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General Article

GENERAL ARTICLE

# Sigma-1 receptor is a key genetic modulator in amyotrophic lateral sclerosis

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## Abstract

Sigma-1 receptor (S1R) is an endoplasmic reticulum (ER) chaperone that not only regulates mitochondrial respiration but also controls cellular defense against ER and oxidative stress. This makes S1R a potential therapeutic target in amyotrophic lateral sclerosis (ALS). Especially, as a missense mutation E102Q in S1R has been reported in few familial ALS cases. However, the pathogenicity of $S1R^{E102Q}$ and the beneficial impact of S1R in the ALS context remain to be demonstrated *in vivo*. To address this, we generated transgenic *Drosophila* that expresses human wild-type S1R or $S1R^{E102Q}$. Expression of mutant S1R in fly neurons induces abnormal eye morphology and locomotor defects in a dose-dependent manner. This was accompanied by abnormal mitochondrial fragmentation, reduced adenosine triphosphate (ATP) levels and a higher fatigability at the neuromuscular junction during high energy demand. Overexpressing IP3 receptor or glucose transporter mitigates the $S1R^{E102Q}$-induced eye phenotype, further highlighting the role of calcium and energy metabolism in its toxicity. More importantly, we showed that wild-type S1R rescues locomotor activity and ATP levels of flies expressing the key ALS protein, TDP43. Moreover, overexpressing wild-type S1R enhances resistance of flies to oxidative stress. Therefore, our data provide the first genetic evidence that mutant S1R recapitulates ALS pathology *in vivo* while increasing S1R confers neuroprotection against TDP43 toxicity.

## Introduction

Sigma-1 receptor (S1R) is a transmembrane protein mostly located in the endoplasmic reticulum (ER), where it is particularly enriched at the mitochondria-associated ER membrane (MAM). Initially confused with opioid receptors, S1R is now well recognized as an unusual molecular chaperone. The topology of S1R has been largely debated but recently using the ascorbate peroxidase 2 (APEX2) tag and electron microscopy it has been demonstrated that S1R presents a main bulk in the ER lumen (1). Thus, S1R might mainly interact with newly synthetized ER proteins and contribute to their proper folding. But also as a resident protein at MAM, S1R modulates the inositol triphosphate receptor (IP3R), regulates calcium signaling from ER to mitochondria and thus enhances adenosine triphosphate (ATP) production (2, 3). Moreover, in stress conditions, S1R enhances nuclear production of antioxidant proteins by chaperoning the ER stress sensor, inositol requiring enzyme 1 (IRE1) or by enhancing nuclear factor erythroid-2 related factor 2 (Nrf2) signaling (4–6). Upon overexpression or stimulation with AQ4

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agonists, S1R can also translocate at the plasma membrane to modulate a wide number of ion channels, receptors and kinases (7, 8). S1R is also localized at the nuclear envelope, where it regulates gene transcription by interaction with chromatin remodeling factors like Emerin (9, 10). All these modulating actions of S1R make it an interesting target for neuroprotection in many neurological diseases. Moreover, despite being found throughout the brain, S1R is strongly expressed in motoneurons of the spinal cord, thus making it particularly attractive for motor neuron diseases. More precisely, S1R is enriched in the ER at the postsynaptic subsurface cisternae of cholinergic C-terminals, a subdomain determinant for motoneuron excitability and survival (11, 12).

AQ5 Numerous exogenous ligands have been identified, acting as S1R agonists, antagonists or modulators but also putative endogenous ligands like neurosteroids (13, 14), N,N-dimethyltryptamine (15) or more recently choline (16). So far, S1R ligands were used in preclinical or clinical studies on Alzheimer's disease, Huntington's disease, schizophrenia, chronic pain and depression (17–20). However, direct evidence using genetic approaches is still lacking. Inversely, S1R dysregulation may play a role in neuropathogenesis. For instance, S1R-binding sites are found decreased in the cerebral cortex and cerebellum of patients with schizophrenic illness (21, 22) or Alzheimer's disease (23, 24). S1R polymorphisms may modify risk of Alzheimer's disease (25–27). In preclinical models, S1R inactivation was found to exacerbate Alzheimer pathology in S1R KO × $APP_{Swe,Lnd}$ mice (28). A more direct link was described between S1R mutations and motor neuron diseases. Several truncations/deletions or point mutations in S1R were reported as a cause for distal hereditary motor neuropathy (29–32). Juvenile cases of amyotrophic lateral sclerosis (ALS) were associated to a missense mutation (c.304G>C, p.E102Q) and a frameshift mutation (c.283dupC, p.L95 fs) in S1R (33, 34). But so far, the deleterious impact of mutant S1R has not been confirmed on models *in vivo*.

ALS is a fatal motor neuron disease. Patients show muscle weakness and atrophy with paralysis, spasticity and dysarthria. Over time, complications appear with dysphagia and respiratory distress. Among treatments, riluzole prolongs life by only a few months but without ameliorating motor functions (35), and the antioxidant edaravone was found to slow disease progression in only few ALS patients (36). More effective cures remain to be developed. About 10% of ALS patients are inherited forms, while sporadic forms with no family history represent the majority of patients. Mutations in the *copper/zinc superoxide dismutase 1* (*SOD1*) gene were first identified as the cause of 20% of familial cases (37), but over the last two decades a major advance was achieved with the identification of more than 25 other ALS-linked genes. Among these, a G4C2 hexanucleotide repeat expansion in a non-coding region of the chromosome 9 open reading frame 72 gene (C9orf72) corresponds to the most common mutation found in 30–40% of familial ALS in Europe and North America. Then, mutations in two DNA/RNA-binding proteins: TAR DNA-binding protein of 43 kDa (TDP43) and fused in sarcoma (FUS) account for 10% of familial cases (38–40). However, strikingly, wild-type TDP43 accumulates in the cytoplasm and forms inclusions in the brains of almost all (97%) patients suffering from ALS (41–43). This implies that deregulation or mislocalization of wild-type TDP43, which is usually mainly localized in the nucleus, mediates both sporadic and familial ALS.

By using *Drosophila* genetic tools, we here investigated how S1R modulates ALS pathology *in vivo*. We showed that expression of $S1R^{E102Q}$ carrying the ALS mutation alters locomotor activity and eye development. $S1R^{E102Q}$-expressing flies displayed a higher fatigability at the neuromuscular junction (NMJ) consecutively to a repetitive stimulation. This was accompanied by abnormal mitochondrial fragmentation and reduced ATP levels. Overexpressing *Drosophila* IP3R or the human glucose transporter GluT-3 both reduced the $S1R^{E102Q}$-induced eye phenotype. More importantly, our data provide direct evidence that wild-type S1R ($S1R^{WT}$) confers protection against TDP43-induced toxicity. $S1R^{WT}$ not only ameliorates locomotion of flies expressing TDP43 but also restores ATP levels. Moreover, the presence of $S1R^{WT}$ in neurons reduces sensitivity to oxidative stress.

