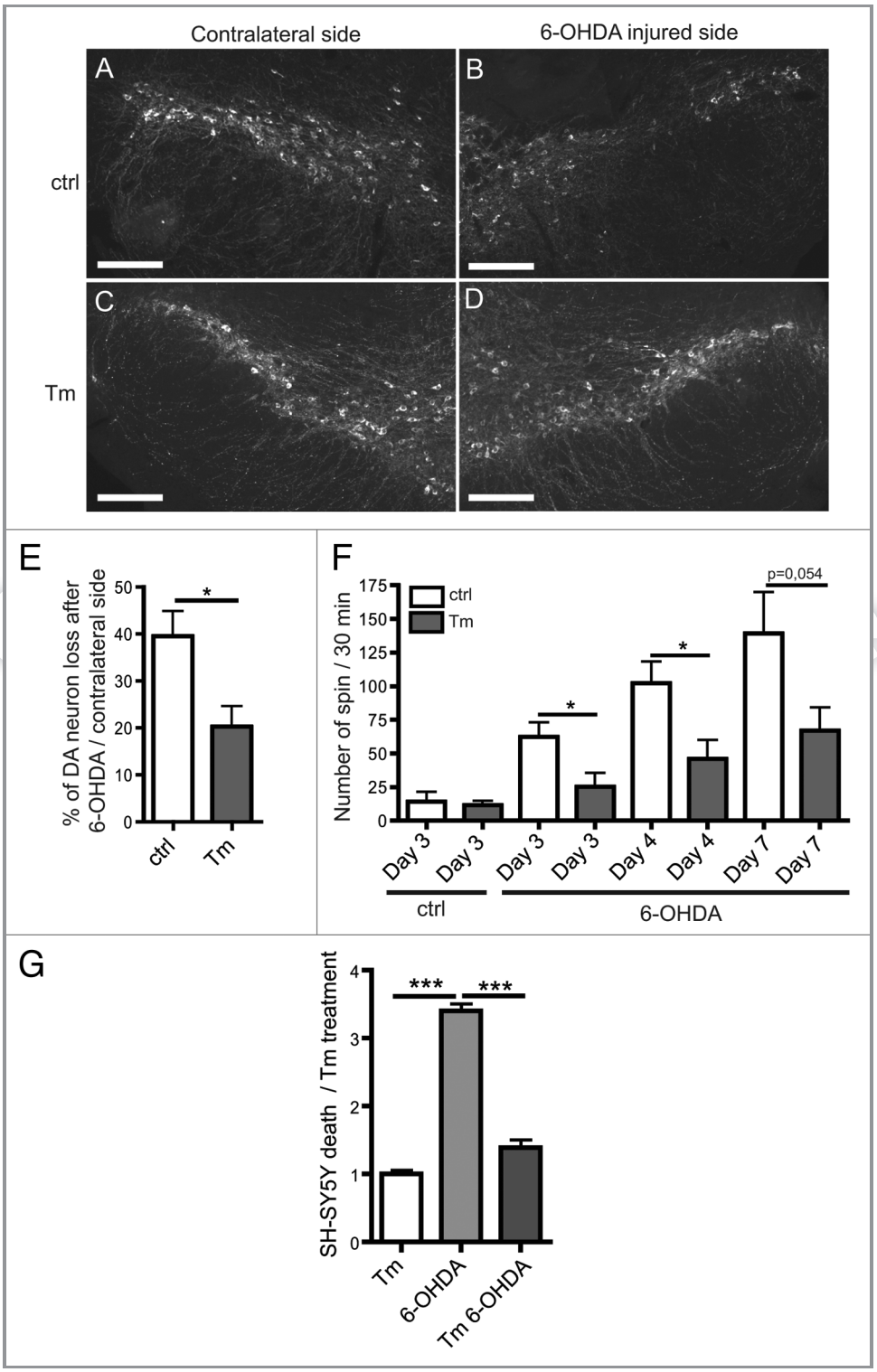


Figure 2. Tm is protective in the 6-OHDA mouse Parkinson disease model. (A–D) Sections of the *substantia nigra* (SN) obtained 4 d after 6-OHDA treatment with or without Tm pre-treatment (0.1 mg/kg). DA neurons are visualized by immunostaining for tyrosine hydroxylase (TH). (E) Quantification of DA neuron loss after 6-OHDA injection normalized to the contralateral side. (F) Rotational behavior of mice after 6-OHDA injection. The graph shows the number of unilateral turns made by mice on days 3, 4 and 7 after the 6-OHDA injection with or without Tm (n = 6–7). (G) In vitro experiments on SH-SY5Y to assess the cell viability after Tm and 6-OHDA treatments. Cell viability was evaluated using trypan blue after Tm and 6-OHDA treatments. Quantification of cell death is normalized to Tm treatment. *$p \leq 0.05$, ***$p < 0.001$ in Student's t-test. Scale bar: 200 μm.

process of autophagy is compromised. Ref(2)P accumulation was observed in *atg8a* mutant as previously described,[37] but not in *ninaA* mutant retina expressing *rpr* (**Fig. S3B**). These results show that the autophagy flux is functional in *ninaA* mutant PRN expressing *rpr* and indicate that mild ER stress activates autophagy, when combined with an apoptotic signal.

