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A dopamine receptor contributes to paraquat-induced neurotoxicity in *Drosophila*

Marlène Cassar[1,†], Abdul-Raouf Issa[1,†], Thomas Riemensperger[1,†,‡], Céline Petitgas[1], Thomas Rival[2], Hélène Coulom[2], Magali Iché-Torres[2], Kyung-An Han[3] and Serge Birman[1,2,*]

[1]Genes Circuits Rhythms and Neuropathologies, Brain Plasticity Unit, CNRS, PSL Research University, ESPCI ParisTech, 10 rue Vauquelin, 75005 Paris, France, [2]Genetics and Physiopathology of Neurotransmission, Developmental Biology Institute of Marseille-Luminy, CNRS, Université de la Méditerranée, 13009 Marseille, France and [3]Department of Biological Sciences, Border Biomedical Research Center, University of Texas at El Paso, El Paso, TX 79968, USA

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Long-term exposure to environmental oxidative stressors, like the herbicide paraquat (PQ), has been linked to the development of Parkinson's disease (PD), the most frequent neurodegenerative movement disorder. Paraquat is thus frequently used in the fruit fly *Drosophila melanogaster* and other animal models to study PD and the degeneration of dopaminergic neurons (DNs) that characterizes this disease. Here, we show that a D_1-like dopamine (DA) receptor, DAMB, actively contributes to the fast central nervous system (CNS) failure induced by PQ in the fly. First, we found that a long-term increase in neuronal DA synthesis reduced *DAMB* expression and protected against PQ neurotoxicity. Secondly, a striking age-related decrease in PQ resistance in young adult flies correlated with an augmentation of *DAMB* expression. This aging-associated increase in oxidative stress vulnerability was not observed in a *DAMB*-deficient mutant. Thirdly, targeted inactivation of this receptor in glutamatergic neurons (GNs) markedly enhanced the survival of *Drosophila* exposed to either PQ or neurotoxic levels of DA, whereas, conversely, *DAMB* overexpression in these cells made the flies more vulnerable to both compounds. Fourthly, a mutation in the *Drosophila* ryanodine receptor (*RyR*), which inhibits activity-induced increase in cytosolic Ca^{2+}, also strongly enhanced PQ resistance. Finally, we found that *DAMB* overexpression in specific neuronal populations arrested development of the fly and that *in vivo* stimulation of either DNs or GNs increased PQ susceptibility. This suggests a model for DA receptor-mediated potentiation of PQ-induced neurotoxicity. Further studies of DAMB signaling in *Drosophila* could have implications for better understanding DA-related neurodegenerative disorders in humans.

INTRODUCTION

Oxidative stress, i.e. the uncontrolled accumulation of reactive oxygen species, plays a central role in aging and major age-related human diseases such as cancer (1), heart failure (2), neurodegenerative syndromes (3,4) and, specifically, Parkinson's disease (PD) (5–8). Parkinson's disease is a multifactorial movement disorder resulting from the dysfunction and loss of dopaminergic neurons (DNs) in the midbrain substantia nigra pars compacta (SNpc) (9–11). No treatment is currently known that could halt or slow the progression of neurodegeneration in this disorder. Parkinson's disease and related diseases are generally sporadic, i.e. without obvious heritability, but familial cases have been described. Several of the ~15 PARK genes whose mutations cause various forms of Parkinsonism were shown to be involved in mitochondrial quality control and oxidative stress pathways (7,12). Oxidation of dopamine (DA) itself, or of its metabolites, produces reactive radicals and quinones that can damage cells and interact with PD-related proteins (13–15). This combined with the physiological features of the SNpc DNs,

*To whom correspondence should be addressed. Tel: +33 140795835; Fax: +33 140794757; Email: serge.birman@espci.fr
†M.C., A.-R.I. and T.R. contributed equally to this work.
‡Present address: Molecular Neurobiology of Behaviour, Johann Friedrich Blumenbach Institute, Georg-August-University of Goettingen, Schwann-Schleiden Research Center, Julia-Lermontowa Weg 3, 37077 Goettingen, Germany.

including prominent Ca^{2+} entry through L-type channels, could account for the specific vulnerability of these cells in PD (6,16,17).

Human epidemiological studies showed that long-term exposure to environmental free-radical generators, such as the herbicide paraquat (PQ, 1,1′-dimethyl-4−4′-bipyridinium) or the insecticide rotenone, is a risk factor for the development of PD (18−21). Although the molecular mechanisms of cytotoxicity of PQ and rotenone appear distinct and have not been fully established *in vivo* (22−24), they are known to increase superoxide ion production by mitochondria and generate oxidative damage (23,25,26). These two neurotoxicants have thus been frequently used to mimic PD and study oxidative stress-induced motor deficits and dopaminergic neurodegeneration in animal models such as rodents (22,27−31) and *Drosophila* (32−38). Melatonin, a potent hydroxyl radical scavenger, efficiently protects the flies against these pesticides (32,39).

In *Drosophila*, DA is both an essential neuromodulator and a precursor of molecules required for hardening and pigmentation of the external cuticle (40,41). DA released from fly neurons interacts with specific G protein-coupled DA receptors, either of the D_1 or D_2 subtypes (42,43), including D_1-like dDA1 and DAMB, which play key roles in arousal and memory (44,45). Here, we show that long-term overexpression of the DA-synthesizing enzyme tyrosine hydroxylase (TH) in the nervous system prolongs the survival of adult PQ-intoxicated flies, and we provide evidence that enhanced PQ resistance results from a down-regulation of the DAMB receptor. Moreover, an augmentation of *DAMB* expression appears to underlie the age-related increase in PQ susceptibility of young adult flies. This suggests that DA signaling modulates oxidative stress resistance in the *Drosophila* nervous system. From these and further results, we propose a model for PQ neurotoxicity in which CNS disturbance would result from the self-reinforcing effects of PQ-induced oxidative stress and a noxious intracellular Ca^{2+} signaling mediated by DA receptor overactivation.

RESULTS

Long-term TH overexpression protects against PQ neurotoxicity

Survival of the wild-type female *Drosophila* fed with 20 mM PQ diluted in a 2% sucrose solution was at most 72 h, with ∼60% of the flies apparently dead after 24 h (Fig. 1A). Because dietary PQ strongly inhibits food intake in flies (46), we alternatively applied the drug directly onto the anterior notum of decapitated adult *Drosophila*, close to the ventral nerve cord (VNC), in order to bypass feeding and digestive tract absorption. Headless flies survive well up to 3 days when conserved in a moist environment at 25°C. They maintain a normal standing posture, a vigorous righting response, and they can respond to mechanical or pharmacological stimulations with grooming and coordinated locomotion (47−50). We observed that death occurred more rapidly when PQ was directly applied to the VNC, compared with oral ingestion. A single 5-s application of a drop of 20 or 80 mM PQ diluted in Ringer's solution killed 60−70% of the decapitated flies in ∼6 and 2 h, respectively (Fig. 1B). No significant difference in PQ vulnerability was observed among various control strains in these conditions (Supplementary Material, Fig. S1). Ingested PQ induced characteristic morphological alterations of brain DNs, whose cell bodies became smaller and rounded, suggestive of an entry of these cells into apoptosis, as shown in Figure 1C for the PPL2 cluster. Similar changes in neuronal morphology were previously described for the PAL, PPL1, and PPM2 clusters (33). Direct application of PQ also altered dopaminergic neuron structure in the VNC, leading, after 2 h, to a severe reduction or loss of their axonal varicosities, which are normally widespread in the ganglion neuropil of untreated flies (Fig. 1D and E).

In *Drosophila*, as in mammals, the TH enzyme is specifically required for DA production in the CNS (41) (Supplementary Material, Fig. S2). To test for an effect of increased DA biosynthesis on PQ susceptibility, we overexpressed genomic *Drosophila TH* (*DTHg*) from the late embryonic stage and thereafter in DNs, under transcriptional control of the *TH-GAL4* driver (51). Resistance of these *TH>THg* flies to PQ intoxication was significantly enhanced compared with the wild type, leading to increased survival rates at 24 h after drug ingestion and 2 h after direct application to the VNC (Fig. 2A and B). Because serotonergic neurons express dopa decarboxylase (Ddc), the second enzyme in the DA biosynthesis pathway (Supplementary Material, Fig. S2), ectopic expression of *DTHg* can force these cells to produce DA. Surprisingly, TH expression selectively in serotonergic neurons with the *TRH-GAL4* driver also resulted in significant protection against PQ (Fig. 2A and B). The survival rate of PQ-ingested *TRH>THg* flies at 24 h was double that of the wild type (Fig. 2A). Thus, enhanced PQ resistance in adult flies can result from a long-term increase in DA synthesis either in DNs or in serotonergic neurons, suggesting that a critical factor for neuroprotection is not the DA level in DNs but rather the amount of DA released in the CNS.

Enhanced PQ tolerance correlates with down-regulation of the DA receptor DAMB

Released DA interacts with specific membrane receptors. We tested whether this interaction plays a role in the control of PQ neurotoxicity. DA receptor antagonists were applied together with PQ to the nerve cord of decapitated *Drosophila*. Application of either SKF-83959 or SKF-83566, two specific antagonists of the D_1 receptor subtypes, markedly enhanced resistance of the flies against PQ. Ninety minutes after PQ application, the survival rate of headless flies increased around twofold in the presence of these antagonists compared with controls treated with PQ alone (Fig. 2C). In contrast, eticlopride, a specific antagonist of D_2 receptors previously shown to be effective in *Drosophila* (47), had no significant effect on PQ toxicity (Fig. 2C). Therefore, D_1 DA receptor antagonists can efficiently protect against PQ poisoning in *Drosophila*.