## Results

### $S1R^{E102Q}$ alters locomotor performances of *Drosophila*

To study the impact of mutant S1R *in vivo*, we generated transgenic flies to express the human full-length cDNA encoding $S1R^{WT}$ or $S1R^{E102Q}$ under the control of the UAS-GAL4 system. This bipartite system allows the expression of genes in specific cell subtypes like here in neurons. Two independently transformed lines expressing $S1R^{WT}$ ($S1R^{WT}$#2 and $S1R^{WT}$#5) or $S1R^{E102Q}$ ($S1R^{E102Q}$#1 or $S1R^{E102Q}$#9) were used. Western blot assay revealed that the levels of mutant S1R were low in both $S1R^{E102Q}$ transgenic lines ($S1R^{E102Q}$#1 and $S1R^{E102Q}$#9 transgenes) as compared to $S1R^{WT}$ flies (Supplementary Material, Fig. S1A–D).

To test whether overexpressing mutant S1R affects locomotor performances of flies, we used the natural negative geotaxis reflex of flies to walk against gravity. The GAL4 activity depends on the temperature and is maximal at 29°C while maintaining *Drosophila* relatively healthy. We thus looked at effects when flies were reared at 25 and 29°C. The presence of either one copy of $S1R^{E102Q}$ transgene ($S1R^{E102Q}$#1 or $S1R^{E102Q}$#9) failed to significantly modify the climbing ability of 15-day-old flies regardless of the temperature (Fig. 1A and Supplementary Material, Fig. S2). This lack of effect is likely due to the low expression level of mutant S1R. Next, we expressed the two transgenes together ($S1R^{E102Q}$#1/#9) by breeding the $S1R^{E102Q}$#1 and $S1R^{E102Q}$#9 lines. Then, protein expression levels of mutant S1R became closer to those measured in flies expressing wild-type S1R at 29°C (Supplementary Material, Fig. S1E and F). Interestingly, flies expressing $S1R^{E102Q}$#1/#9 exhibited progressive locomotor deficits (Fig. 1B). At 9 days old, >60% of control flies reached the top of the column. In contrast, only 36% of $S1R^{E102Q}$#1/#9 flies succeeded to reach the top and 44% were unable to climb. The defects were more pronounced at 15 days old with 68% of $S1R^{E102Q}$#1/#9 flies that remained at the bottom. The decline in climbing performances results from the presence of the E102Q mutation since $S1R^{WT}$ overexpression did not induce locomotor defects at 29°C (Fig. 1C). Spontaneous locomotor activity was also monitored during 15 min with 1-min time interval, by using a videotracking system (Viewpoint). Control flies were active for 15–25 s/min, i.e. 25–40% of the time spent in the Petri dish (Fig. 1E). In contrast, $S1R^{E102Q}$#1/#9 expressing flies were quite immobile since they were found active only 8% of the time. Altogether, our data indicate that the presence of the E102Q mutation in S1R alters locomotor activity of flies, whereas overexpression of wild-type S1R has no effect on locomotor performances.

In order to determine whether locomotor defects were associated to alteration at the NMJ, we set up electrophysiological