In the light of our findings in the Drosophila PRN, we examined autophagy activation of DA neurons in the SN of mice subjected to Tm and 6-OHDA treatments (**Fig. 3G–K**). Basal LC3 levels were observed in DA neurons in both control mice and mice treated with Tm or 6-OHDA alone (**Fig. 3G'–I'**). By contrast, punctate LC3 staining was higher in DA neurons in animals subjected to both Tm and 6OHDA treatments (**Fig. 3J' and K**). We also examined autophagy activation in SH-SY5Y cells submitted to Tm and 6-OHDA treatments. 6-OHDA induced an increased of LC3II form compared with untreated cells. Moreover, we detected a 2-fold increase in LC3II form by combined treatment with Tm and 6-OHDA compared with 6-OHDA alone (**Fig. 3L**). The increase of LC3II form was only observed in the presence of bafilomycin A_1 that alters the lysosomal pH and prevents lysosomal degradation (**Figs. 3L and S3C**). This result indicates that the autophagic flux is functional in SH-SY5Y cells submitted to Tm and 6-OHDA treatments. Thus, autophagy is specifically increased in DA and SH-SY5Y neurons in response to a combination of Tm and 6-OHDA and may therefore be responsible for the neuroprotection.

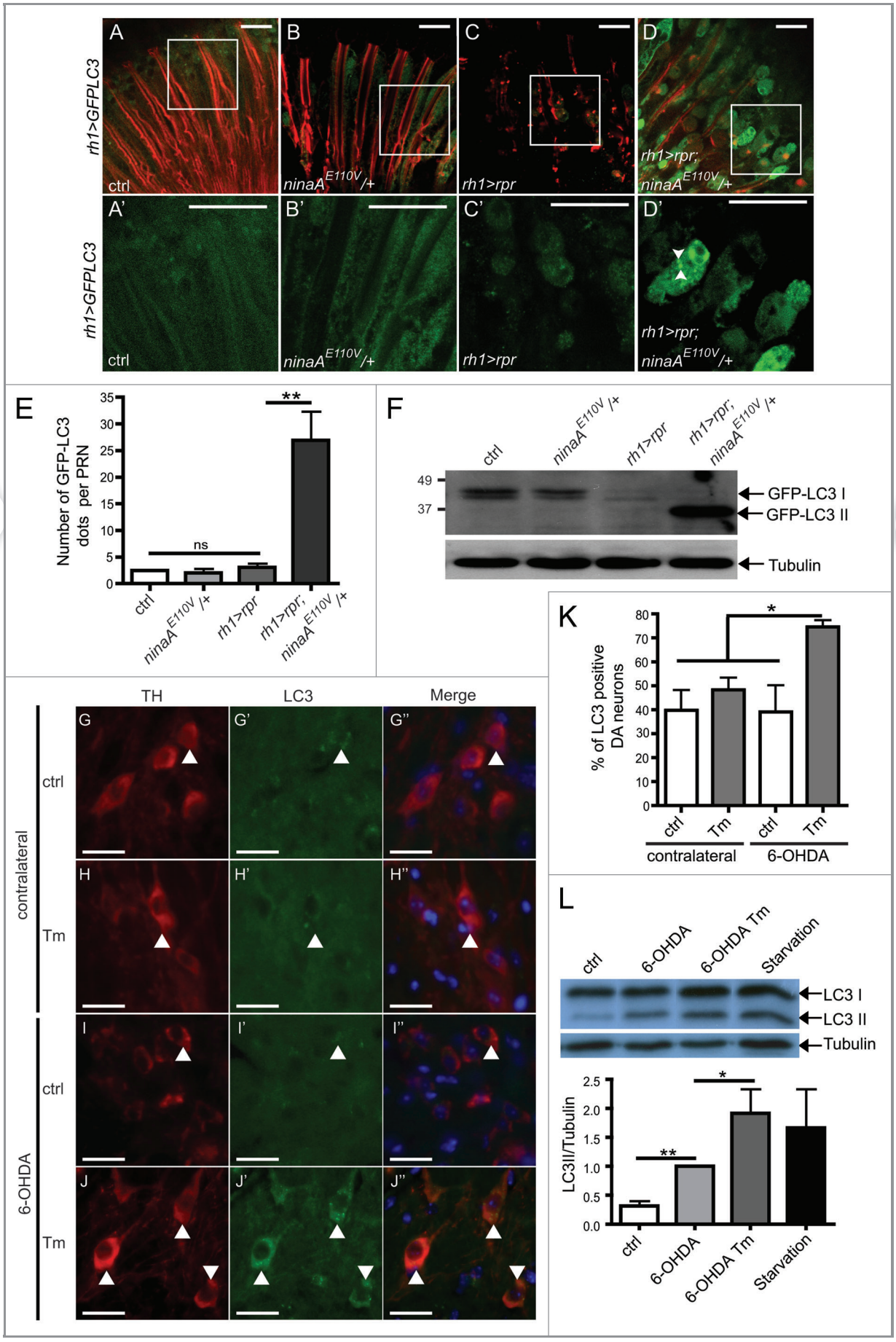
We then investigated whether autophagy activation is required for the ER-mediated protection in Drosophila PRN and in human SH-SY5Y cell line. We first inactivated autophagy in Drosophila PRN with mutations and RNAi knockdown of components of the autophagy pathway. We used the cornea neutralization technique to evaluate PRN viability in living flies.[38,39] We found that in the presence of ectopic *rpr* expression, PRN loss was greater in double-mutant $ninaA^{E110V}$/*atg1*Δ*3D* flies than in flies carrying only $ninaA^{E110V}$ mutation (**Fig. 4A–F**). Similarly, expression of a transgenic *atg6-IR* abolished *ninaA* mutant-mediated protection in Drosophila PRN (**Fig. S3D**). We then examined the role of Drosophila Ref(2)P/p62, a protein required for the formation of protein aggregates that are eliminated by autophagy in Drosophila brain.[37] We found that the expression of transgenic *ref(2)P-IR* suppressed PRN protection in *ninaA* mutant (**Fig. 4G–L**). These results indicate that autophagic clearance contributes to the ER-mediated protection. Next, we examined if Tm-mediated protection required autophagy in SH-SY5Y cell line. To achieve this goal, we performed a siRNA treatment against *atg8/LC3*, which knocked down LC3 expression on a western blot (**Fig. 4M**). We found that LC3 knockdown abolished Tm-mediated protection in SH-SY5Y cell submitted to 6-OHDA (**Fig. 4N**). In addition, we used 3MA which inhibited autophagy induced by starvation or 6-OHDA/Tm treatments (**Fig. S3E and S3F**). We observed that the 3MA treatment also suppressed Tm-mediated protection in SH-SY5Y cell submitted to 6-OHDA (**Fig. 4O**). Altogether these results demonstrate that autophagy is required for ER-mediated protection.