Two D_1-like receptors are known in *Drosophila*: dDA1 and DAMB. We determined the PQ resistance of $Damb^1$, a viable null *DAMB* mutant, and $dumb^2$, a hypomorphic dDA1 mutant (Fig. 2D). After 2 h of PQ poisoning, the survival rate of decapitated $Damb^1$ flies was found to be ∼2.5 times higher than that of the wild-type Canton S (CS) flies. In contrast, survival of $dumb^2$ flies was similar to CS. $Damb^1$ flies were also much more resistant than the wild type when PQ intoxication was carried out by dietary ingestion (Fig. 2E). The comparable stress-protective effects of *DAMB* inactivation and long-term enhanced DA suggested that DAMB expression could be altered in a compensatory manner by TH overexpression. To test this hypothesis, we

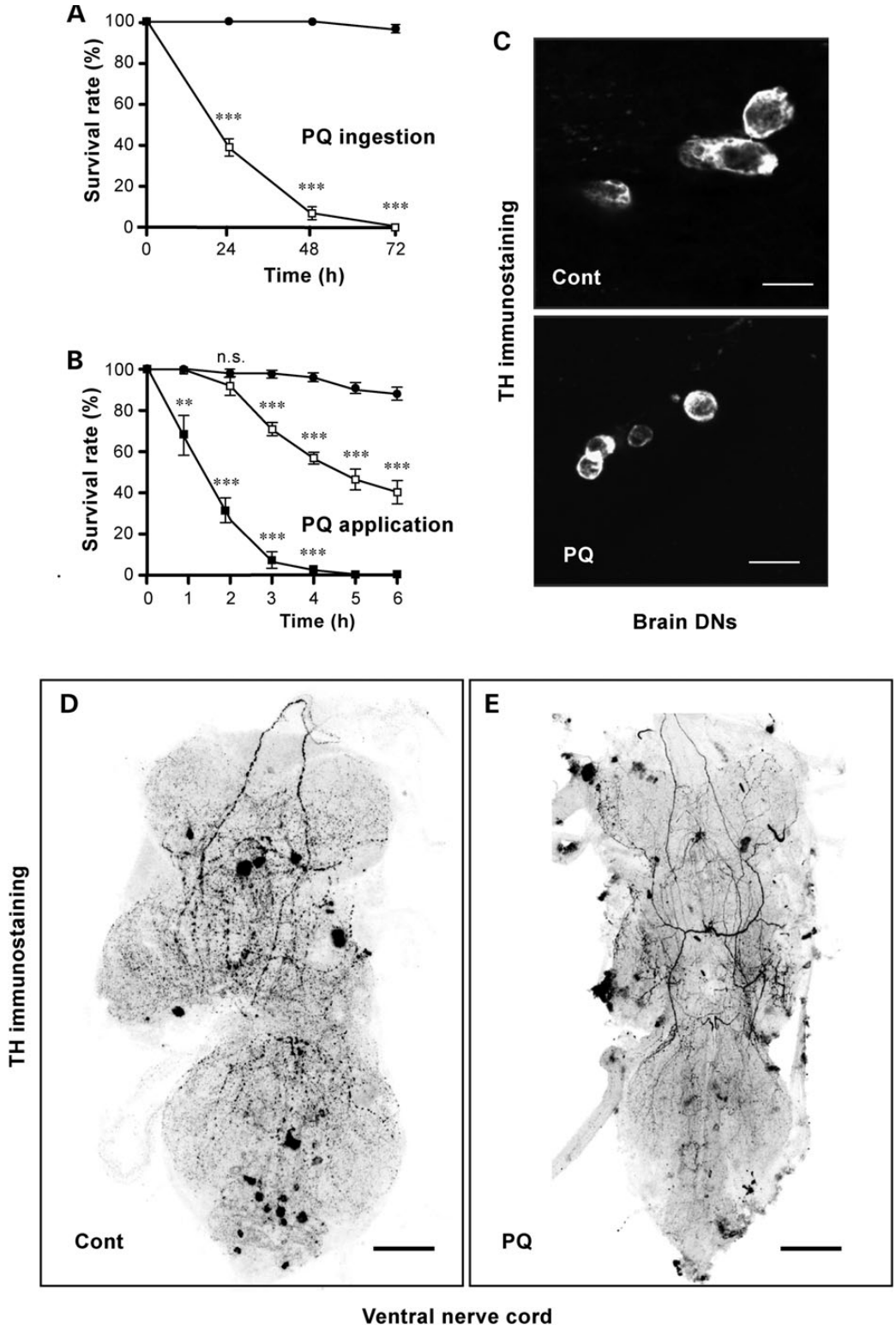

Figure 1. Paraquat-induced lethality and morphological alterations of *Drosophila* DA neurons. (**A** and **B**) Survival rate of wild-type Canton-S *Drosophila* either fed with a sucrose solution containing PQ (dietary ingestion) (A) or after a 5-s application of the drug diluted in Ringer's solution to the VNC of decapitated flies (direct application) (B). PQ concentrations were as follows: 0 (closed circles), 20 (open squares), or 80 mM (closed squares). (**C**) Structure of brain DNs from wild-type *Drosophila* fed for 24 h on a sucrose solution containing either no PQ (control, upper panel) or 20 mM PQ (lower panel). Brains were then dissected and stained with anti-TH antibodies. The pictures show part of the dorsolateral posterior protocerebral 2 (PPL2) neuronal cluster. Dopaminergic cell bodies of PQ-treated flies appeared smaller and rounder with shrunken nuclei. (**D** and **E**) Whole-mount VNC of decapitated *Drosophila* dissected 2 h after a 5-s application of either Ringer's alone (control) (D) or 80 mM PQ (E) and then immunostained for TH. Confocal projections were converted into negative images to improve visibility. DN axonal varicosities that are widespread in the VNC of control flies appear to be much reduced in size or lost (98.4 $\pm$ 0.005%) in PQ-treated VNC. Scale bars: (C) 10 μm, (D and E) 50 μm.

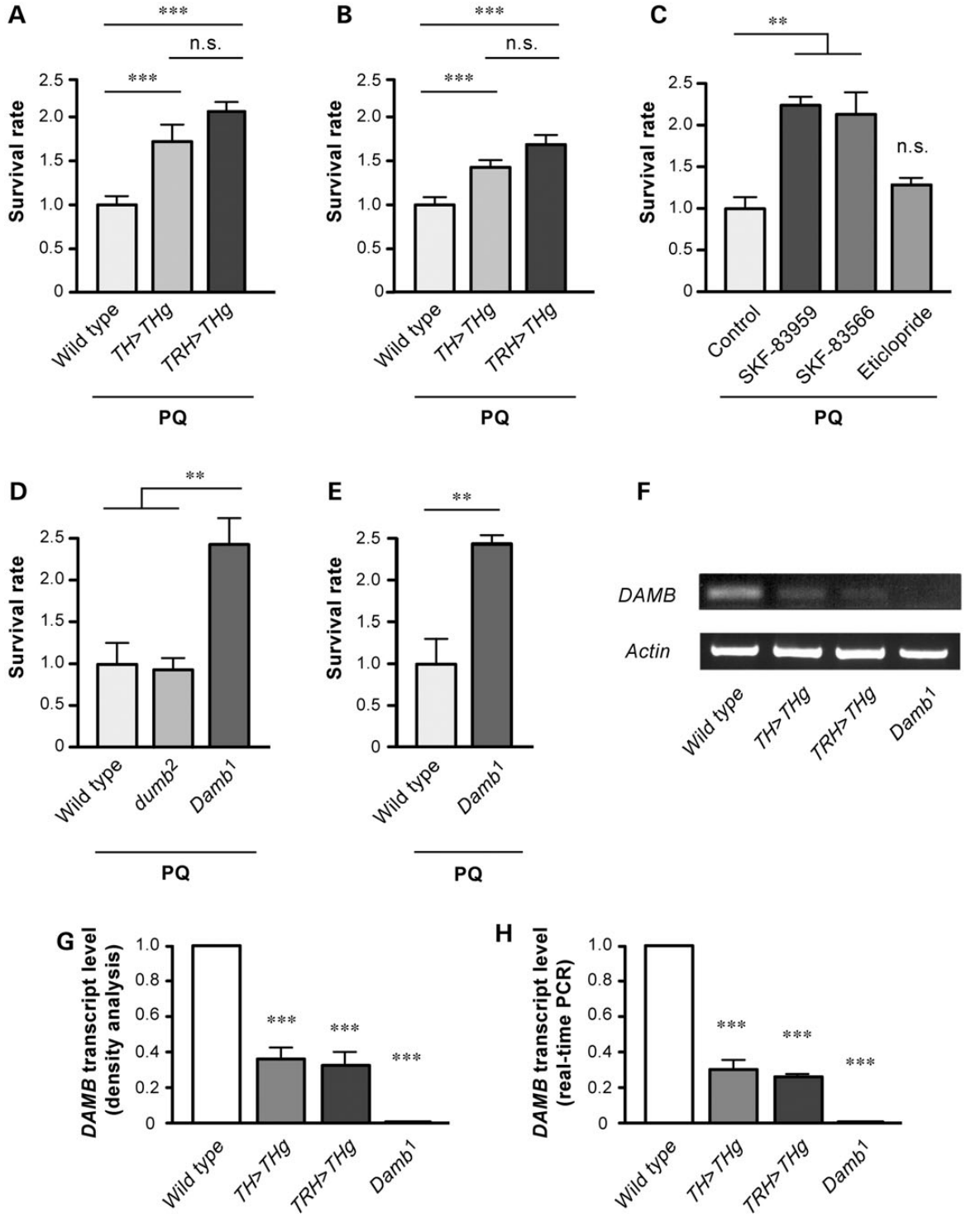

Figure 2. Down-regulation of the DA receptor DAMB protects *Drosophila* against PQ-induced oxidative stress. (**A**) Survival rate of wild-type flies fed for 24 h with sucrose plus 20 mM PQ, compared with *TH-GAL4*; *UAS-DTHg* flies overexpressing *TH* in DNs (*TH>THg*), and *TRH-GAL4*; *UAS-DTHg* flies ectopically expressing *TH* in serotonergic neurons (*TRH>THg*). (**B**) Survival rate of decapitated flies of similar genotypes as in (A) 2 h after a 5-s application of 80 mM PQ dissolved in Ringer's to the VNC. (**C**) Effect of DA receptor antagonists. Survival rate of decapitated flies was monitored 1 h 30 min after brief application of a drop of Ringer's solution containing either PQ alone (control) or PQ plus D_1 antagonists SKF-83959 or SKF-83566, or D_2 antagonist eticlopride. Protection was observed with the D_1-specific antagonists. (**D**) PQ susceptibility of decapitated D_1-like DA receptor mutants. Whereas the survival rate of *dumb*^{2} is comparable with wild type, the *Damb*^{1} strain shows higher resistance to PQ. (**E**) Survival of intact *Damb*^{1} flies after PQ ingestion is similarly increased compared with wild type. In (A)–(E), values were normalized to the mean survival rate of wild-type controls. (**F**–**H**) TH overexpression decreases the *DAMB* transcript level in adult flies. (F) RT-PCR from head RNAs (30 cycles of amplification) showed reduced level of *DAMB* transcripts, compared with wild type, after long-term *TH* overexpression in dopaminergic (*TH>THg*) or serotonergic (*TRH>THg*) neurons, and their absence in the *Damb*^{1} mutant. Density analysis of RT-PCR products (G) or real-time PCR experiments (H) yielded similar quantitative results.