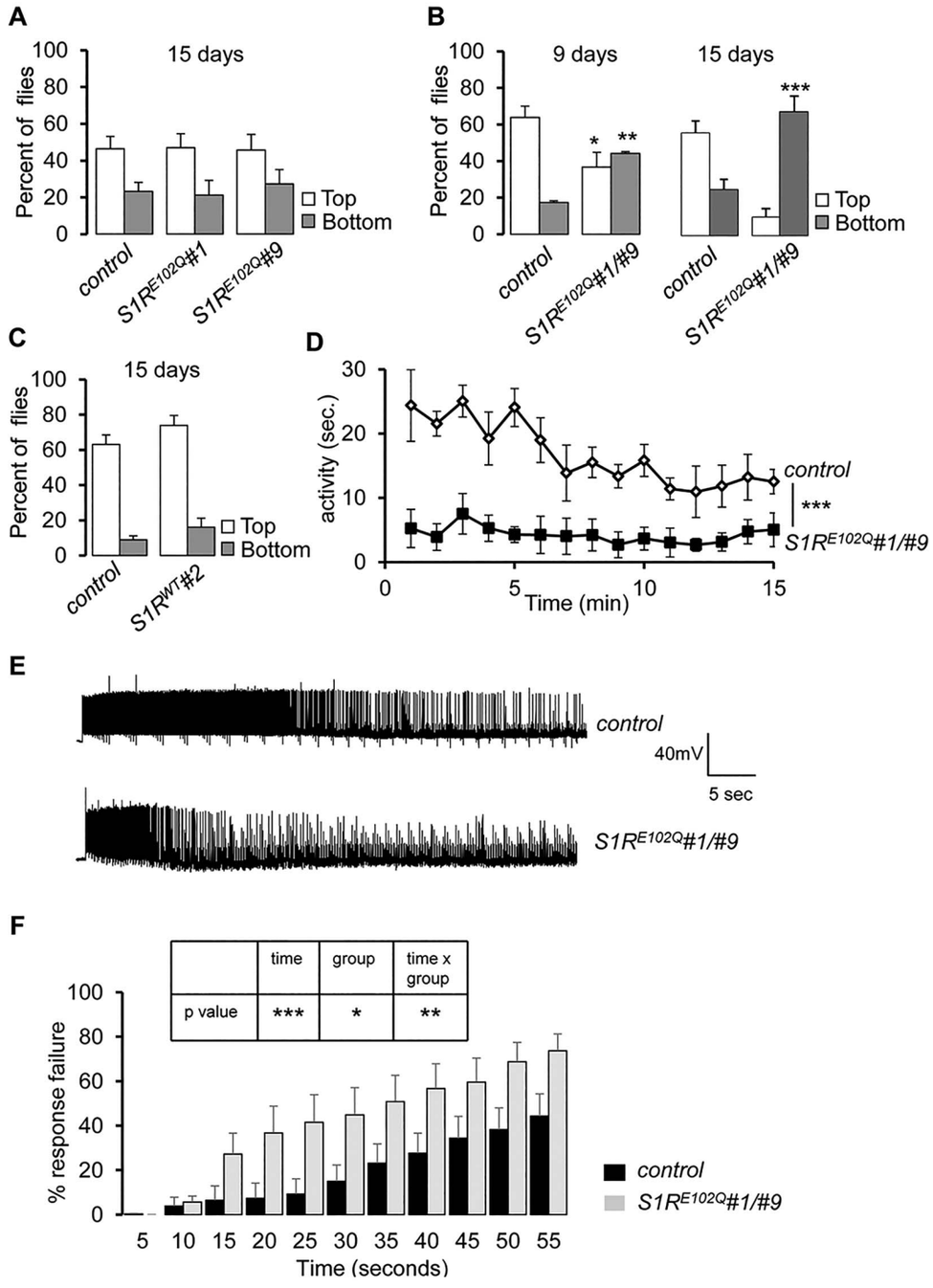


**Figure 1.** ALS-linked S1R variant alters locomotor activity and JNM transmission. (**A**) Impact of S1R$^{E102Q}$ on locomotor behaviour of 15-day-old flies reared at 29°C. Climbing performances of flies expressing no transgene (control) or one copy of mutant S1R variant (S1R$^{E102Q}$#1 or S1R$^{E102Q}$#9) in neurons. In each experiment, the proportions of flies that climbed to the top of the column or that remained at the bottom were determined after 1 min. Statistical significance was assessed by analysis of variance (ANOVA; $n = 4$–6). (**B**) Climbing performances of 9- or 15-day-old flies expressing no transgene (control) or the two S1R$^{E102Q}$ transgenes together (S1R$^{E102Q}$#1/#9) in neurons. Flies were reared at 29°C. Statistical significance was assessed by Student's t-test ($n = 5$–6, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (**C**) Climbing performances of 15-day-old flies expressing no transgene (control) or S1R$^{WT}$#2 in neurons. Flies were reared at 29°C. Statistical significance was assessed by Student's t-test ($n = 7$). (**D**) Duration of active behaviour of 19-day-old flies expressing no transgene (control) or S1R$^{E102Q}$#1/#9. Flies were reared at 29°C. The locomotor activity was measured every 60 s. from five flies in a petri dish ($n = 5$ independent measures). Statistical significance was assessed by a repeated-measure ANOVA test ($F_{(14,120)} = 2.28$, $^{***}P < 0.001$). (**E**) Representative traces of evoked action potentials following a repetitive stimulation at 15 Hz (15 V for 55 s) of giant fibers in control or S1R$^{E102Q}$#1/#9 flies. Flies expressing S1R$^{E102Q}$#1/#9 display higher response failures through time. (**F**) Quantitative analysis of the response failure percentage following repetitive stimulation of giant fibers in control or S1R$^{E102Q}$#1/#9 flies. Data from 13 flies were averaged and presented as mean ± SEM. Statistical comparisons were performed using a repeated-measure ANOVA ($n = 13$, genotype × time: $F_{(10,250)} = 2.76$, $P < 0.01$).

recording of evoked responses after stimulation of the giant fiber circuit that controls jump and flight muscles. Threshold potentials to elicit an evoked response were similar between control and S1R$^{E102Q}$#1/#9 flies at 15 days of age (control: 6.25 ± 0.69 V and S1R$^{E102Q}$#1/#9: 6.19 ± 0.39 V). Next, we evaluated the fatigability of giant fiber circuit consecutively to a repetitive stimulation at 15 Hz. Control flies progressively displayed response failures that increased to attaint 44% after a repetitive stimulation of 55 s (Fig. 1E and F). In contrast, S1R$^{E102Q}$#1/#9 flies presented much more response failures as compared to control. Whereas less than 10% of stimulation failed to elicit evoked response at 25 s for control flies, this proportion already reached 41% in S1R$^{E102Q}$#1/#9 flies. Similarly, higher fatigability of S1R$^{E102Q}$#1/#9 flies was observed when they were stimulated at 20 Hz (Supplementary Material, Fig. S3). Our data demonstrate that S1R$^{E102Q}$ perturbs NMJ functioning, resulting in a higher fatigability during high energy demand.

## S1R$^{E102Q}$ interferes with *Drosophila* eye development

Compound eye morphology in adult *Drosophila* is commonly used to evaluate the impact of pathological genes involved in neurodegenerative defects. At 25°C, no modification in the regular arrangement of ommatidia was detected in flies expressing one S1R$^{E102Q}$ transgene alone or both S1R$^{E102Q}$#1/#9 transgenes together in neurons (Data not shown). However, when flies were reared at 29°C, eye structure was morphologically aberrant for flies expressing S1R$^{E102Q}$#1 transgene alone or both transgenes (Fig. 2A). Flies exhibited a rough eye phenotype with 100% penetrance. In contrast, flies expressing S1R$^{E102Q}$#9 or S1R$^{WT}$#2 did not exhibit eye malformation. Again the impact of mutant S1R on eye development seems to depend on the expression level of the mutant allele, S1R$^{E102Q}$ transgene being more expressed in S1R$^{E102Q}$#1 line than in S1R$^{E102Q}$#9 line (Supplementary Material, Fig. S1A and B). Observations by scanning electron microscopy indicate that S1R$^{E102Q}$ overexpression resulted in collapsed eye with mild ommatidium fusion and bristle loss (Fig. 2B). A *Drosophila* eye develops as a regular cluster of eight photoreceptors among which only seven are visible on tangential sections (Fig. 2C). As a consequence of the eye malformation, S1R$^{E102Q}$#1/#9-expressing flies showed variable position and number of visible photoreceptors. Our findings suggest that the presence of the E102Q mutation impairs signaling pathways normally required for proper ommatidial organization. Overexpression of wild-type S1R failed to rescue the rough eye phenotype of flies expressing S1R$^{E102Q}$#1 transgene (Fig. 2D).

## S1R$^{E102Q}$ induces mitochondrial fragmentation and ATP depletion

We determined whether S1R$^{E102Q}$ leads to mitochondrial abnormalities in *Drosophila*. Mitochondrial shape and ultrastructure were assessed by transmission electron microscopy examination in photoreceptor neurons. Flies expressing S1R$^{E102Q}$#1/#9 at 11 days of age did not show pathological accumulation of cytoplasmic vacuoles or electron dense structures. However, mitochondria seemed overall smaller compared to control flies (Fig. 3A). Accordingly, quantitative analysis showed that the area and perimeter of mitochondria were significantly decreased in the presence of the two S1R$^{E102Q}$ transgenes (Fig. 3B and C). The mean surface was 0.27 µm$^2$ for S1R$^{E102Q}$#1/#9 flies versus 0.33 µm$^2$ for control flies.

