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## **Neuroprotective effects of PACAP against paraquat-induced oxidative stress in the *Drosophila* central nervous system**

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## 24 **Abstract**

25 Parkinson's disease (PD) is a progressive neurodegenerative movement disorder that can arise  
26 after long-term exposure to environmental oxidative stressors, such as the herbicide paraquat  
27 (PQ). Here we investigated the potential neuroprotective action of vertebrate pituitary adenylate  
28 cyclase-activating polypeptide (PACAP) against PQ in *Drosophila*. We found that pretreatment  
29 with this neuropeptide applied to the ventral nerve cord (VNC) at low doses markedly extended  
30 the survival of wild-type decapitated flies exposed to neurotoxic levels of PQ or dopamine (DA).  
31 In contrast and interestingly, application of a PACAP receptor antagonist, PACAP-6-38, had  
32 opposite effects, significantly decreasing the resistance of flies to PQ. PACAP also reduced PQ-  
33 induced caspase activation and reactive oxygen species (ROS) accumulation in the VNC. We  
34 then searched for the endogenous neuropeptide receptor potentially involved in PACAP-mediated  
35 neuroprotection in *Drosophila*. Knocking down the gene encoding the receptor Han/PDFR of the  
36 neuropeptide pigment-dispersing factor (PDF) in all neurons conferred to flies higher resistance  
37 to PQ, whereas PDFR downregulation restricted to PDF or DA neurons did not increase PQ  
38 resistance, but remarkably suppressed the neuroprotective action of PACAP. Further experiments  
39 performed with *Pdf* and *Pdfr*-deficient mutant strains confirmed that PDF and its receptor are  
40 required for PACAP-mediated neuroprotection in flies. We also provide evidence using split-  
41 GFP reconstitution that PDF neurons make synaptic contacts onto DA neurons in the abdominal  
42 VNC. Our results, therefore, suggest that the protective action of PACAP against PQ-induced  
43 defects in the *Drosophila* nervous system involves the modulation of PDFR signaling in a small  
44 number of interconnected neurons.

45

46

## 47 **Introduction**

48        Parkinson's disease (PD) is a progressive neurodegenerative movement disorder believed to  
49        develop as a result of an interplay between genetic and environmental factors (1). The  
50        identification of genetic factors that modulate an individual's sensitivity to environmental agents  
51        can lead to better risk assessment and understanding of the disease mechanisms (2, 3). The motor  
52        symptoms of PD are primarily caused by the degeneration of dopaminergic (DA) neurons  
53        innervating the striatum whose cell bodies are located in the midbrain substantia nigra pars  
54        compacta (SNpc) (4). Epidemiological studies have suggested that long-term exposure to  
55        pesticides such as the herbicide paraquat (1,1'-dimethyl-4,4'-bipyridinium, PQ) is associated with  
56        an increased risk of developing PD (5–7). In accordance with this, PQ has been shown to induce  
57        the degeneration of nigrostriatal DA neurons in rodents (8–12), due to increased production of  
58        superoxide ions and oxidative damage (13–19).

59        The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP), originally  
60        identified as a highly potent adenylate cyclase-stimulating peptide with two biologically active  
61        isoforms, PACAP-38 and PACAP-27, exerts a wide range of actions *via* the activation of three G  
62        protein-coupled receptors (GPCRs): PAC1-R, which is PACAP-specific, and VPAC1-R and  
63        VPAC2-R indifferently activated by PACAP or vasoactive intestinal polypeptide (VIP) (20).  
64        PACAP is known as a powerful anti-apoptotic, anti-inflammatory and anti-oxidative agent (20–  
65        22). By reducing apoptosis, PACAP exhibits neuroprotective effects in diverse cellular and  
66        animal models of neuronal damage, including cerebral ischemia, brain injury, Alzheimer's  
67        disease and PD (23–27). In particular, it has been reported that PQ-induced loss of DA neurons  
68        was more severe in the SNpc of PACAP knock-out mice compared to wild-type animals,  
69        associated with increased microglial activation (25). This result indicates that endogenous

70 PACAP normally acts to maintain the integrity of SNpc DA neurons, an action that may be in  
71 part linked to its ability to regulate microglia and immune cells (25, 28).