measured the *DAMB* transcript level in adult heads of flies over-expressing TH in dopaminergic or serotonergic neurons: these transgenic flies showed marked down-regulation of *DAMB* expression to ~30% of the wild type, and no *DAMB* mRNA was detected in *Damb*^{1} mutants (Fig. 2F–H). Two independent sets of experiments using different techniques, i.e. density analysis of RT-PCR products (Fig. 2G) and real-time PCR (Fig. 2H), showed similar quantitative results. This strengthens the conclusion that DAMB is down-regulated in adult flies when TH is over-expressed in dopaminergic or serotonergic neurons and that the *DAMB* transcript is absent in *Damb*^{1} mutants (as also shown in Fig. 4B). Thus, various treatments or mutations that resulted in inactivation or down-regulation of DAMB, either application of D_1 antagonists, *DAMB* mutation or long-term augmentation of DA synthesis, all led to the common effect of increased PQ tolerance. Therefore, DAMB signaling appears to contribute significantly to PQ-induced neurotoxicity in *Drosophila*.

Age-related decline in PQ resistance correlates with *DAMB* expression

Newly eclosed 1-day-old wild-type *Drosophila* have been reported to be more resistant to PQ exposure than 5- or 10-day-old flies, which were more sensitive to the toxin (52,53), despite no significant difference in food intake between PQ-treated 1- and 5-day-old flies (52). We, indeed, found that the PQ resistance of wild-type flies strongly decreased during the first 2 weeks of adult life: ~95% of the 1-day-old flies survived 24 h after PQ exposure against only 8% of the 15-day-old flies (Fig. 3A). Interestingly, *DAMB* expression level was found to augment progressively during the same period, reaching twice as high in 15-day-old flies compared with newly eclosed flies (Fig. 3B and C). We therefore tested the evolution with age of PQ susceptibility in the *Damb*^{1} mutant. Strikingly, the mutant flies were strongly resistant at all ages between 1 and 15 days after eclosion (Fig. 3A). This suggests that DAMB is required for the progressively higher sensitivity of aging flies to PQ-induced oxidative stress.

DAMB inactivation in glutamatergic neurons alleviates PQ neurotoxicity

The DAMB receptor is known to be expressed in the mushroom bodies (45,54), a fly brain center involved in the regulation of associative learning and sleep. As described earlier, *DAMB* inactivation enhanced the resistance of PQ-fed *Drosophila* or of decapitated flies when PQ was applied to the nerve cord.

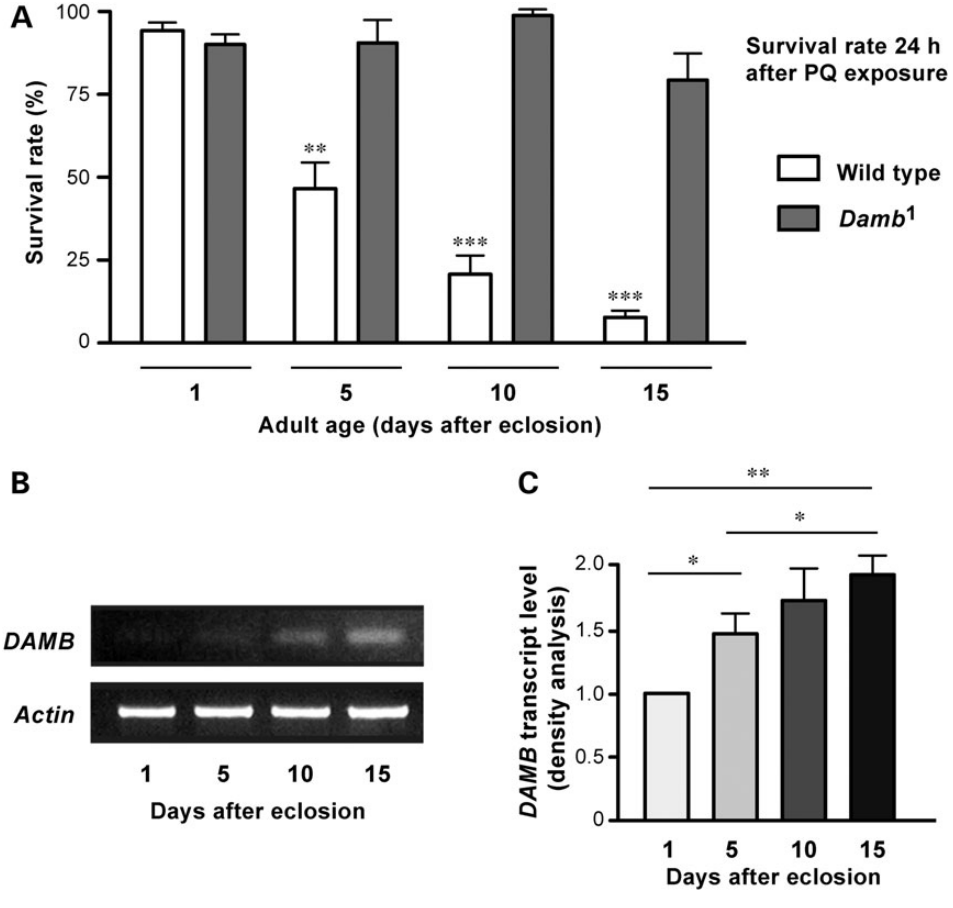

Figure 3. Age-related increase in *Drosophila* PQ susceptibility appears to be DAMB dependent. (**A**) Whereas newly eclosed (1-day-old) wild-type (w^{1118}) *Drosophila* were quite resistant to PQ exposure, susceptibility markedly increased during the first 2 weeks of adult life. In contrast, resistance of *w*; *Damb*^{1} mutant flies after PQ ingestion was similarly high at all ages during this period. (**B**) RT-PCR experiments (30 cycles of amplification) demonstrated that *DAMB* expression progressively increased with age in wild-type flies during the first 2 weeks after adult eclosion. (**C**) Quantification of the RT-PCR products from 3 to 5 independent experiments by density analysis. The relative transcript abundance of the DA receptor in 15-day-old flies was found to be about twice as much as in 1-day-old flies.