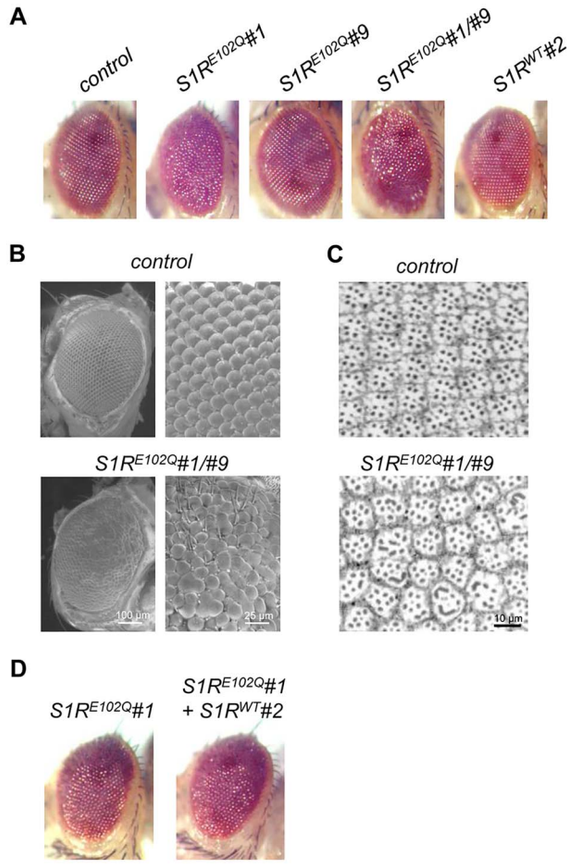


**Figure 2.** ALS-linked S1R variant alters compound eye morphology. (**A**) Photographs of eyes from 1-day-old flies expressing no transgene (control), one copy of mutant S1R variant (S1R$^{E102Q}$#1 and S1R$^{E102Q}$#9), the two copies together (S1R$^{E102Q}$#1/#9) or wild-type S1R (S1R$^{WT}$#2) in neurons. Flies were reared at 29°C. (**B**) Scanning EM photographs of eyes from control or S1R$^{E102Q}$#1/#9 flies. Overexpression of S1R$^{E102Q}$ resulted in collapsed eye with mild ommatidium fusion and bristle loss. (**C**) Semithin eye sections showing the position of photoreceptors in ommatidia of flies that express no transgene (control) or S1R$^{E102Q}$#1/#9. (**D**) Photographs of eyes from flies expressing S1R$^{E102Q}$#1 or S1R$^{E102Q}$#1 together with S1R$^{WT}$#2 in neurons.

To detect potential alteration of energy production, ATP levels were measured. We observed a 46% decrease in ATP levels in the brain of the S1R$^{E102Q}$#1/#9 flies compared to the control condition (Fig. 3D). Thus, S1R$^{E102Q}$ perturbs mitochondrial dynamics and respiration, indicating a mitochondrial dysfunction.

## Overexpressing the IP3 receptor or the glucose transporter mitigate S1R$^{E102Q}$ toxicity in eyes

In an attempt to better understand how S1R$^{E102Q}$ may be detrimental, we overexpressed one of its key partners: the IP3 receptor. In *Drosophila*, only a single copy of IP3R gene, so-called *ITPR*, is present and shares about 60% of sequence identity with mammalian IP3R isoforms. Interestingly, overexpression of wild-type ITPR (ITPR$^{WT}$) seemed to reduce the eye phenotype in flies expressing S1R$^{E102Q}$#1 (Fig. 4A–C). The coefficient of distance variation between ommatidia was quantified to evaluate the eye disorganization. While flies expressing S1R$^{E102Q}$ presented

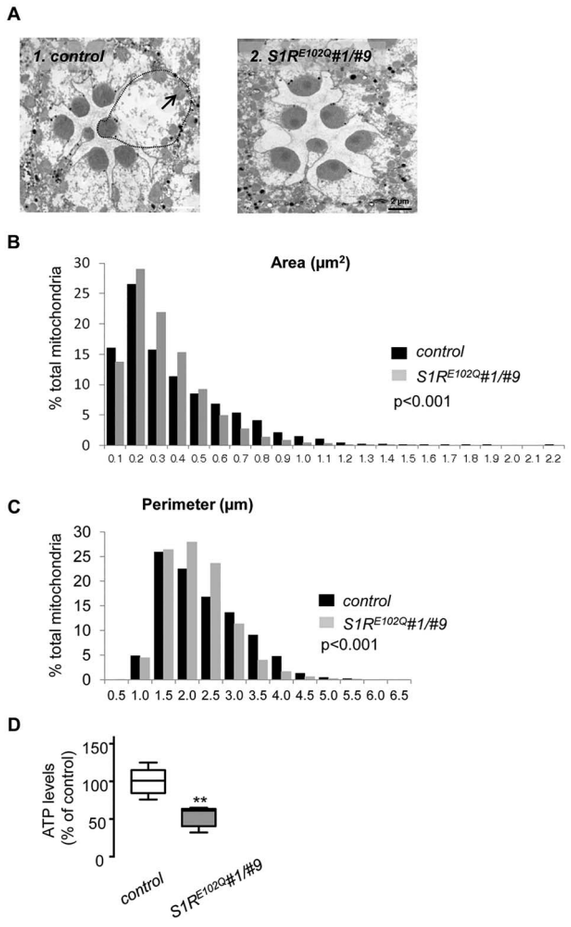


**Figure 3.** S1R$^{E102Q}$ expression alters mitochondria morphology. (**A**) Transmission EM photographs of eye sections of 12-day old flies expressing no transgene (control) or S1R$^{E102Q}$#1/#9 in neurons. Arrow: mitochondria, dotted line: one photoreceptor. (**B**, **C**) Quantitative analyses of frequency distribution of mitochondrial surface area (B) and perimeter (C) in control or S1R$^{E102Q}$#1/#9 flies. Statistical comparisons between control ($n = 2802$ mitochondria) versus S1R$^{E102Q}$#1/#9 flies ($n = 3076$ mitochondria) were performed using the Mann–Whitney *U* test ($P < 0.001$). (**D**) ATP levels of 15-day-old flies expressing no transgene (control) or S1R$^{E102Q}$#1/#9 in neurons. Mutant S1R reduces ATP levels in the brain of flies. Data from five independent experiments were averaged and presented as median and interquartile range. Statistical analysis was performed using a Mann–Whitney test (**$P < 0.01$).