Last, we investigated the controversial issue of whether autophagy inhibits apoptosis.[16,40] We found that ectopic *atg1* expression rescued the viability of Drosophila PRN from *rpr*-induced apoptosis (**Fig. 5A–D**). In addition, rapamycin, an activator of autophagy,[41] inhibited Drosophila S2 cell death induced by cycloheximide (CHX) or UVC and suppressed caspase activation (**Fig. 5E and F**). These results demonstrate that autophagy inhibits apoptosis.

Discussion

Our study provides new insight that mild ER stress promotes neuroprotection via the activation of autophagy. We have defined in vitro and in vivo experimental conditions in which the activation of UPR does not induce cell or organism lethality but rather promotes an adaptive response that protects from apoptotic stimuli. We show that a mild dose of tunicamycin (Tm) activates Ire1-Xbp1 and promotes protective autophagy in response to apoptotic stimuli. We have previously proposed that a preferential activation of Ire1-Xbp1 is responsible for protection in Drosophila S2 cells.[10] This hypothesis is supported by our new results in which we show that mild dose of Tm induces the Ire1-Xbp1 pathway but not *chop* expression in mouse brains (**Fig. S1**). It is also possible that upon mild ER stress, *chop* is induced with a different kinetic than Ire1-Xbp1 and leads to a partial activation of its transcriptional targets as previously proposed.[42] Thus, an adaptive response to mild ER stress may alter *chop* expression or Ddits/Chop activity and promotes survival. In a recent study, it was shown that adaptive suppression of the Atf4/Chop branch by toll-like receptor engagement, promotes survival in response to prolonged ER stress.[43,44] It remains to be demonstrated whether selective activation of Ire1-Xbp1 or suppression of Atf4/Chop promotes neuroprotection in Drosophila and mouse Parkinson disease models.