72 One model system that has proven surprisingly tractable for neurodegenerative diseases is the  
73 fruit fly *Drosophila melanogaster*, which is now widely used to search for novel therapeutic  
74 targets and neuroprotective compounds (29–32). Previous reports have demonstrated that  
75 *Drosophila* exposed to environmental pro-oxidant toxins, such as rotenone or PQ, reproduce PD  
76 features, including selective degeneration of DA neurons (33–40). Neuropeptides are abundant in  
77 the *Drosophila* brain where they play essential roles in various physiological functions and  
78 behavior neuromodulation (41, 42). The fly *amnesiac* (*amn*) gene potentially encodes three  
79 neuropeptides, one of which displays a low (10%) identity to PACAP-38 and was previously  
80 considered as a possible PACAP orthologue (43–45), but there is still no direct evidence for the  
81 presence of *amn*-encoded neuropeptides in *Drosophila* (41). Nevertheless, a PACAP-like  
82 immunoreactivity was detected in the nervous system of various invertebrates (45, 46), and at the  
83 *Drosophila* larval neuromuscular junction (47). Focal application of vertebrate PACAP-38 to this  
84 glutamatergic synapse triggered two temporally distinct responses: an immediate depolarization  
85 and a large enhancement of potassium current (47). PACAP-38 also enhanced L-type calcium-  
86 current in larval muscles via the adenylate cyclase-cAMP-protein kinase A (PKA) transduction  
87 pathway (48). No specific receptor has been described to date that could mediate these effects.  
88 However, the receptor Han/PDFR of the neuropeptide pigment-dispersing factor (PDF), is  
89 homologous to the mammalian VIP/PACAP receptor VPAC2-R (49–51), and it has binding  
90 affinity for PACAP (51). Interestingly, both PACAP and PDF are “circadian neuropeptides” that  
91 play important roles in the regulation and entrainment of circadian rhythms in mammals and  
92 insects, respectively (42, 52, 53). Circadian clocks are highly conserved from flies to humans.  
93 They control rhythms in most physiological function, with free-running periods close to 24 h.

94 Clock disruption is suspected to reduce lifespan and quality of life during aging, and  
95 neurodegenerative disorders, like PD, often disrupt biological clocks early on (54, 55).

96 Here we assessed the protective effect of PACAP against PQ neurotoxicity in *Drosophila* and  
97 we searched for the G protein-coupled receptor (GPCR) mediating the action of PACAP in the  
98 central nervous system (CNS). We show evidence that the neuroprotective action of PACAP is  
99 conserved in *Drosophila* and specifically involves PDFR signaling in PDF and DA neurons.

100

## 101 **Results**

### 102 **PACAP protects wild-type flies from PQ-induced neurotoxicity**

103 To test the potential neuroprotective action of PACAP against PQ, administration via diet was  
104 not feasible because neuropeptides are vulnerable to intestinal peptidases. Therefore, we applied  
105 the neuropeptide onto the exposed ventral nerve cord (VNC) of decapitated flies, which can  
106 maintain a normal standing posture and survive up to 3 days when kept in a humid environment  
107 at 25°C (29, 39, 56, 57). We have previously shown in this preparation that a 5-s application of a  
108 drop of 80 mM PQ diluted in Ringer's solution onto the exposed VNC killed about 30-40% of  
109 the flies in 2 h (39).

110 Consistent with our previous report, PQ application decreased significantly the survival of  
111 decapitated flies and this effect was observed from the first 30 min after exposure (Fig. 1A).  
112 Remarkably, a 5-s application of a drop of 2  $\mu$ M PACAP (P) diluted in Ringer's solution, but not  
113 of Ringer's only (R), 30 min before that of PQ, markedly protected flies against PQ-induced  
114 lethality (Fig. 1A). The protective action of PACAP remained significant until 3 hours after PQ  
115 exposure. A maximal effect was observed at 120 min (+30.3%,  $p < 0.001$ ) for the survival of P +  
116 PQ vs R + PQ flies. Note that PACAP had no effect by itself on fly survival (P + R vs R + R, Fig.