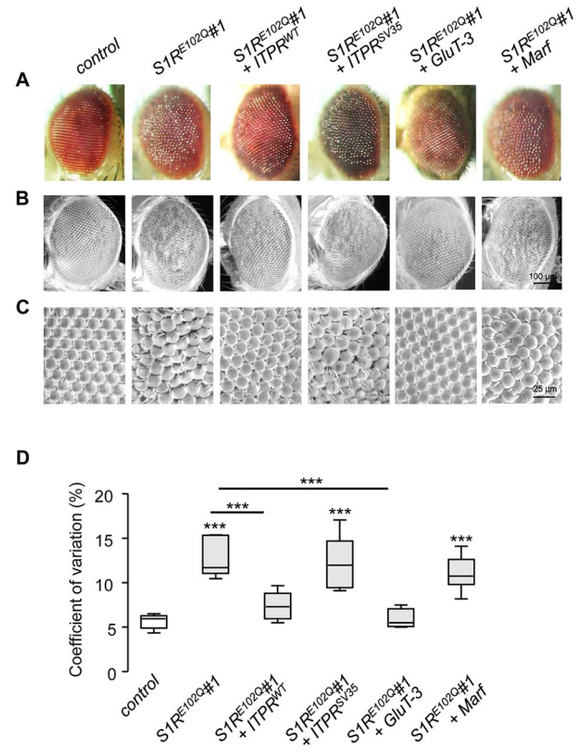


**Figure 4.** S1R$^{E102Q}$-induced eye malformation is reduced by overexpressing wild-type IP3R/ITPR or the glucose transporter GluT-3. (**A**) Photographs of eyes from flies expressing S1R$^{E102Q}$#1 alone or together with wild-type ITPR (ITPR$^{WT}$), one mutant allele of ITPR (ITPR$^{SV35}$), GluT-3 or Marf. (**B**, **C**) Scanning EM photographs of the same conditions as in (A). (**D**) Coefficient of distance variation between ommatidia of flies expressing no transgene (control), S1R$^{E102Q}$#1 alone or together with ITPR$^{WT}$, ITPR$^{SV35}$, GluT-3 or Marf. Data from at least five flies were averaged and presented as median and interquartile range. Statistical analysis was performed using ANOVA followed by Tukey's multiple-comparison test (***$P < 0.001$).

increased coefficient of variation compared to control flies, the overexpression of ITPR$^{WT}$ significantly restored it (Fig. 4D). In contrast, flies expressing S1R$^{E102Q}$#1 with a mutant allele of ITPR$^{WT}$, ITPR$^{SV35}$, showed similar eye malformation as compared to flies expressing mutant S1R alone (Fig. 4). As controls, flies with one ITPR$^{SV35}$ allele or overexpressing ITPR$^{WT}$ did not exhibit abnormal eye morphology (Supplementary Material, Fig. S4). Thus, our data indicate that increasing IP3R counteracts S1R$^{E102Q}$ toxicity in eyes. We next tested whether or not increasing glucose metabolism, as a source of energy, may influence eye phenotype. Of interest, flies that overexpressed the human glucose transporter GluT-3 in the presence of S1R$^{E102Q}$#1 had relatively normal eyes (Fig. 4A–C). In this context, ommatidia arrangement was not affected by S1R$^{E102Q}$ (Fig. 4D). Overexpressing GluT-3 by itself had no impact on eye morphology (Supplementary Material, Fig. S4). Since Mitofusin proteins are known to promote mitochondrial fusion, we overexpressed the *Drosophila* Mitofusin homolog, Marf. Unfortunately, increasing Marf failed to significantly reduce deleterious effect of S1R$^{E102Q}$ on eye morphology.

## Wild-type S1R rescues TDP43-induced pathology

TDP43 dysregulation is now considered to be involved not only in the majority of ALS cases but also in some cases suffering from frontotemporal dementia (41–43). Previous studies have reported that TDP43 mislocalization compromises respiratory chain activity and thereby ATP production (44, 45). Since S1R is supposed to regulate mitochondrial metabolism, we asked whether or not wild-type S1R may have beneficial effects on TDP43 toxicity. We previously reported that flies expressing wild-type human TDP43 (TDP43$^{WT}$) exhibited a decline in their climbing performances (46). Only 26% of flies expressing TDP43$^{WT}$ succeeded to go over the top mark at 11 days of age at 25°C (Fig. 5A). In contrast, more than 60% of flies co-expressing TDP43$^{WT}$ and S1R$^{WT}$ attained the top of the column (Fig. 5A). Similar results were observed with both independent S1R$^{WT}$ transgenes, indicating that the rescue was not due to the insertion site in the *Drosophila* genome. The impact of S1R$^{WT}$ was also evaluated in the presence of the G298S mutant ALS