Several previous studies investigated the link between the UPR and autophagy but a lot remains to be understood on how the UPR activates autophagy and promotes neuroprotection. Yeast cells subjected to severe ER stress manifest an autophagic response, which counterbalances ER expansion.[13] In this model, severe ER stress alone induces autophagy, which in turn limits ER expansion. In a recent study, it was shown that mild ER stress promotes cardioprotection against an ischemic/reperfusion injury.[45] In this model, autophagy activation could reduce subsequent lethal ischemic reperfusion injury. Another study reports that *xbp1* deficiency induces autophagy in a mouse model of the amyotrophic lateral sclerosis.[46] The *xbp1* deficiency leads to an unexpected rescue of *sod1* mutant motor neurons. Although several interpretations have been suggested to explain this result, it is possible that *xbp1* deficiency induces an increase in basal ER stress leading to autophagy. From our results, we propose a model in which mild ER stress primes the cells to trigger neuroprotective autophagy upon an apoptotic stimulus. Our results also indicate that autophagy is neuroprotective, and we further delineate a distinct mechanism by which UPR regulates autophagy. The understanding of the complex relationship between UPR, autophagy and apoptosis probably resides in the identification


A
B
C
D
rh1>GFPLC3
ctrl
ninaA E110V /+
rh1>rpr
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A'
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Number of GFP-LC3 dots per PRN
ns
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F
49
37
GFP-LC3 I
GFP-LC3 II
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and characterization of key factors that integrate these stress responses. In a recent study, it was shown that these responses are controlled by Bax inhibitor-1 (BI-1). BI-1 is a factor that inhibits IRE1-α, controls autophagy and apoptosis.[47] In cells lacking BI-1, IRE1-α is activated and induces autophagy, promoting cell survival. A role of BI-1 remains to be investigated in the control of autophagy in cells submitted to mild ER stress and apoptotic signal.

Previous work has identified a link between apoptosis and autophagy.[48] The authors have shown that stimulating cell death with TNFα in the presence of caspase inhibitors induced autophagy in L929 fibroblastic mouse cells.[48] Based on their hypothesis, TNFα stimulates an alternative autophagic death program when caspases are inhibited. In our hands, we found that inhibition of caspases with p35 did not induce autophagy in PRN submitted to apoptosis by *rpr* expression (data not shown). We favor a model in which combined signals of the UPR and apoptosis induce autophagy. In addition, our results differ from the one presented by Lenardo and col.,[48] as we demonstrated in several in vivo and in vitro models that autophagy is protective in cells submitted to mild ER stress. As discussed elsewhere, the opposite functions of autophagy on survival and death may depend on cell type and the level of autophagy activation.[49]

Autophagy has been proposed to inhibit cell death, however its role in the inhibition of apoptosis is a controversial subject.[16,40] We have shown that autophagy inhibits caspase activation and apoptosis in several in vitro and in vivo paradigms (**Figs. 4 and 5**). We have observed a concomitant increase of autophagic markers and decrease of caspase activation in cell submitted to both mild ER stress and apoptosis (**Figs. 2, 3 and S2**). Our findings suggest that cells switch from an apoptotic to an autophagic response when submitted to both mild ER stress and apoptotic signal. However, how autophagy inhibits apoptosis remains to be uncovered. Autophagosomes could engulf and degrade impaired mitochondria (mitophagy) to prevent the subsequent activation of apoptotic pathway.[50] Another hypothesis is that autophagy could directly sequester pro-apoptotic factors, such as caspases, and promote their degradation as previously proposed in a mouse model of Alzheimer disease.[51] Further work is required to elucidate this mechanism.

Relevance to pathology. We found that mild ER stress is protective in the Parkinson 6-OHDA mouse model, showing that maintaining UPR at a moderate level could protect against Parkinson disease. After injection in the striatum, 6-OHDA is selectively taken in DA by retrograde transport.[52] 6-OHDA induces an oxidative burst and caspase activation, which leads to DA death.[28] We show that Tm treatment activates mild UPR responses, correlates to reduced DA death and improved locomotor function in mice bearing 6-OHDA lesions. Moreover, mild ER stress protects DA neurons of the SN from 6-OHDA-induced death by limiting caspase activation (**Fig. S2**) as previously observed in human neuroblastoma cell lines.[28] As in the fly paradigm, the increased autophagy in DA submitted to Tm and 6-OHDA suggests that autophagy is an active player of neuroprotection in mice. Our results incite new investigations into therapeutic possibilities to trigger and maintain ER stress at a moderate level, so that the stress response protects against or delay the onset of neurodegeneration, or retard the disease progress.