117 1A). We have previously reported that DA itself can also be lethal when applied at 20 mM or  
118 higher to VNC in the same way as PQ (39). Here we observed that PACAP pretreatment fully  
119 rescued the flies from the lethality induced by the application of a drop of DA at 35 mM to the  
120 VNC (Supplementary Material, Fig. S1). These results show that PACAP is protective against  
121 both PQ- and DA-induced neurotoxicity in the *Drosophila* CNS.

122 The N-terminal residues of PACAP (PACAP-38 and PACAP-27) are essential for its  
123 biological activity. Consequently, the deleted peptide PACAP-6-38 is inactive and it is also a  
124 potent antagonist of the PACAP-specific receptor PAC1-R (58). We therefore investigated the  
125 effect of PACAP-6-38 on PQ sensitivity in *Drosophila*. Strikingly, we found that decapitated  
126 flies pre-treated for 30 min with 2  $\mu$ M PACAP-6-38 were significantly more susceptible to PQ  
127 than control flies (Supplementary Material, Fig. S2). In the presence of PACAP-6-38, fly  
128 survival was reduced by 20% and 19% ( $p < 0.001$ ) 60 and 90 min after PQ application,  
129 respectively. This shows that PACAP and PACAP6-38 applied to the VNC have opposite effects  
130 on oxidative stress resistance in *Drosophila*.

131 To learn more about the mechanisms of PACAP-mediated protection, we analyzed the effects  
132 of this neuropeptide on PQ-induced increase in reactive oxygen species (ROS) and caspase  
133 activation levels in the VNC. We estimated ROS levels by dihydroethidium (DHE) staining in  
134 wild-type Canton-S (CS) flies pre-treated or not with PACAP. We found that PACAP-treated  
135 flies exhibited significantly lower dye fluorescence in the VNC after 90 min of PQ exposure ( $-$   
136 70%;  $p < 0.01$ ) (Fig. 1B and C). Similarly, using the CaspaseTracker biosensor system (59), which  
137 detects activation of the effector caspases, such as DrICE, that cleave protein substrates at a  
138 DQVD motif, we observed that PACAP pre-treatment significantly decreased the number of  
139 activated caspase-positive VNC cells in PQ-exposed decapitated flies ( $-40\%$ ;  $p < 0.01$ ) (Fig. 1D  
140 and E). Therefore, PACAP efficiently reduced the level of oxidative stress and caspase activation

141 under acute PQ intoxication in the *Drosophila* CNS.

## 142 **Neuronal downregulation of the PDF receptor decreases PQ susceptibility**

143 In order to search for the neuropeptide receptor potentially involved in PACAP-mediated  
144 neuroprotection, genes encoding G protein-coupled receptors for several neuropeptides of the  
145 *Drosophila* CNS were downregulated by targeted RNA interference (RNAi) with the pan-  
146 neuronal driver *elav-Gal4* and co-expression of the RNAi booster *Dcr-2*, and adult fly survival  
147 was monitored 90 min after application of PQ (Table 1). Downregulation of *Dh31-R*, *Dh44-R1*,  
148 *Dh44-R2*, *FMRFaR*, and *sNPF-R* did not affect fly PQ susceptibility, or the protective effect of  
149 PACAP from PQ-provoked neurotoxicity. In contrast, silencing the gene *han/Pdfr*, which  
150 encodes the receptor for the neuropeptide pigment-dispersing factor (PDF), significantly  
151 increased *Drosophila* resistance to PQ, and PACAP pre-treatment did not further increase the  
152 survival rate of these flies (Table 1). We then analyzed the survival kinetics of flies in which *Pdfr*  
153 was either downregulated (Fig. 2A) or overexpressed (Fig. 2B) in all neurons, respectively. This  
154 confirmed that *Pdfr* silencing by RNAi significantly counteracted PQ-induced lethality from the  
155 first hour of exposure, while there was no detectable effect of PACAP in these conditions (Fig.  
156 2A). In contrast, *Pdfr* overexpression in all neurons did not cause a noticeable effect on PQ  
157 susceptibility (Fig. 2B).