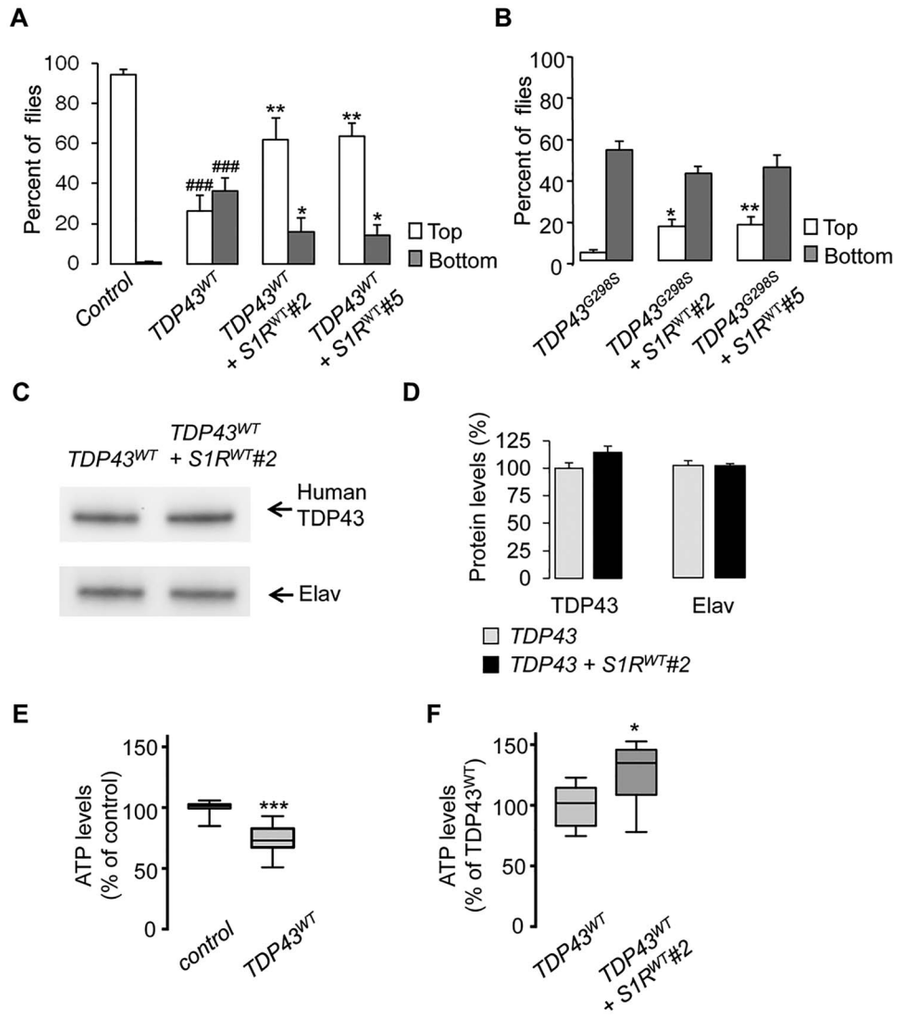


**Figure 5.** Wild-type S1R overexpression rescues climbing performances and ATP levels of flies expressing TDP43. (**A**) Climbing performances of 11-day-old flies expressing wild-type human TDP43 ($TDP43^{WT}$) alone or together with wild-type S1R (two independent transgenes: $S1R^{WT}$#2 and $S1R^{WT}$#5) in neurons. Flies were reared at 25°C. Statistical significance was assessed by ANOVA followed by Tukey's multiple-comparison test ($n = 6–7$, $^{*}P < 0.05$; $^{**}P < 0.01$ versus TDP43-expressing flies; $^{\#\#\#}P < 0.001$ versus control). (**B**) Climbing performances of 11-day-old flies expressing mutant TDP43 ($TDP43^{G298S}$) alone or with $S1R^{WT}$ in neurons. Statistical significance was assessed by ANOVA followed by Tukey's multiple-comparison test ($n = 7–9$, $^{*}P < 0.05$; $^{**}P < 0.01$ versus $TDP43^{G298S}$-expressing flies). (**C**) Western blot analysis of TDP43 protein levels from head extracts of 12-day-old flies expressing $TDP43^{WT}$ alone or with $S1R^{WT}$ in neurons. Elav protein was used as a loading control. (**D**) Densitometric measurements of TDP43 or Elav protein levels in flies expressing $TDP43^{WT}$ alone or with $S1R^{WT}$. Data are expressed in percent relative to TDP43-expressing flies. Data from four independent samples were averaged and presented as mean ± SEM. Statistical analysis was performed using a Student's *t*-test. (**E, F**) ATP levels of 15-day-old flies expressing no transgene (control), $TDP43^{WT}$ alone or together with $S1R^{WT}$ in neurons. Data from eight to nine independent experiments were averaged and presented as median and interquartile range. Statistical analysis was performed using Mann–Whitney *U* test ($^{***}P < 0.001$; $^{*}P < 0.05$).

variant of human TDP43. Flies expressing $TDP43^{G298S}$ exhibited strong locomotor deficits with less than 4% of them that could reach the top (Fig. 5B). Again, overexpressing $S1R^{WT}$ partially improved locomotor performances since 17–18% of flies co-expressing $TDP43^{G298S}$ and $S1R^{WT}$ climbed over the top mark. The presence of $S1R^{WT}$ did not modify expression levels of TDP43 (Fig. 5C and D). More importantly, while flies expressing $TDP43^{WT}$ showed a 27% decrease in ATP levels versus controls, overexpressing $S1R^{WT}$ rescued by 26% ATP levels in the presence of $TDP43^{WT}$ (Fig. 5E and F). Thus, it is tempting to propose that $S1R^{WT}$ rescued TDP43 toxicity at least in part by increasing ATP production.

## Wild-type S1R increases resistance to oxidative stress

In an attempt to better understand how $S1R^{WT}$ may be neuroprotective, we also tested the resistance of flies to oxidative stress. Flies were exposed to hydrogen peroxide ($H_2O_2$) and survival rates were evaluated over 4 days. The presence of 20% $H_2O_2$ during 4 days reduced survival of control flies to 5%. Overexpressing $S1R^{WT}$ in neurons strongly enhanced resistance to $H_2O_2$ with 39% of flies still alive at 4 days (Fig. 6A and B). Paraquat is known to induce $O_2^{-\cdot}$ superoxide anion production through the mitochondrial respiratory chain. Control flies exposed to 13 mM paraquat drastically died, reaching 22% of flies alive after 23 h

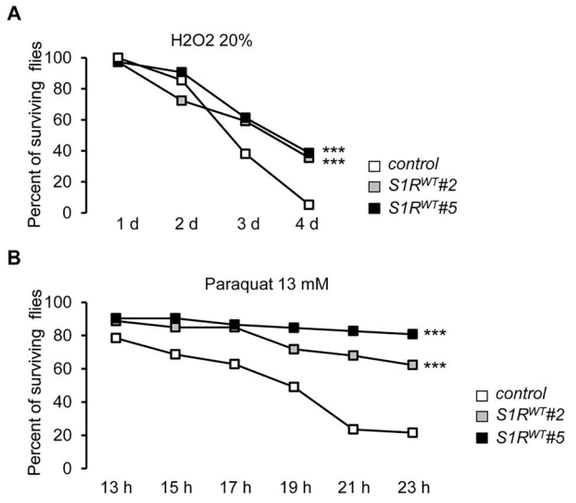


**Figure 6.** SIR overexpression protects from oxidative stress. (**A**) Survival rate of flies expressing or not $S1R^{WT}$ in the presence of 20% hydrogen peroxide. Data are presented as percent of surviving flies. Statistical analysis was performed using log-rank test ($P < 0.01$). (**B**) Survival rate of flies expressing or not $S1R^{WT}$ in the presence of 13 mM paraquat. Data are presented as percent of surviving flies. Statistical analysis was performed using log-rank test ($P < 0.001$).

of exposure. In contrast, 62–81% of flies expressing one $S1R^{WT}$ transgene were still alive after 23 h (Fig. 6C). Altogether, our results demonstrate *in vivo* that $S1R^{WT}$ improves resistance to oxidative stress.

## Discussion

The purpose of this study was to use genetic tools in *Drosophila* to provide direct evidence of the importance of S1R in ALS. For the first time, we demonstrate that expression of S1R carrying the E102Q mutation is sufficient to induce toxicity *in vivo*. $S1R^{E102Q}$ alters *Drosophila* photoreceptor organization, spontaneous walking behavior and startle-induced climbing response. At cellular levels, this is accompanied by mitochondrial fragmentation and ATP depletion, indicating that $S1R^{E102Q}$ challenges mitochondrial function. Additionally, we demonstrate that increasing wild-type S1R confers neuroprotection against one of the major ALS proteins, TDP43. We found that $S1R^{WT}$ ameliorates climbing performances of TDP43-expressing flies. This likely results from a better production of mitochondrial ATP as well as increased resistance to oxidative stress. Altogether, these findings highlight the complex role of S1R in ALS pathogenesis, inducing the disease when mutated or protecting from it in its wild-type form.