Materials and Methods

Drosophila genetics. Flies were maintained at 25°C in a 12:12 h light cycle. The wild-type flies used for this study were Canton^{S} strain. The *ninaA^{E110V}* fly stock is a kind gift from Charles Zuker.[53] The *rh1-gal4* fly stock is a generous gift from Jessica Treisman.[38] *UAS-reaper* (*rpr*), *UAS-lacZ* and *atg8^{KQ70569}, elav-gal4* and *th-gal4* were obtained from Bloomington stock. *UAS-atg1* and *atg1Δ3D* stocks were a kind gift from Thomas Neufeld,[54] *UAS-GFP-LC3* was a kindly provided by Harald Stenmark,[36] *UAS-atg6-IR* were kindly obtained from Udai Bhan Pandey and *UAS-hu-α-syn* was kindly given by Mel Feany. The following genetic combinations were used to express transgenes in adult outer PRN: (1) *rh1-gal4; UAS-GFP; UAS-rpr*, (2) *rh1-gal4; UAS-GFP*, (3) *rh1-gal4; UAS-GFP-LC3*, (4) *rh1-gal4; UAS-GFP-LC3; UAS-rpr*, (5) *rh1-gal4; UAS-atg1*, (6) *rh1-gal4; ninaA^{E110V}/UAS-GFP-LC3*, (7) *rh1-gal4; ninaA^{E110V}/UAS-GFP-LC3*; *UAS-rpr*, (8) *rh1-gal4; UAS-rpr*, (9) *rh1-gal4; ninaA^{E110V}/+; UAS-rpr*, (10) *rh1-gal4;ninaA^{E110V}; UAS-rpr*, (11) *rh1-gal4; ninaA^{E110V}/ UAS-GFP*; *UAS-rpr*, (12) *rh1-gal4; ninaA^{E110V}/UAS-GFP*; *UAS-rpr/atg1Δ3D*, (13) *rh1-gal4; UAS-GFP/ UAS-lacZ; UAS-atg1*, (14) *rh1-gal4; UAS-GFP; UAS-atg1/ UAS-rpr*. (15) *elav-gal4; UAS-hu-α-syn;* (16) *elav-gal4; UAS-GFP;* (17) *UAS-hu-α-syn; th-gal4* (18) *UAS-lacZ; th-gal4.*

Drosophila pharmacological treatments. ER stress was pharmacologically induced using tunicamycin (Tm; Covalab, 11089-65-9), an inhibitor of protein glycosylation. Twenty males and 20 females aged for 24 h were collected and starved for 5 h on 0.8% agarose, 1X PBS medium. Flies were then transferred in vial containing food (0.8% agarose, 10% sucrose, 1X PBS medium) supplemented with Tm (1μg/ml; 10μg/ml) or vehicle solution (Dimethyl sufoxide, 2% Sigma Aldrich, D8418) for 4 h.

Immunostaining on eye whole mount. GFP-LC3 labeling was performed on Drosophila whole-mount retina as previously

Figure 3 (See opposite page). Activation of autophagy by combined ER stress and cell death signals. (A–D′) Whole-mount adult retina from flies expressing GFP-LC3 in PRN. GFP-LC3 is in green and phalloidin labels PRN rhabdomeres in red. (A′–D′) Higher magnification view of GFP staining in the area surrounded by a white rectangle in (A–D). Scale bar: 10 μm. (E) Quantification of the number of GFP-LC3 dots per PRN. (F) western blot with anti-GFP antibody showing the conversion of GFP-LC3-I to GFP-LC3-II in Drosophila retina. The western blots shown are representative of three independent experiments. (G–J″) *Substantia nigra* sections after Tm and 6-OHDA injections in mice. (G–J) Anti-TH in red. (G′–J′) Anti-LC3 in green. (G″–J″) The merge shows TH, LC3 and DAPI (blue) for nuclei. (K) The graph shows the percentage of TH positive cells with LC3 punctates. Between 220 and 250 neurons from each of three different mice were assessed. Scale bar: 20 μm. (L) western blot and quantification showing the conversion of LC3-I to LC3-II in SH-SY5Y cells treated or not treated with Tm and 6-OHDA in the presence of bafilomycin A_1. Cells under starvation are used as a positive control. The western blots shown are representative of three independent experiments. *p ≤ 0,05, **p ≤ 0.01 in Student's t-test.