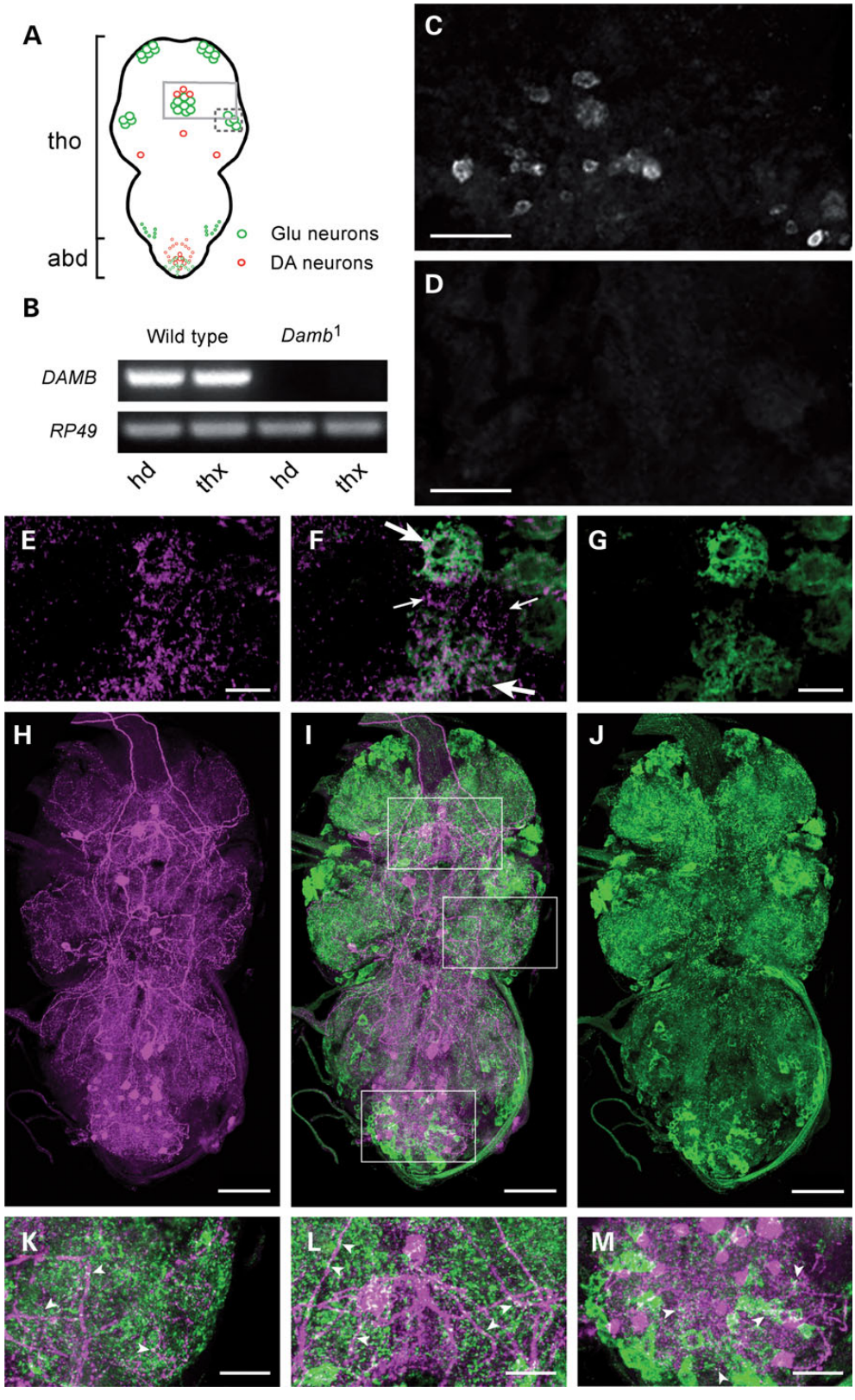

Figure 4. DAMB is expressed in GNs of the VNC. (**A**) Sketch of Glu- (green circles) and DA- (red circles) synthesizing cells in the VNC. The regions corresponding to the thoracic ganglia (tho) and fused abdominal neuromeres (abd) are marked. Circles represent neuronal cell bodies. The gray rectangle indicates the region magnified in panels (C) and (D), and the dashed rectangle does the same for panels (E) and (G). (**B**) RT-PCR experiment (40 cycles) showing that *DAMB* is expressed both in the head and thorax of wild-type Canton-S flies and is absent from both tissues in *Damb*^{1} mutant. (**C**) Presence of DAMB immunoreactivity in adult VNC of wild-type flies. DAMB is expressed particularly in neuronal cell bodies that may correspond to Glu-releasing motor neurons. (**D**) No DAMB immunoreactivity was detected in VNC

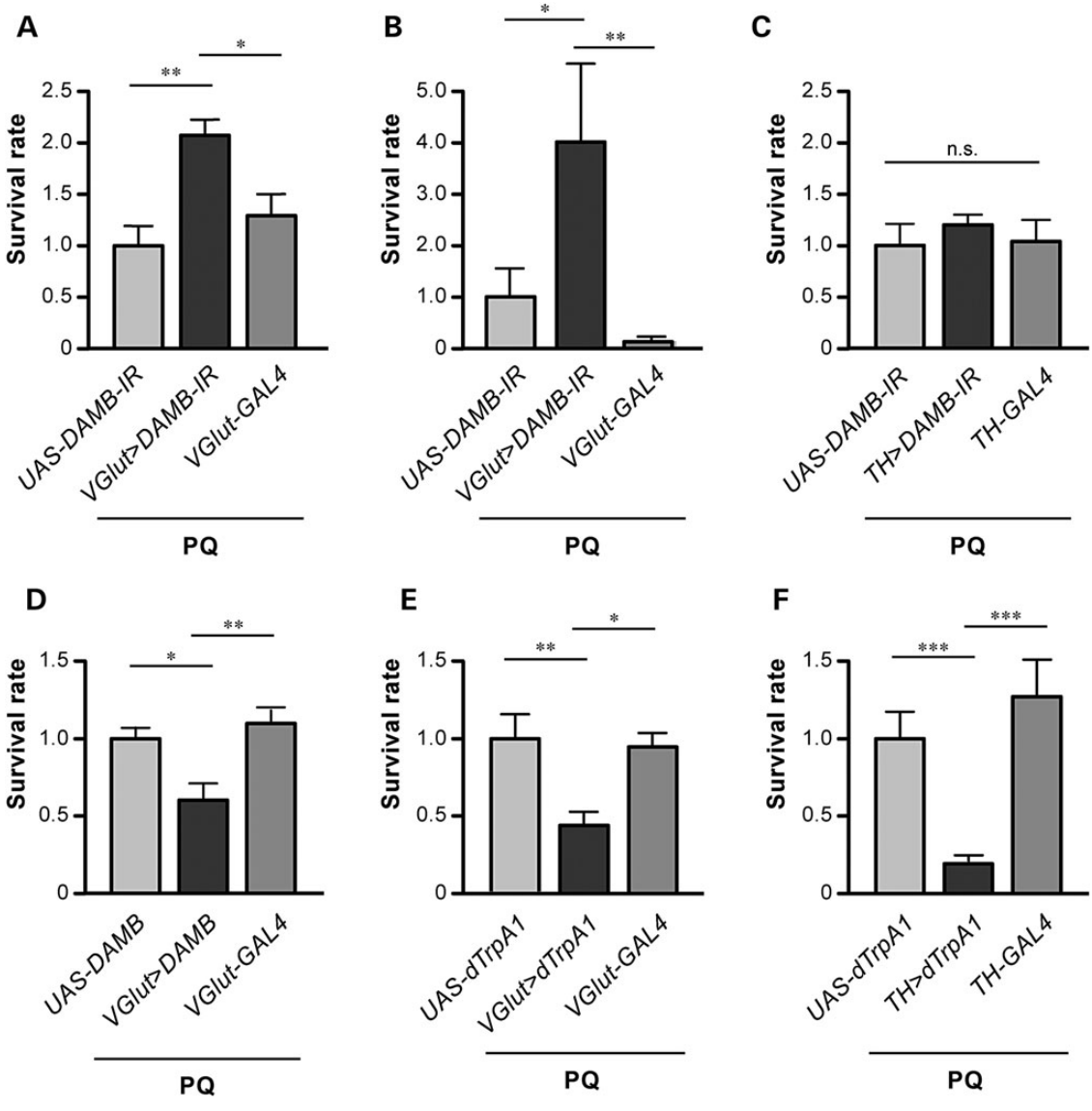

Figure 5. *DAMB* inactivation in GNs enhances PQ tolerance. (**A** and **B**) Survival of decapitated (A) and intact (B) flies at 2 and 48 h, respectively, after PQ exposure. The survival rate is greater for *VGlut-GAL4*; *UAS-DAMB-IR Drosophila* (*VGlut>DAMB-IR*), which express *DAMB* interfering RNA (IR) in GNs compared with control flies carrying one copy of *VGlut-GAL4* or *UAS-DAMB-IR* alone. (**C**) Survival of decapitated *TH-GAL4*; *UAS-DAMB-IR* flies (*TH>DAMB-IR*) that express *DAMB* IR in DNs is, in contrast, similar to controls. (**D**) PQ susceptibility of *VGlut-GAL4*; *UAS-DAMB* flies (*VGlut>DAMB*) that overexpress *DAMB* in GNs is significantly increased compared with controls. (**E** and **F**) Effect of *in vivo* stimulation of Glu- or DA-releasing neurons on PQ resistance. (E) Survival of decapitated *VGlut-GAL4*; *UAS-dTrpA1* (*VGlut>dTrpA1*) *Drosophila* incubated for 2 h at 31°C in the presence of PQ is decreased compared with similarly treated control flies carrying one copy of either *VGlut-GAL4* or *UAS-dTrpA1* alone. (F) Same experiment as in D with *TH-GAL4*; *UAS-dTrpA1* flies (*TH>dTrpA1*). Short-term stimulation of DNs also markedly increased PQ neurotoxicity. In all panels, values were normalized to the mean survival rate of respective *UAS* strain controls.

We looked, therefore, for *DAMB* expression in the VNC as well. The structure of the VNC with the relative localization of Glu- and DA-releasing neurons is schematically depicted in Figure 4A. RT-PCR experiments showed that *DAMB* mRNA is detectable in both the head and thorax of adult wild-type *Drosophila* but not in the *Damb*^{1} mutant (Fig. 4B). Note that the *DAMB* signal appears stronger in this figure because 40 cycles of amplification were performed instead of 30 in Figures 2F and 3B, as described in Materials and Methods. DAMB-specific immunostaining confirmed that this receptor

of *Damb*^{1} mutant. (**E**–**G**) Double staining with anti-DAMB (magenta) and anti-GFP (green) antibodies in VNC of *VGlut-GAL4*; *UAS-mCD8::GFP* flies, (E) anti-DAMB, (F) merge and (G) anti-GFP. Large arrows show cell bodies of GNs expressing DAMB at various levels. Thin arrows indicate cells expressing DAMB and not GFP. (**H**–**J**) Double staining with anti-TH (magenta) and anti-GFP (green) antibodies in VNC of *VGlut-GAL4*; *UAS-VGlut::GFP* flies. (H) anti-TH, (I) merge and (J) anti-GFP. DN axonal varicosities and GN nerve endings are widely distributed and overlap in several regions of the VNC neuropil. (**K**–**M**) Magnification of the regions indicated by white rectangles in I. Examples of overlapping neuronal domains are marked (arrowheads). Scale bars: (C and D) 25 μm, (E–G) 5 μm, (H–J) 50 μm and (K–M) 20 μm.

Table 1. Lethal effect of DAMB expression in selective neuronal subpopulations

Driver name	Driver pattern	Score of adult females		Score of adult males[b]		Phenotype
		Cy	Cy^{+a}	*Cy*	Cy^{+a}	
elav-GAL4[b]	All neurons	35	0 (*0*)	33	98 (*74.8*)	Lethal
repo-GAL4	All glial cells	17	11 (*39.3*)	12	13 (*52.0*)	Not lethal
TH-GAL4	Dopaminergic	49	0 (*0*)	63	0 (*0*)	Lethal
TRH-GAL4	Serotonergic	56	45 (*44.5*)	48	27 (*36.0*)	Not lethal
Cha-GAL4	Cholinergic	71	0 (*0*)	57	0 (*0*)	Lethal
VGlut-GAL4	Glutamatergic	26	38 (*59.4*)	23	28 (*54.9*)	Not lethal

Female *UAS-DAMB/CyO* flies were mated to male flies with hemizygous or homozygous insertions of the indicated *GAL4* drivers. Survival was determined by scoring the relative number of Cy^+ adult flies in the progeny. The DAMB receptor did not hinder *Drosophila* survival when it was expressed in either glial cells, or selectively in glutamatergic or serotonergic neurons. In contrast, *DAMB* expression in all neurons, or restricted to dopaminergic or cholinergic neurons, caused lethality prior to adult eclosion.