So far, S1R mutations have been mainly related to motor neuron diseases, including ALS and distal hereditary motor neuropathy (29–34). Whereas S1R is expressed throughout the nervous system, the highest levels are found in motor neurons of the brainstem and spinal cord (11, 47, 48). This specific expression pattern may explain the higher vulnerability of motor neurons. Neuronal functioning and health mainly depend on mitochondrial ATP due to their high energy demand. This is especially true for motoneurons that have particularly long axons and require energy for action potential and synaptic transmission. We here demonstrate *in vivo* that the presence of $S1R^{E102Q}$ induces ATP stock reduction. This is accompanied by a higher fatigability during a repetitive stimulation that requires high energy. Reduced ATP mitochondrial production was also previously reported in $S1R^{E102Q}$-transfected cells (3, 49). Altogether, these observations suggest that altered energetic metabolism plays a critical role in $S1R^{E102Q}$-induced phenotype. In accordance with this view, we found that increasing glucose metabolism prevents eye malformation in flies expressing $S1R^{E102Q}$. Mitochondrial calcium homeostasis and oxidative phosphorylation are closely linked. $S1R^{E102Q}$ may perturb calcium mobilization between ER and mitochondria through IP3R. Accordingly, while expression of $S1R^{WT}$ in transfected cells stimulated IP3R-mediated calcium transport, $S1R^{E102Q}$ resulted in reduced calcium mobilization upon stimulation of IP3R (3, 49). The rescuing effect we observed when *Drosophila* IP3R was overexpressed is also in line with this hypothesis. Moreover, we also discovered that $S1R^{E102Q}$ alters mitochondrial dynamics with a tendency of mitochondria towards fragmentation. Conversely, overexpression of $S1R^{WT}$ was reported to promote mitochondrial elongation in transfected cells (50). Balance between fusion and fission events is essential to maintain mitochondrial functional integrity and cell survival during stress (51). Expanded mitochondrial network is preferred in respiratory active cells where optimal mitochondrial function is needed. Fragmented mitochondria are more observed in resting cells and represent an abnormal morphological state during high energy demand (52). We found that *Drosophila* Mitofusin/Marf overexpression failed to rescue mutant $S1R^{E102Q}$ toxicity in *Drosophila* eye. This suggests that mitochondrial fragmentation may be a secondary event due to mitochondrial dysfunction. Mitochondrial fission is known to facilitate elimination of mitochondria when they are irreversibly damaged (53). Whether or not mitophagy is affected by $S1R^{E102Q}$ remains to be clarified. Both enhanced and defective autophagy were reported in $S1R^{E102Q}$-transfected cells (3, 49). Nonetheless, $S1R^{E102Q}$ might induce energy stock reduction due to abnormal ER-mitochondria calcium crosstalk.

Considering the recessive mode of the disease inheritance (33), $S1R^{E102Q}$-induced alterations could be attributed to a loss of function. Accordingly, S1R knockout mice display locomotor alterations but they are subtle (11, 54, 55). A combination with a gain of toxic functions of $S1R^{E102Q}$ cannot be excluded. Overexpressing $S1R^{E102Q}$ protein is found deleterious in transfected cells, and this even for MCF-7 cancer cells that express very little or no S1R (3, 49, 56). We here demonstrate that overexpressing human $S1R^{E102Q}$ in flies is neurotoxic in a dose-dependent manner. To our knowledge, a S1R homolog has not been found in *Drosophila*, while most of S1R key partners or S1R-regulated signaling pathways are conserved in this species (57–62). Moreover, overexpression of wild-type S1R fails to counteract $S1R^{E102Q}$-rough eye phenotype. Taken together, these observations argue the existence of a concomitant toxic gain and loss of function of $S1R^{E102Q}$.

S1R agonist administration was previously reported to partially alleviate ALS pathology in animal models. Most observations were performed on mutant SOD1-expressing murine models (63, 64). Administration of S1R agonist pridopidine to mutant SOD1 mice reduces mutant SOD1 aggregation and preserves NMJ and muscle wasting (64). Conversely, knockdown of S1R enhanced $SOD1^{G93A}$ or $SOD1^{G85R}$ mouse pathology (34, 65). To our knowledge, the potential benefits of S1R on TDP43-induced toxicity remained to be demonstrated. For the first time, we here provide evidence of a neuroprotective role of S1R in TDP43-induced pathology. We show direct genetic demonstration that S1R confers benefits by ameliorating ATP production. TDP43 was previously reported to impair OXPHOS complex 1 activity (44, 45). Interestingly, brain mitochondria of mice treated with S1R agonists showed an increased activity of respiratory

complex 1 (66). As a possible mechanism, S1R adapts calcium transfer from ER into mitochondria and thereby modulates intra-mitochondrial dehydrogenases, including pyruvate, isocitrate or $\alpha$-ketoglutarate dehydrogenases (67). The activation of these enzymes is considered important to generate NADH cofactor, which stimulates the respiratory complex 1 and hence ATP supply under conditions of increased ATP demand (68). Enhancing glucose energy metabolism was also previously reported to prevent TDP43-induced phenotype in *Drosophila* (69), further emphasizing the importance of energy metabolism in TDP43-induced toxicity. As another possibility, mitochondria require cholesterol to function and S1R is known to influence cholesterol biosynthesis and transport to mitochondria (70). Moreover, we showed that flies overexpressing S1R had better resistance to free radicals. S1R was reported to enhance ER–nuclear interaction to respond to oxidative stress through IRE1-X-box binding protein 1 (XBP1) signaling pathway (4). Additionally, S1R activation induces NRF2 expression and reduces oxidative stress (5, 6). So far, increased NRF2 signaling was only reported in retinal cells and further analyses are needed to extend this observation to other cell types. However, since S1R can interact with numerous other proteins, neuroprotection by S1R chaperone may simply result from a more broad impact to prevent protein misfolding in stress conditions (71). In particular, in the mammalian spinal cord, S1R is strongly localized in the ER at the postsynaptic subsurface cisternae of cholinergic C-terminals (11, 12). The C-terminal postsynaptic membrane is particularly enriched in hyperpolarization channels like potassium Kv2.1 and SK. S1R might thus protect motoneurons by interacting with those channels and thereby reducing excitability. More works are needed to elucidate how S1R confers protection in *Drosophila* models of ALS.

In conclusion, for the first time, we demonstrated *in vivo* that $S1R^{E102Q}$ is neurotoxic, leading to mitochondrial dysfunction. We also provide the proof of concept by direct genetic approaches that S1R influences TDP43 toxicity, then highlighting the therapeutic value of S1R in ALS.

## Materials and Methods

### *Drosophila* strains

Flies were reared on a standard agar medium containing cornmeal and yeast at either 25 or 29°C as mentioned in the text. Flies carrying UAS-$TDP43^{WT}$ were generated as previously described (46). $Elav^{C155}$-GAL4 driver line, ITPR mutant flies ($ITPR^{SV35}$; BL30740) were obtained from Bloomington *Drosophila* stock center (BDSC, Bloomington, Indiana). UAS-$ITPR^{WT}$ (72) flies were a gift from Dr Gaiti Hasan (National Centre for Biological Sciences, Bangalore, India). Flies expressing the human neuronal glucose transporter GLUT3 were previously generated (73). Flies carrying UAS-Marf were a gift from Pr H. Bellen (Baylor College of Medicine). In accordance with the genetic background, $w^{1118}$ flies were used to generate controls ($w^{1118}$/$Elav^{C155}$-GAL4). All analyses were performed on female *Drosophila*.