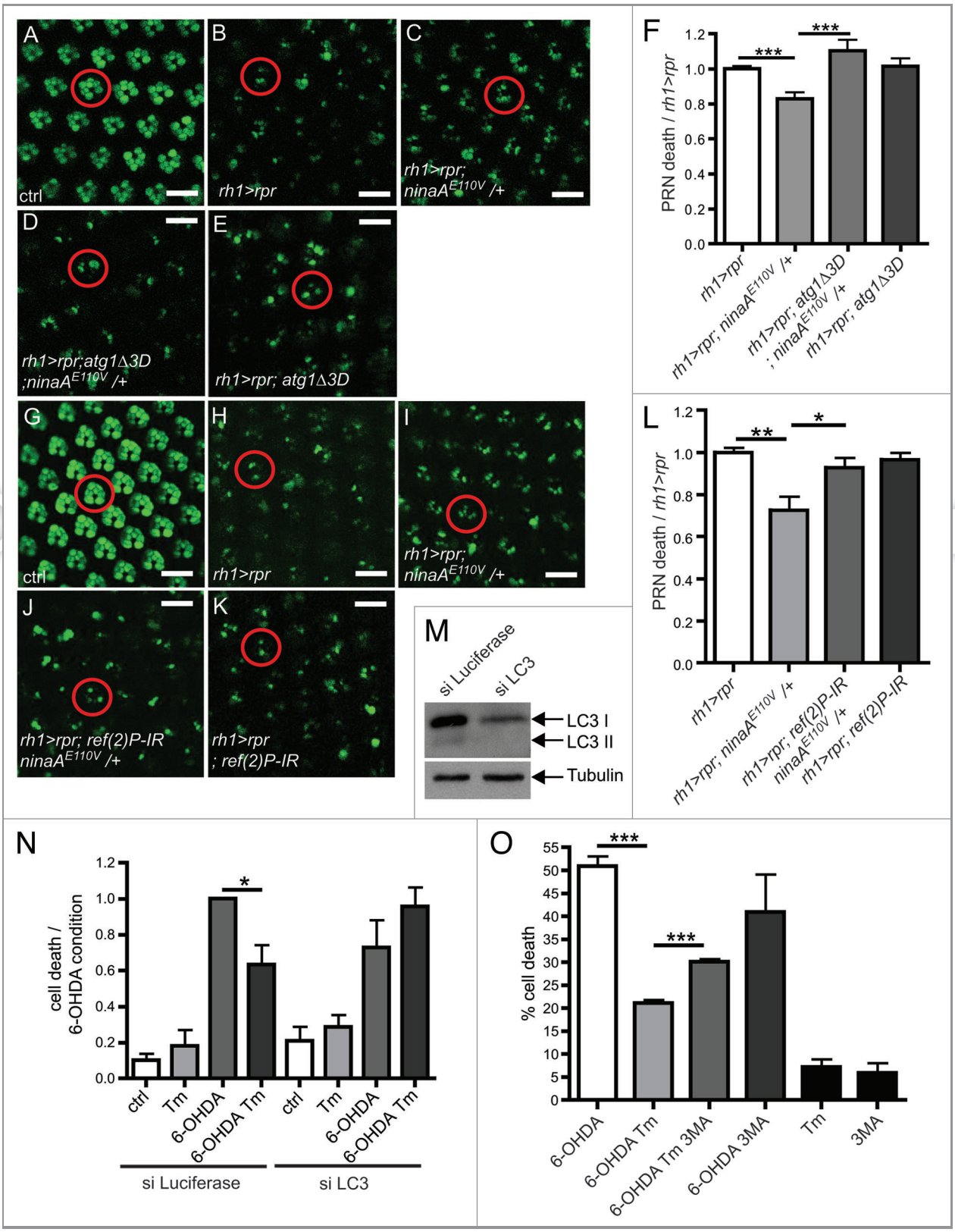

Figure 4. For figure legend, see page 923.

Figure 4 (See opposite page). Autophagy is required for ER-mediated neuroprotection. (A–E and G–K) Visualization of PRN viability in 16 h-old living flies expressing *rh1 > GFP*. (A–E) Visualization of PRN in retina overexpressing *rpr* (*rh1 > rpr*) and mutant for *ninaA*$^{E110V/+}$ and *atg1Δ3D*. (F) Quantification of PRN loss in the various mutants (B–E) relative to *rh1 > rpr* (n = 10). (G–K) Visualization of PRN in retina overexpressing *rpr* (*rh1 > rpr*), *ref(2)P-IR* and mutant for *ninaA*$^{E110V/+}$. (L) Quantification of PRN loss in the various mutants (H–K) relative to *rh1 > rpr* (n = 10). (M) western blotting showing LC3I/II levels after siRNA against LC3 in SH-SY5Y cell compared with control (siRNA luciferase). (N) SH-SY5Y cell viability was assessed by trypan blue exclusion after treatments with Tm, 6-OHDA and siRNA against LC3 or luciferase as control (n = 3). (O) SH-SY5Y cell viability was assessed by trypan blue exclusion after Tm and 6-OHDA treatments. 3-MA treatment is used to block autophagy. *p ≤ 0.05, **p < 0.01, ***p < 0.001 in Student's t-test. Scale bar: 10 μm. The abbreviations used: *rh1-gal4; UAS-GFP* (*rh1 > GFP*), *rh1-gal4;UAS-rpr* (*rh1 > rpr*).