[a]Percent of Cy^+ progeny are in *italics*.

[b]Because *elav-GAL4* is inserted on the X chromosome and male driver flies were used, only the female progeny of this cross inherited the GAL4 transgene.

is widely expressed in the VNC, specifically in areas containing glutamatergic neurons (GNs) (Fig. 4C). No immunostaining was observed in the VNC of mutant $Damb^1$ flies (Fig. 4D). We used the *VGlut-GAL4* driver to express GFP selectively in GNs. Double staining for DAMB and GFP showed that DAMB could be detected in GNs (large arrows) and in other cells (thin arrows) in the VNC (Fig. 4E–G). Expression of a synaptic vesicle marker, VGlut::GFP, in GNs revealed that glutamatergic nerve endings are widely distributed in the VNC neuropil in regions that also contain dense dopaminergic arborizations (Fig. 4H–J, showing co-immunostaining against GFP and TH). Higher magnification demonstrates that dopaminergic projections and glutamatergic nerve terminals are often overlapping (Fig. 4K–M, arrowheads). Previous studies in *Drosophila* and other insects suggest that these glutamatergic terminals could originate from Glu-releasing CNS interneurons or, alternatively, from motor axon collaterals (55–57).

To determine whether DAMB signaling in GNs contributes to PQ-induced lethality, we inactivated *DAMB* selectively in GNs by targeted RNA interference (RNAi). This led to a significant increase in fly survival under PQ poisoning, by either dietary ingestion or after application of the drug to decapitated flies, compared with the control GAL4 and UAS-RNAi strains (Fig. 5A and B). In contrast, expression of the double-stranded *DAMB* RNAi in DNs with *TH-GAL4* had no effect on PQ susceptibility (Fig. 5C). Conversely, we overexpressed *DAMB* in GNs and this indeed caused a higher susceptibility of the flies to PQ lethal effects (Fig. 5D). We then tested the effect of *DAMB* overexpression on *Drosophila* survival in the absence of PQ. This led to contrasting results depending on the cell type. Overexpression either in GNs, where *DAMB* is normally expressed, in serotonergic neurons, or in glial cells was without effect. In contrast, *DAMB* expression in all neurons as well as in dopaminergic or cholinergic neurons resulted in fly death at the pupal stage (Table 1). However, we observed that panneuronal expression of dDA1, the other D_1-like receptor in flies, did not alter *Drosophila* survival in any case. This suggests that DAMB signaling can be specifically neurotoxic, either when this receptor is overexpressed in certain neuronal subpopulations, leading to hampered fly development, or in the adult stage when its activation is combined with an exposure to pro-oxidant molecules such as PQ.

Short-term stimulation of GNs or DNs increases PQ susceptibility

DAMB activation elevates intracellular cAMP and Ca^{2+} levels (58), which in turn could potentiate neuronal activity and neurotransmitter release. dTRPA1 is a cation-permeant thermal sensor channel that depolarizes neurons when the ambient temperature is raised above 25°C (59). We expressed dTRPA1 in GNs to assess whether the activity of these neurons can modulate PQ toxicity. Headless adult flies were treated with PQ or plain Ringer's solution and then incubated at 31°C for 2 h. We observed that the expression of dTRPA1 in GNs did not trigger paralysis of the flies at 31°C, indicating that neuromuscular junctions were still functional under this condition. In the absence of PQ treatment, most of the dTRPA1-expressing flies survived the 2 hours of neuronal overstimulation (80% compared with 93% for control flies that did not express dTRPA1). In contrast, the survival rate of PQ-intoxicated dTRPA1-expressing flies was reduced by half compared with that of the control flies containing *UAS-dTRPA1* or *VGlut-GAL4* alone (Fig. 5E). Similarly, overstimulation of DNs with dTRPA1 for 2 h heavily decreased the resistance of the flies against PQ (Fig. 5F): survival rate dropped to ~5 times lower than the *UAS-dTRPA1* and *TH-GAL4* controls. This last result contrasts with the protective effect observed in adult flies after a long-term augmentation of DA biosynthesis in the CNS during development (see Fig. 2A and B). Although short-term stimulation of DNs in the adult fly CNS aggravates the detrimental effects of oxidative stress (Fig. 5F), a long-term increase in DA release compensates for this effect by constitutively down-regulating the DAMB receptor (Fig. 2F–H).

DAMB inactivation protects against DA neurotoxicity

After PQ exposure, DAMB could be overactivated by the large amounts of DA likely to be released from PQ-damaged DNs, thus impairing *Drosophila* survival under oxidative stress. As a further test of this hypothesis, we applied various dilutions of DA directly to the nerve cord of decapitated *Drosophila* in the absence of PQ. Interestingly, DA levels of >10 mM were found to be lethal in these conditions in a dose-dependent manner, with a median lifespan of ~45 min at 50 mM DA

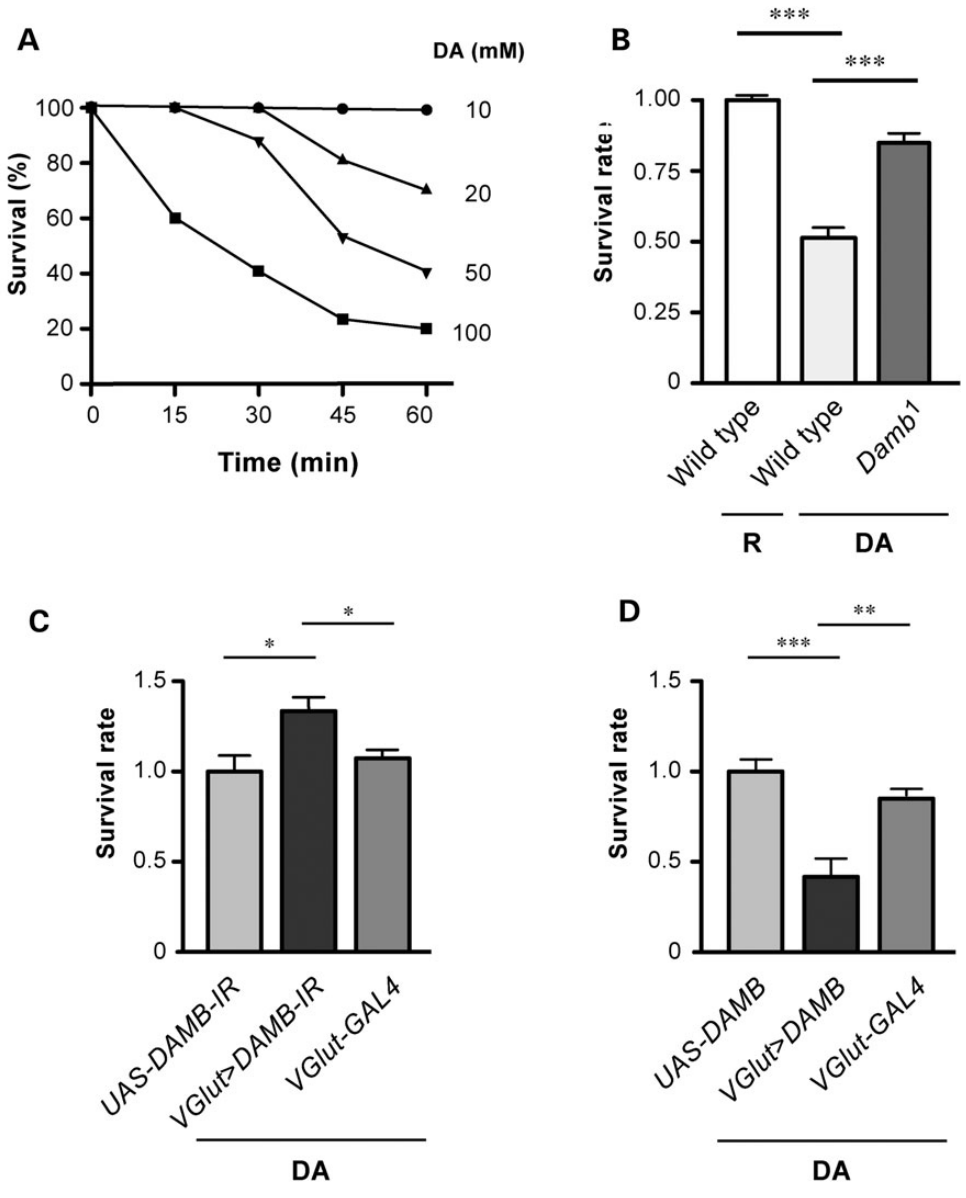

Figure 6. *DAMB* inactivation protects decapitated *Drosophila* against DA neurotoxicity. (**A**) Survival of decapitated wild-type (Canton-S) flies was monitored at various times (15, 30, 45 and 60 min) after a 5-s application of a drop of DA dissolved in Ringer's at the indicated concentration (10, 20, 50 or 100 mM). DA levels of >10 mM were found to be toxic in a dose-dependent manner under these conditions. (**B**) Survival rate of decapitated flies 2 h after a 5-s application of Ringer's solution alone (R) or 35 mM DA dissolved in Ringer's (DA). *Damb*^{1} mutants resist DA toxicity better than wild-type flies. (**C**) Survival of decapitated *Drosophila* 2 h after a 5-s exposure to 35 mM DA is significantly prolonged for *VGlut-GAL4*; *UAS-DAMB-IR* flies (*VGlut>DAMB-IR*) that express *DAMB* interfering RNA in GNs as compared with control flies carrying one copy of *VGlut-GAL4* or *UAS-DAMB-IR* alone. (**D**) DA susceptibility of *VGlut-GAL4*; *UAS-DAMB Drosophila* (*VGlut>DAM*B) that overexpress *DAMB* in Glu neurons is markedly increased compared with controls. Values were normalized to the mean survival rate of *UAS* strain controls. Note that all experiments in this figure were performed in the absence of PQ.