### *Drosophila* S1R generation

The cDNA encoding human wild-type S1R was initially inserted in pCI-neo vector (generous gift from Dr Timur Mavlyutov, University of Wisconsin Medical School, Madison, Wisconsin). After digestion by *Eco*R1 and *Xho*I restriction enzymes, the purified S1R fragment was inserted between the *Eco*R1 and *Xho*I sites of the pUAST plasmid. The G304C mutation was generated by using Quickchange mutagenesis accordingly to the manufacturer's instructions (Agilent Technologies, Santa Clara, California). Selected clones were checked by DNA sequencing (Genewiz, Leipzig, Germany). Then purified plasmids were used for germ line-mediated P-element transformation by BestGene Inc. (Chino Hills, California). AQ8

### Climbing performances

Fly climbing performances were determined using the reflex of flies to walk against gravity (negative geotaxis). Eight flies were placed in a clean plastic column (1.3 cm diameter). After being gently tapped, flies that remained at the bottom or crossed the top mark at 22 cm were counted after 1 min. The test was repeated three times for each batch of flies. The data are the mean of at least five trials and are expressed as percent ± SEM of the total number of flies.

### Spontaneous walking activity

Eight flies were placed overnight in a petri dish (9 cm diameter) containing a solid agarose/6% sucrose medium in order to allow flies to walk only in the horizontal plane. The next day flies were recorded with a digital camera and locomotor activity was measured using the Videotrack software (Viewpoint, Lyon, France). Data are the mean of three repeated recordings of 15 min from at least five trials.

### Western blot analysis

Heads of flies ($n = 5$) were dissected and homogenized in 65 µl RIPA lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Igepal CA-630) containing cOmplete™ protease inhibitor cocktail (Merck, Darmstadt, Germany). Following a 1-min centrifugation, 1/4 (v/v) sample Laemmli buffer was added to supernatants. Total proteins were separated through a 4–15% Mini-Protean® TGX resolving gel (Bio-Rad, Hercules, California). Then proteins were transferred to nitrocellulose membranes (Amersham™, Merck). Membranes were blocked for 1 h in the blocking solution (1X PBS, 0.1% Tween 20, 5% dry milk) and incubated overnight with primary antibodies at 4°C. The following primary antibodies were used: rabbit anti-S1R antibody (1/200, HPA018002, Merck), rabbit anti-TDP43 antibody (1/1000, 10 782-2-AP, Proteintech, Rosemont, Illinois), rabbit anti-translocase of the outer mitochondrial membrane 20 kDa (Tom20) antibody (1/1000, sc-11 415, Santa Cruz Biotechnology, Dallas, Texas), rat anti-Elav antibody (1/700, 7E8A10, Developmental Studies Hybridoma Bank, Iowa City, Iowa). Secondary peroxidase-conjugated antibodies (1/5000, Jackson ImmunoResearch, Cambridge, UK) were incubated for 2 h in blocking solution. Chemiluminescence was revealed by using the Clarity™ Western ECL Blotting substrates (Bio-Rad) and the ChemiDoc$^2$ Touch Imaging System (Bio-Rad). Quantitative analysis was performed using ImageJ software.

### Electron microscopy

Adult fly heads were fixed in 0.1 M sodium cacodylate containing 2% paraformaldehyde and 2.5% glutaraldehyde and 5 mM $CaCl_2$ for 1 h at RT and overnight at 4°C. Postfixation was performed in 0.1 M sodium cacodylate containing 2% glutaraldehyde and 0.8% osmium tetroxide for 2 h at 4°C.

For observation by scanning electron microscopy, samples were then progressively dehydrated in 30–100% ethanol and dried using HMDS (*hexamethyldisilazane*). Observations were performed on the FEI Quanta 200 FEG microscope at 10 kV. The distance between ommatidia was evaluated from 49 ommatidia per fly by using ImageJ software. The coefficient of distance variation was defined as the ratio of the standard deviation to the mean for each fly.

For transmission electron microscopy, fixed heads were stained in 2% uranyl acetate, dehydrated and included in Epon resin. Semithin and ultrathin sections were prepared using an UC7 ultramicrotome (Leica, Nussloch, Germany). Semithin sections were stained in Toluidine blue and washed in water. Examination on transmission electron microscopy was performed using the JEOL 1400 Plus at 80 kV. Quantitative analysis was achieved by using the ImageJ software on five flies for each condition. At least 100 photoreceptors per animal and 500 mitochondria per animal were examined.

### ATP measurement

ATP measurements were essentially performed according to the manufacturer's instructions (Luminescent ATP Detection Assay Kit from Abcam, Cambridge, UK). For each experiment, five adult fly brains were dissected and homogenized. Luminescence was measured with a Centro LB 960 luminometer (Berthold Technologies, Calmbacher, Germany). Data from at least five independent experiments were averaged and were presented as mean ± SEM. Statistical analysis was performed using a Student's *t*-test.

### Electrophysiological recordings

Neuromuscular responses were recorded on 15-day-old adult flies as previously described (74). Briefly, flies were glued on a needle under $CO_2$ anesthesia. A bipolar tungsten electrode was introduced into the head to stimulate the giant fiber circuit. An Ag/AgCl reference electrode was placed into the abdomen. Borosilicate glass micropipettes filled with 3 M KCl were used to impale fibers of the dorsal longitudinal indirect flight muscles. Stimulation was performed by a Grass S88 Stimulator (GRASS Instruments, West Warwick, Rhode Island). Recordings were made with an Intracellular Electrometer IE-210 amplifier (Warner Instruments, Hamden, Connecticut) connected to a PowerLab 4/35. Recordings were digitized at 20 KHz frequency. Stimuli at 15 and 20 Hz were delivered to the brain during a period of 60 s at 15 V (0.1 ms pulse duration). Analysis was achieved by using LabChart 8 software (ADInstruments, Sydney, Australia). Data were averaged and presented as mean ± SEM. Statistical analysis was performed using a repeated measures analysis of variance test.

### Oxidative stress resistance

Adult *Drosophila* was tested at 25°C for resistance to $H_2O_2$ (Merck) or paraquat dichloride hydrate (Merck) treatment. Flies ($n > 40$) were placed into vials containing Whatman paper pieces saturated with $H_2O_2$ (20%) or paraquat (13 mM) in 2% sucrose. Numbers of dead flies were recorded after the beginning of the treatment. Statistical analysis was performed using the Log rank test.

## Supplementary Material

Supplementary Material is available at HMG online.

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## Conflict of interest

The authors have no actual or potential conflicting or financial interest to disclose.

## Authors' contributions

J.-C.L. and T.M. initiated the project. J.C.L. supervised the project. S.C., B.K., V.V., J.R. and J.-C.L. carried out the experiments. S.C., B.K. and J.-C.L. analyzed the data. S.C., B.K., T.M. and J.-C.L. wrote the manuscript. All authors read and approved the final manuscript.

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