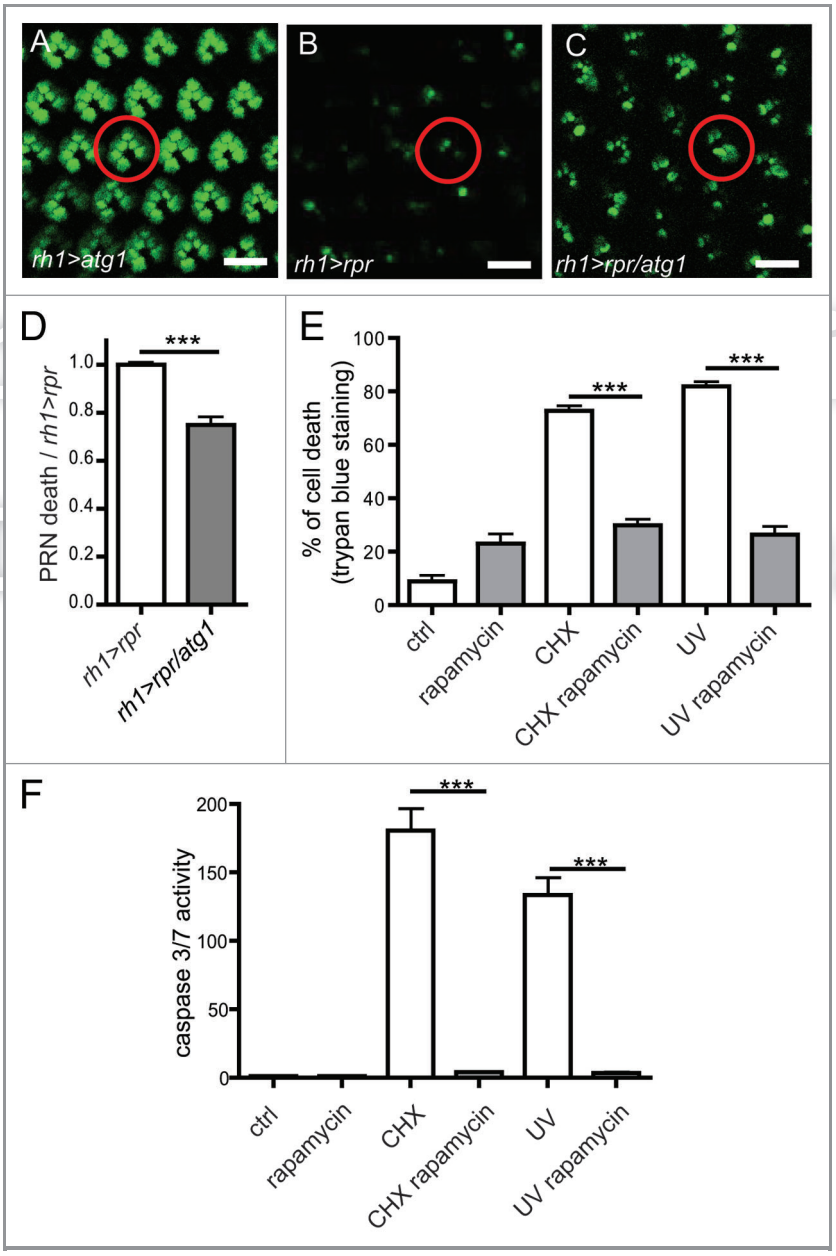

Figure 5. Autophagy inhibits cell death. (A–C) Visualization of PRN viability in 16 h-old living flies expressing GFP. PRN express *rpr* (*rh1 > rpr*) and *atg1* (*rh1 > atg1*). (D) Quantification of PRN loss in (B and C) relative to *rpr* (n = 6–7). (E) In vitro experiments on S2 cells to assess the cell viability after rapamycin and cycloheximide (CHX) treatments and UVC irradiation. Cell death was monitored by FACS analysis after incorporation of propidium iodide. (F) Caspase activity in S2 cells subjected rapamycin and cycloheximide (CHX) treatment and UVC irradiation. Results are expressed as ratio of caspase activity relative to control values (n = 3). Scale bar: 10 μm. ***p < 0.001 in Student's t-test. The abbreviations used: *rh1-gal4;UAS-rpr* (*rh1 > rpr*), *rh1-gal4; UAS-atg1* (*rh1 > atg1*).

described.[55] Briefly, Drosophila heads were bisected in the middle with a scalpel. Brain tissue was removed to expose retina underneath. The retinae were fixed in 4% PFA for 15min. GFP-LC3 was revealed by immunostaining using a rabbit anti-GFP antibody (1/200, Invitrogen, A-6455) followed by an anti-rabbit secondary antibody (Alexa 488 1/400, Invitrogen, A-21206). Photoreceptor rhabdomeres were visualized using Actin coupled with phalloidin staining (1/400, Sigma Aldrich, 77418-1EA). Retinae were mounted in DAPI mounting media (Vectashield, AbCys, H1500). Fluorescent images were obtained using a Leica SP5 confocal microscope.