(Fig. 6A). Here again, the *Damb*^{1} mutant strain was markedly more resistant than the wild type to DA toxicity (Fig. 6B): survival rate of *Damb*^{1} *Drosophila* 2 h after exposure to 35 mM DA was ~1.7 times higher than that of similarly treated wild-type flies and close to the survival rate of the untreated wild type. Similar to the results of experiments conducted with PQ (Fig. 5A and B), we found that targeted inactivation of *DAMB* by RNAi in GNs and *DAMB* overexpression in GNs, respectively, increased and decreased the survival of headless *Drosophila* 2 h after DA exposure (Fig. 6C and D). This suggests that DA neurotoxicity in *Drosophila* in part results from DAMB overactivation in GNs.

Decreased cytosolic Ca^{2+} protects against PQ neurotoxicity

As DAMB activation can elevate both cytosolic cAMP and Ca^{2+} levels, we tried to determine which of these second messengers could be involved in the detrimental effects induced by the DA receptor under oxidative stress. To raise the level of cAMP in cells, we applied PQ together with forskolin and IBMX to the

trunk of decapitated flies. Compared with controls with PQ only, we found that the resistance of the flies with a higher level of cAMP was increased (Fig. 7A). This result agrees with the view that cAMP generally has a neuroprotective effect in the nervous system (38,60–62). This suggests that cAMP may not take part in the neurotoxic signaling triggered by the DAMB receptor.

As shown in Figure 5E and F, we have found that increasing the level of cations in neurons using the dTRPA1 channel made the flies more susceptible to PQ, arguing for a role of Ca^{2+} in PQ neurotoxicity. To test in reverse for an effect of reduced intracellular Ca^{2+}, we determined the PQ resistance of a heterozygous mutant of the single ryanodine receptor (RyR) in flies, RyR^{16} (63). Ryanodine receptor channels are major mediators of activity-induced increase in cytosolic Ca^{2+} (64,65), and the RyR^{16} mutation was previously shown to suppress Aβ neurotoxicity in a *Drosophila* model of Alzheimer's disease (66). Interestingly, we found that the RyR^{16} mutant flies were markedly resistant to PQ exposure, to an extent comparable to that of the *Damb1* mutants (Fig. 7B). This shows that RyR is a component of the PQ-induced cell death pathway and suggests that DAMB contributes to PQ toxicity at least in part by increasing the level of cytosolic Ca^{2+} in neurons.

DISCUSSION

Neuronal targets of PQ in *Drosophila*

Paraquat has long been used in *Drosophila* and other species to study mechanisms of oxidative stress resistance and to model aging or PD (26,33,39,53,67–73). Advantages of this compound include water solubility, its ability to cross the digestive and blood–brain barriers and its ability to quickly generate reactive oxygen and nitrogen species, which cause oxidative damage. In *Drosophila*, PQ is generally administered by feeding for 1–2 days (74). However, this drug was previously shown to have a strong anorexigenic effect in flies (46). Here, we introduce an alternative technique for PQ exposure, i.e. its direct application onto the anterior notum of decapitated *Drosophila*. Such a behaviorally active preparation was used in previous works for pharmacological studies of biogenic amines (47–49,75,76). This method allows for PQ to diffuse rapidly into the VNC, thus avoiding the use of sucrose solution and digestive tract absorption and also minimizing the time for experiments because lethality occurs a few hours after the brief (5-s) drug application. A potential concern is that the brain and other parts of the CNS located in the head are also oxidatively damaged following PQ ingestion and are absent in decapitated flies. Although it is likely that the head tissues contribute to PQ susceptibility in entire flies, we always found qualitatively similar results in survival tests when comparing PQ ingestion and direct application to decapitated animals, as shown here in Figures 1, 2 and 5. This shows that both methods can be used with confidence to analyze PQ neurotoxicity effects.

Because PQ is an environmental risk factor for PD (18,77) that causes selective loss of SNpc DNs in animal models (78–80), it is essential to investigate the relationship between PQ-induced toxicity and the neural dopaminergic system. The selective susceptibility of SNpc neurons to PD factors is thought to arise in part from the pro-oxidant properties of DA and of its metabolites (13–15,17). This suggests that increasing DA levels in neurons should enhance PQ susceptibility. Intriguingly, a previous study in *Drosophila* showed that mutations that augment DA levels have the opposite effect, causing enhanced PQ tolerance, whereas mutations that diminish DA pools increase fly vulnerability (33). Here, we found that increasing DA synthesis in neurons, by overexpressing the *TH* gene during fly development, extended PQ tolerance both after ingestion and direct application of the toxin. PQ-induced neurotoxicity appears therefore to be a primary cause of death in *Drosophila* that precedes a slower systemic organ failure.

Surprisingly, a similar protective effect was observed when DA synthesis was increased either in dopaminergic or in serotonergic neurons, suggesting that a critical factor for DA-mediated neuroprotection is the amount of DA released over time in the extracellular space rather than the level of DA inside DNs. Consistent with this interpretation, it was reported that flies overexpressing throughout life the vesicular monoamine transporter in DNs, a genetic way to increase DA release, showed enhanced resistance to PQ (36). We subsequently gathered an array of evidence agreeing with this hypothesis and arguing for the involvement of a DA receptor in PQ susceptibility (Figs 2 and 3): (1) PQ tolerance is enhanced in the presence of DA receptor antagonists, (2) increased DA synthesis in neurons down-regulates the D_1-like DA receptor DAMB, (3) *DAMB*-deficient mutants are more tolerant to PQ than wild-type flies, and (4) *DAMB* expression is required for the age-related decrease in PQ resistance that occurs during the first 2 weeks of adult life. This last observation suggests, quite interestingly, that the expression of a DA receptor underlies aging-associated changes in oxidative stress susceptibility in young adult flies. All together, these results lead to conclude that DAMB significantly contributes to PQ neurotoxicity and that the amount of DA released over the long term in the CNS controls *Drosophila* PQ resistance by modulating the expression of this receptor.

On the other hand, we also present evidence that a sudden increase in extracellular DA triggered by acute PQ intoxication in naïve flies directly contributes to fly death. PQ-induced DA release could not be demonstrated here because of the small size and low accessibility of the adult *Drosophila* CNS. However, it was demonstrated in mammals (see below) and suggested by the rapid morphological alterations of DNs in PQ-intoxicated flies (Fig. 1). Here, we provide two arguments in favor of this hypothesis. First, short-term stimulation of DNs by dTRPA1 activation in the adult fly CNS strongly aggravated the detrimental effects of PQ (Fig. 5F). Second, brief exposure of headless flies to DA levels of >10 mM, in the absence of PQ, was enough to induce progressive lethality in the following hours (Fig. 6A). These experiments show that a sudden increase in the extracellular DA level is neurotoxic in *Drosophila*. Interestingly, $Damb^1$ mutant flies appear much less susceptible to DA toxicity than wild-type flies (Fig. 6B), as is the case with PQ susceptibility. This indicates that a significant portion of DA neurotoxicity results from the activation of DAMB. Therefore, PQ could primarily target DNs, causing abnormal extracellular DA, which would then aggravate oxidative damage by overactivating the DAMB receptors located on nerve terminals of neighboring neurons.

Because headless $Damb^1$ mutants are also less susceptible to PQ toxicity, we searched for the presence of the DAMB receptor in the VNC. *DAMB* is indeed expressed in neuronal subtypes in this tissue, including glutamatergic motor neurons (Fig. 4C–G).

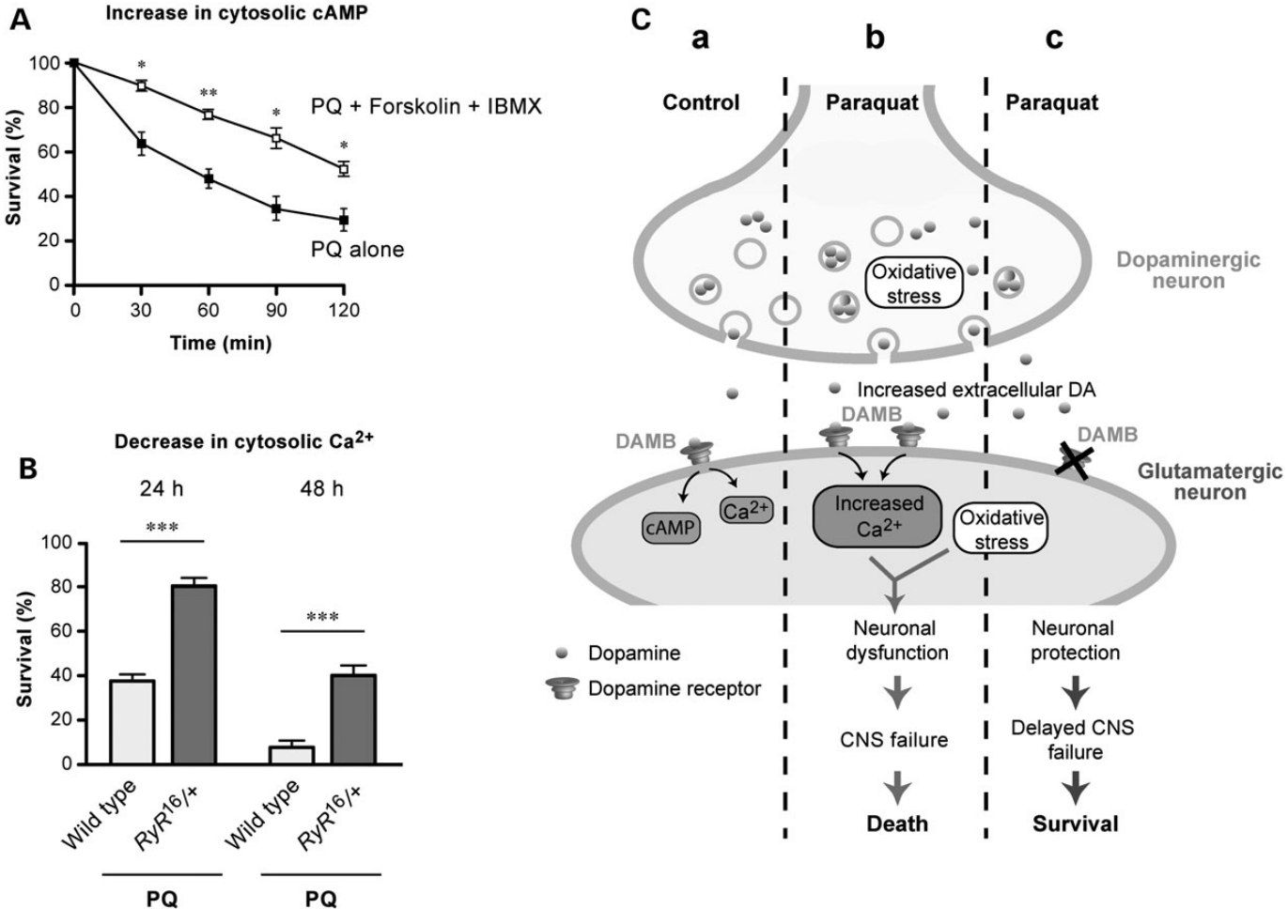

Figure 7. Mechanisms of DAMB-mediated potentiation of PQ susceptibility in *Drosophila*. (**A**) Survival of decapitated wild-type *Drosophila* was monitored at various times after a 5-s application of a PQ solution mixed (open squares) or not (closed squares) with forskolin plus IBMX, as described in Materials and Methods. Increased cAMP levels appeared to protect the flies against PQ neurotoxicity. (**B**) Heterozygous RyR^{16} mutants that have a reduced level of ryanodine receptor calcium channels survived in significantly higher numbers compared with wild-type flies either at 24 or 48 h after PQ ingestion. This suggests a primary role for cytosolic Ca^{2+} in the PQ toxicity pathway. (**C**) Proposed model of PQ neurotoxicity in flies. (a) In physiological conditions, DA release and DAMB-mediated signaling are not neurotoxic. (b) In naïve flies exposed to PQ, high amounts of DA released from oxidative stress-injured DNs would overactivate DAMB. Neuronal dysfunction would then result from two synergistic causes: on the one hand, elevated oxidative stress caused by PQ absorption, and on the other hand, aberrant DAMB activation leading to increased cytosolic Ca^{2+} through the ryanodine receptor. This would ultimately cause CNS failure and precipitate organism death. (c) In $Damb^{1}$ mutants or in flies with constitutively higher DA synthesis, *DAMB* deficiency or DAMB down-regulation, respectively, alleviate PQ neurotoxicity, mediating CNS protection and prolonged *Drosophila* survival. Such a modulation of DAMB expression might be used in wild-type flies as an adaptive mechanism to increase oxidative stress tolerance.

We also find that glutamatergic projections are widespread in the VNC neuropil and often located close to dopaminergic varicosities (Fig. 4H–M), such that when large amounts of DA are released, diffusion could ensure that the DAMB receptors expressed in GNs are overactivated. Accordingly, we show that *DAMB* inactivation by RNAi targeted to GNs did partially protect the flies against both PQ- and DA-induced toxicity, indicating that DAMB activation in these cells contributes to the detrimental effects of those compounds (Figs 5A and B and 6C). This conclusion is strengthened by the fact that, in contrast, overexpressing *DAMB* in GNs increased susceptibility to either PQ or DA (Figs 5D and 6D). In accordance with these results, other reports demonstrated a central role for GNs in *Drosophila* PQ susceptibility. Thus, targeted expression of either the human superoxide dismutase, SOD1, or the mitochondrial heat shock protein, Hsp22, within glutamatergic motor neurons significantly increased *Drosophila* survival after PQ poisoning (68,81).

Mechanisms of DAMB-mediated potentiation of PQ susceptibility

How can DAMB overactivation trigger CNS failure and death under PQ exposure? It is known that increased cytosolic Ca^{2+} in neurons can mediate or potentiate the damaging effects of oxidative stress and excitotoxicity (82–84). One possibility, therefore, is that DAMB signaling in PQ-intoxicated flies abnormally elevates cytosolic Ca^{2+} and cAMP levels in neurons expressing the receptor, leading to aberrant release of Glu and other neurotransmitters and enhanced excitotoxic defects (85,86). Accordingly, we previously showed that flies with reduced Glu

buffering capability are more susceptible to PQ exposure (85). This hypothesis also agrees with novel observations reported here, e.g. the localization of DAMB in GNs of the VNC, the enhanced PQ susceptibility of flies in which GNs are stimulated with the cation channel dTrpA1 (Fig. 5E) and the striking resistance of a ryanodine receptor mutant to PQ neurotoxicity (Fig. 7B). A raised level of cytosolic Ca^{2+} appears, therefore, to be an important component of the neurotoxic effects of PQ in *Drosophila* and could lead to aberrant Glu release. In contrast, an increase in cAMP level appears to be neuroprotective in the same conditions (Fig. 7A). However, more experiments are needed before we could conclude that DAMB-mediated activation of cAMP signaling does not play a role in PQ neurotoxicity. Finally, we also report that *DAMB* overexpression in all neurons or in specific neuronal subpopulations, i.e. dopaminergic or cholinergic cells, in the absence of PQ, arrests development of the flies during the final steps of metamorphosis (Table 1), indicating that DAMB signaling can be by itself potently neurotoxic. It can be noted that a comparable lethal effect was previously reported following panneuronal expression of the *Drosophila* adenosine receptor that also activates cAMP and calcium signaling (87). The reasons why *DAMB* overexpression in other cell types, such as GNs, serotonergic neurons and glial cells, has no detrimental effect on fly survival are still to be unraveled.

Further data from the literature support the excitotoxicity hypothesis of PQ action. In *Drosophila*, a hypomorphic mutant of *methuselah* (*mth*), a gene encoding a peptide-binding G protein-coupled receptor, has an increased average lifespan and enhanced resistance to various stress, including dietary PQ (53,88). Interestingly, evoked Glu release from motor neurons in this *mth* mutant is decreased by ~50% owing to reduced synaptic area and synaptic vesicle density (89). Such a decrease in Glu release capability could be an advantage under oxidative stress conditions by delaying excitotoxic insults. In another system, i.e. the striatum of freely moving rats, it was demonstrated that PQ dose dependently elevates extracellular Glu and DA levels. The increase in extracellular DA level lasted for >24 h after the PQ treatment. Pharmacological evidence showed that PQ-stimulated Glu efflux initiates a cascade of excitotoxic reactions eventually leading to damage of striatal dopaminergic terminals (90).

A proposed model for PQ neurotoxicity

Overall, our results suggest that the activation state of a DA receptor contributes to oxidative stress susceptibility in *Drosophila* and lead to a proposed model for PQ neurotoxicity, schematically depicted in Figure 7C. Large amounts of DA released under acute oxidative stress in naïve flies would overactivate DAMB, triggering Ca^{2+} release in the cytosol through the ryanodine receptor, leading to neuronal dysfunction and finally causing nervous system failure. This hypothesis is supported by the following experimental evidence: extracellular DA is by itself lethal at high doses, short-term stimulation of DNs or GNs increases PQ susceptibility and, in GNs, *DAMB* inactivation and overexpression, respectively, protects against and sensitizes to, either PQ- or DA-induced toxicity. The fact that acute stimulation of specific neuronal subsets increased PQ susceptibility in flies importantly correlates neuronal activity and oxidative stress tolerance. In contrast, moderate and long-term augmentation of DA synthesis in the CNS down-regulate *DAMB*, leading to better resistance against oxidative stress.

We may infer from this model that modulating downstream DAMB signaling in response to a mild and prolonged oxidative stress exposure could elevate resistance in adult flies. Such an adaptive 'hormetic' effect might be used in *Drosophila* and other insects to survive under conditions of raised environmental stress. An adaptive increase in oxidative stress resistance was indeed recently demonstrated in *Drosophila,* and it was shown to depend on the induction of the Nrf2 antioxidant response pathway (91). A potential relation between DAMB signaling and the Nrf2 pathway remains to be investigated.

Parallel with human disease conditions

A role for postsynaptic D_1 DA receptors in neuronal cytotoxicity is not restricted to *Drosophila* and has been previously reported in mammalian models in the case of striatal neurodegeneration, a symptom associated with major neurological disorders such as multiple system atrophy and Huntington's disease. The striatal GABAergic medium spiny neurons are indeed uniquely vulnerable to elevated extracellular DA levels (92,93). In these cells, D1 receptor stimulation potentiates Ca^{2+} influx via Glu receptors of the NMDA subtype (94,95) and stimulates the phosphorylated form of the extracellular signal-regulated kinase (p-ERK) via cAMP-dependent signaling (96). Both mechanisms contribute to the activation of cellular toxicity pathways, ultimately resulting in neuronal death. Such a toxic convergence of DA and Glu signals is quite similar to the mechanism of PQ neurotoxicity observed here. The *Drosophila DAMB*-expressing neurons and human striatal medium spiny neurons share the property of being potentially overloaded with cAMP and Ca^{2+} ions in conditions that abnormally increase extracellular DA levels. Further investigations on the *DAMB*-mediated neurotoxic pathways in *Drosophila* could thus lead to a better understanding of major DA-related disorders in humans.