Mouse protocol. All animal protocols were approved by the regional ethics committee for animal experiments, Rhônes Alpes (authorization n°153). C57Bl6/J female mice (10 weeks old) were used for this study. Tm (Covalab, 11089-65-9) was administered by intraperitoneal (I/P) injection (0.01 mg/kg, 0.1mg/kg or 4.5 mg/kg). Eighteen hours after Tm treatment, 8 µg of 6-hydroxydopamine (6-OHDA, 4 µg/µl, Tocris, 2547) in 0.02% ascorbic acid was injected stereotaxically into the left striatum of the mouse brain to induce Parkinson disease-like injury.[56] Rotational behavior tests were performed on 6-OHDA-treated mice to evaluate alterations of the nigra-striatal pathway. Rotational asymmetry was induced by I/P injection of apomorphine at 0.6 mg/kg (Sigma Aldrich, A4393) on days 3, 4 and 7 as described.[30] Motor behavior was tested to assess motricity following Tm injection. Walking distance (cm) was measured over a period of 2 min three times for Tm-treated (n = 7) and control mice (n = 8).

Immunostaining on mice brain sections. Visualization of dopaminergic neurons was performed in mice substantia nigra sections. Mice were sacrificed by lethal I/P injection of pentobarbital, then perfused intracardiacaly with saline solution and 4% PFA for fixation. Brains were extracted, further post-fixed in 4% PFA for 2h, transferred to 30% sucrose solution at 4°C, and serially freezed-sectioned. Fourteen µm-thick floating brain sections were transferred into blocking solution (PBS-triton 0.1%, 4% BSA, 10% normal goat serum) for 1h at RT. DA neurons were visualized using an anti-Tyrosine Hydroxylase antibody (α-TH, 1:2000, Millipore, Ab152) and anti-LC3 antibody (1:800, Cell Signaling, 2775S). Specifically, brain sections were incubated with the primary antibodies at 4°C overnight and with secondary antibodies Alexa 555 (1: 500, Invitrogen, A21424) and Alexa 488 (1: 500, Invitrogen, A-21206) at RT for 2h in the dark. Fluorescent images were taken using an ApoTome Imager M2 with an AxioCam MRm (Zeiss). The loss of DA neurons after 6-OHDA treatment was defined as the percent of TH positive cells in the 6-OHDA injured side compared with the contralateral side. Autophagy level in DA neuron was defined as the percent of TH positive cells with LC3 punctates.

Cell culture. Drosophila S2 cells were cultured in Drosophila Schneider medium (Invitrogen, 21720024) supplemented with 10% fetal bovine serum. Cells were pre-incubated with 0.4 µg/ml of rapamycin for 40 h, then subjected to treatment with 10 µM cycloheximide (Sigma-Aldrich, C1988) or 300 mJ/cm^2 UV C (UVC) with a UV irradiator (Vilber Lourmat 254 nm, LBX). After 8 h, the cells were stained with 50µg/ml propidium iodide and analyzed by flow cytometry (FACSCalibur4C) to measure cell death.

SH-SY5Y neuroblastoma cell line were cultured in DMEM: HamF12 (1:1) supplemented with L-Glu plus nonessential amino acid (1%) and 10% FCS (Invitrogen, 10270106). Cells were pre-incubated with 0.5 µg/ml of Tm for 4 h, then subjected to treatment with 50 µM 6-OHDA (Tocris, 2547). After 16 h the cells were stained with 50 µg/ml propidium iodide and analyzed by flow cytometry (FACSCalibur4C) to measure cell death. For starvation, SH-SY5Y were maintained for 24 h in DMEM: HamF12 (1:1) supplemented with L-Glu plus nonessential amino acid (1%) without FCS.

Inhibition of autophagy in SH-SY5Y. Inhibition of autophagy was performed via RNAi as previously described.[57] Briefly, 40 nM of small interfering RNA (siRNA) sequences targeting LC3 (5'-GAAGGCGCUUACAGCUCAA-3') or siRNA targeting luciferase, used as negative control (5'-CGUACGCGGAAU ACUUCGA-3'), were introduced in 0,1% lipofectamine 2000 (Invitrogen, 11668019) at day 1. siRNA experiment was repeated 48 h following the first siRNA. Inhibition of autophagy was assessed by western blotting experiment using LC3 antibody (Cell Signaling, 2775S). Autophagy was also inhibited by 3-methyladenine (3-MA; Sigma Aldrich, M9281). Cells were incubated with Tm (0.5 µg/ml) for 4 h, then subjected to 3-MA treatment at 10 µM and 6-OHDA at 50 µM (Tocris, 2547) for 16 h. Cell viability was assessed by trypan blue staining (Sigma Aldrich, T8154). In **Figure 3L**, autophagy flux was inhibited by adding bafilomycin A_1 to the cells at 10 nM for 12 h.

Statistical analysis. Data from mRNA expression, photoreceptor cell survival, autophagy activation, 6-OHDA cytototoxicity, rotatory and motricity behavior assays were analyzed using Student's t-test (2-group comparison). Level of significance was set at $p \leq 0.05$.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Materials

Additional Material and Methods may be found here:
www.landesbioscience.com/journals/autophagy/article/19716

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