MATERIALS AND METHODS

Drosophila culture and strains

Fly stocks were raised at 25°C on standard cornmeal-yeast-agar medium supplemented with methyl-4-hydroxy-benzoate as a mold protector, under 12 h:12 h light/dark cycle and ~70% humidity. The following *Drosophila* strains were used: Canton S or w^{1118} as wild type; *y*, *w* for germ-line transformation; *UAS-DTHg* (40) to express the *Drosophila* TH gene; *UAS-VGlut::GFP* to express GFP fused to the *Drosophila* vesicular Glu transporter gene (*VGlut*), used as a synaptic vesicle marker (97); *Cha-GAL4* (98), *TH-GAL4* (51), *TRH-GAL4 and VGlut-GAL4* that drive gene expression in cholinergic, dopaminergic, serotonergic and GNs, respectively; *elav-GAL4* (99) that expresses in all neurons and *repo-GAL4* (100) in all glial cells in adult flies; $Damb^{1}$, a null mutant of the DopR2/DAMB receptor generated by P element imprecise excision (45,101); $dumb^{2}$, a hypomorphic DopR/dDA1 allele (102); *UAS-DAMB/CyO* (provided by Kyung-An Han); *UAS-dDA1* (50); *UAS-DAMB RNAi* (*UAS-DAMB-IR*) (Vienna *Drosophila* RNAi center, stock #v3391); RyR^{16}, a mutant of the ryanodine receptor (63), *UAS-dTrpA1* (59) (described in Result section), *UAS-mCD8::GFP* (103) and *UAS-GFP-S65T*

(Bloomington *Drosophila* Stock Center). Constructions of the *TRH-GAL4*, *VGlut-GAL4* and *UAS-VGlut::GFP* transgenes used in this study are described in the Supplementary Materials and Methods and in Supplementary Material, Figures S3 and S4.

PQ intoxication and survival score

Unless otherwise indicated, PQ treatment was performed on 7- to 10-day-old adult females, either by dietary ingestion or by direct application of the drug to decapitated flies. For dietary ingestion, ~100 flies per condition were incubated at 25°C in 2-inch (5.2 cm) diameter Petri dishes (10 flies per dish) containing two layers of Whatman paper soaked with 600-µl PQ (methyl viologen, Sigma) diluted in 2% (wt/vol) sucrose or sucrose only for controls. To avoid dehydration, dishes were enclosed in a plastic box layered with moist paper. Survival was monitored after 24 or 48 h. Generally, 20 mM PQ was used in ingestion experiments, which yielded ~40% survival after 24 h for wild-type flies (Fig. 1A).

For direct application, flies were anesthetized on ice for 10 min and their heads were cut off with 7-mm blade spring scissors (Fine Science tools). About 100 decapitated flies per condition were transferred to a 2-inch Petri dish (10 flies per dish) and allowed to recover for a few minutes until they stood on their legs. A 5-µl droplet of PQ diluted in *Drosophila* Ringer's solution (in mM: 130 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 0.5 Na_2HPO_4, 0.35 KH_2PO_4, pH 7.4 adjusted with 150 Na_2HPO_4) or Ringer's only for controls was applied for 5 s with a P10 Pipetman to the VNC at the anterior notum as described (47). The same droplet was successively used for 10 flies. Then, flies were incubated at 25°C in the same conditions as those intoxicated by ingestion. A concentration of 80 mM PQ was generally used that gave 30–40% survival with 7- to 10-day-old wild-type flies after 2 h (Fig. 1B and Supplementary Material, Fig. S1). Flies were considered as dead when they laid on the side or back and did not react to a light mechanical stimulus. Survival was monitored every 30 min, and graphs present survival at 2 h unless otherwise noted. A similar procedure was used to assay for DA toxicity.

DA receptor antagonists were purchased from Tocris and added to the PQ solution used for direct application at the following concentrations: SKF83959, 50 µM; SKF83566, 5 mM; eticlopride, 5 mM. To increase cAMP level in cells, 25 µM forskolin plus 100 µM 3-Isobutyl-1-methylxanthine (IBMX) (both from Sigma–Aldrich) were similarly added to the PQ solution and controls received equivalent volumes of DMSO. For *in vivo* neuron activation, 30 decapitated *Drosophila* expressing *dTrpA1* in neuronal subsets were intoxicated as usual at room temperature and then immediately incubated at 31°C for 2 h before fly survival was scored.

Survival rates were normalized to the mean value of internal controls, most often wild-type or *UAS* control strains. Statistical analyses were performed with Prism (GraphPad Software, La Jolla, CA, USA), using ANOVA with *post hoc* Tukey–Kramer or Student's *t*-test. Unless otherwise stated, errors bars represent standard errors of the mean (SEM) of 9 or 10 independent determinations. Statistical significance in all figures: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, n.s. not significant.

RNA extraction

Total RNA was extracted by the TrIzol (Invitrogen) method. Adult flies were anesthetized on ice and decapitated with a sterile scalpel. Twenty heads (or 5 thoraxes) were dissolved in 500 µl TriZol and kept at −80°C overnight. Remaining tissues were then crushed with a mini-Potter. The mixture was extracted with 100 µl chloroform and centrifuged at 2320*g* (5000 rpm) in an Eppendorf 5415 R microcentrifuge for 10 min at 4°C. The supernatant was collected, mixed with 250 µl isopropanol, and stored for 30 min to 2 h at −20°C. After 15-min microcentrifugation at 2320*g*, the pellet was washed twice with 250 µl 75% (vol/vol) ethanol prepared with diethylpyrocarbonate-treated water followed by 4 min of microcentrifugation at 2320*g*. The dried pellet was suspended in 20 µl of RNAse-free water. One microliter of the solution was used to determine RNA concentration with a NanoDrop spectrophotometer (ThermoScientific).

Reverse transcription–PCR

For retrotranscription, 1 µg of total RNA was mixed with 0.5 µg of oligo$(dT)_{15}$ (Promega) and 10 mM dNTP mix (Promega) in RNAse-free water. After heating at 65°C for 5 min, the tube was placed on ice and supplemented with 4 µl of 5 × First-Strand Buffer (Invitrogen), 2 µl of 0.1 M DTT, and 1 µl of RNAsin (Promega). After 2 min pre-incubation at 42°C, 1 µl of SuperScript II Reverse Transcriptase was added and the reaction mix (final volume: 20 µl) was incubated for 50 min at 42°C. The reaction was stopped by heating at 70°C for 15 min. PCR was performed as previously described (41) in a Techne TC-312 thermal cycler in 20 µl of final volume. The program included a cycle of 40 s of denaturation at 94°C, 40 s of annealing at 62°C and 40 s of elongation at 72°C, repeated 35 or 40 times. Alternatively, PCR was performed using PrimeSTAR Max DNA Polymerase (Takara). In this case, the program included a cycle of 10 s of denaturation at 98°C, 10 s of annealing at 55°C and 30 s of elongation at 72°C, repeated 30 times only to allow comparison of transcript expression levels. Band density analysis of the PCR products was performed with the Fiji software, and data were normalized to the internal controls (either *rp49* or *actin*). Results are mean ± SEM of 3–5 independent experiments. The following primers were used: for *DAMB*, sense: 5′ TTTGACTCCTCTGTCGCTCA, antisense: 5′ CAAAGAACGTAATGGAGGATG (amplicon: 232 bp); for *rp49*, sense: 5′ GACGCTTCAAGGGACAGTATC, antisense: 5′ AAACGCGGTTCTGCATGAG (amplicon: 126 bp); for *Actin 5C*, sense: 5′ CGACAACGGCTCTGGCATGT, antisense: 5′ TCCATTGTGCACCGCAAGTG (amplicon: 1094 bp).

Real-time PCR

For real-time PCR, RNA extracted from adult heads was cleaned from contaminant DNA prior to retrotranscription by treatment with RQ1 RNAse-free DNAse (Promega) according to the manufacturer's protocol. PCR was carried out in a MyiQ2 two-color real-time PCR detection system (Bio-Rad) with the SYBR GreenER qPCR SuperMix (Invitrogen) in 96-well plates. Each well contained 2.5 µl of the retrotranscription product and 0.1 µM sense and antisense primers in 1 × Syber Green Mix, in a final volume of 25 µl. The ribosomal gene *rp49* was used as an internal control. The program was: 95°C denaturation for 10 min, followed by 40 amplification cycles (15 s at 95°C, 15 s at 55°C and 45 s at 60°C), and ended by 10-s ramping from 50

to 90°C to run a melting curve. The *DAMB* and *rp49* primers were the same as those used for the RT-CPR experiments.

Immunohistochemistry

Whole adult CNS was dissected at room temperature in *Drosophila* Ringer's solution and fixed for 2 h on ice in a watch glass in 4% (wt/vol) paraformaldehyde in PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM KH_2PO_4) before being transferred with Dumont #5 forceps to 24-well plates (5–10 brains per well). After three 20-min washes in 0.5 ml PBS plus 0.5% (vol/vol) Triton X-100 (PBT), tissues were pre-incubated for 2 h in PBT + 2% (wt/vol) bovine serum albumin as blocking solution, transferred again to 96-well plates and incubated overnight at 4°C with agitation in the presence of primary antibodies diluted in blocking solution (final volume: 100 µl). The primary antibodies used were: mouse monoclonal anti-TH (1:50; ImmunoStar), mouse anti-GFP (1:500; Invitrogen Molecular Probes), rabbit anti-serotonin (1:500; Sigma), rabbit anti-DAMB (1:100) (54) and rabbit anti-synaptotagmin (Syt-1) (1:100, generous gift of Hugo Bellen). After three 20-min washes in PBT, brains were incubated for 2 h at room temperature with 0.5 ml of secondary antibodies: Alexa Fluor 555 anti-mouse, Alexa Fluor 488 anti-rabbit (Invitrogen Molecular Probes), or FITC anti-rabbit, TRITC anti-mouse (Jackson ImmunoResearch) diluted to 1:250. Tissues were washed three times again in PBT for 20 min and finally mounted in Vectashield (Vector Laboratories) or Mowiol 4–88 (Polysciences). Images were collected on a Nikon A1R confocal microscope and processed with ImageJ and Adobe Photoshop. The dopaminergic nerve-ending varicosities were quantified by measuring the respective area they covered in TH-immunostained whole VNCs as total pixel number using Adobe Photoshop.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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