158 The receptor PDFR is widely expressed in the *Drosophila* clock neurons, and it is also  
159 expressed in non-clock brain cells as well as in peripheral clock cells (60, 61). Knocking down  
160 *Pdfr* by RNAi with the clock cell driver *tim-Gal4* (62, 63) conferred to decapitated flies a higher  
161 resistance to PQ, an effect that was highly significant between 90 and 150 min after PQ  
162 application (Fig. 2C). Such a protection (*tim>Pdfr*<sup>RNAi</sup>, *Dcr-2* flies, R + PQ) was comparable to  
163 that induced by PACAP pre-treatment on the controls (*Pdfr*<sup>RNAi/+</sup>, *Dcr-2/+*, P + PQ). Combining



164 the effects of PACAP pre-treatment and *Pdfr* knock down (i.e. *tim>Pdfr<sup>RNAi</sup>*, *Dcr-2*, P + PQ) did  
165 not further increase neuroprotection, indicating that these effects are apparently not additive (Fig.  
166 2C). Note that a similar level of protection against PQ was observed when *Pdfr* RNAi was  
167 expressed with *elav-Gal4* or *tim-Gal4* in a parallel experiment (Fig. 2D). We also tested the effect  
168 of silencing the PDF receptor in glial cells using *repo-Gal4*, but the survival rate of these flies  
169 was not increased, suggesting that this cell type is not involved in the protective effect  
170 (Supplementary Material, Fig. S3).

171 It may seem surprising that knocking down *Pdfr* with the clock cell driver *tim-Gal4* had a  
172 protective effect on decapitated flies, because no clock neurons are present in the adult  
173 *Drosophila* VNC (63). However, it has been reported that *tim-GAL4* drives GFP expression in  
174 some neurons that do not express the TIM protein at detectable levels in the adult brain (63, 64).  
175 We have checked that this is this also the case in the adult VNC. The pattern of membrane-  
176 associated GFP in the CNS of *tim>mCD8::GFP, nSyb::GFP* flies revealed that *tim-Gal4*  
177 expresses in several subsets of neurons in the abdominal ganglia (Supplementary Material, Fig.  
178 S4A). This region of the VNC also contain the abdominal (Abd) PDF neurons (65) and subsets of  
179 DA neurons (39). PDF co-immunostaining showed that *tim-Gal4* apparently does not label the  
180 Abd PDF neurons (Supplementary Material, Fig. S4B), in agreement with the observation that  
181 these cells do not express a molecular clock (65).

182

### 183 **Neuroprotection by PACAP requires PDF and the PDF receptor**

184 We then tested the PQ resistance of a *Pdfr* null mutant strain, *Pdfr<sup>5304</sup>* (49). This strain  
185 appeared slightly more resistant than wild-type CS controls between 30 and 90 min after PQ  
186 application, but the effect was not statistically significant (Fig. 3A). Strikingly, we observed that  
187 PACAP pre-treatment did not induce protection against PQ in *Pdfr<sup>5304</sup>* mutant flies, suggesting

188 that PDF receptor signaling is required for the protective action of PACAP (Fig. 3A). To further  
189 assess this idea, we used the *Pdf<sup>01</sup>* null mutant line that does not express PDF (66). PACAP-  
190 mediated protection was indeed abolished in this strain (Fig. 3B). Therefore, PACAP requires  
191 both PDF and PDFR expression to promote neuroprotection.

192 We noted that the lack of PDF or PDFR did not increase PQ resistance (Fig. 3B), in contrast to  
193 the effect induced by RNAi-mediated *Pdfr* knock down in all neurons or clock cells, suggesting  
194 that *Pdfr* downregulation in neuronal subsets can induce different effects than its total absence.  
195 This result may suggest that it is a constitutive, and not PDF-stimulated, activity of PDFR that  
196 increases PQ susceptibility in wild-type flies. Alternatively, spontaneous mutations accumulated  
197 over generations (67) might have compensated for the lack of PDFR signaling for this specific  
198 phenotype in the *Pdfr<sup>5304</sup>* and *Pdf<sup>01</sup>* mutants. To test these possibilities, we silenced PDF neuron  
199 activity by expressing the hyperpolarizing potassium channel Kir2.1 with the *Pdf-Gal4* driver  
200 (68). Interestingly, this led to significantly increased PQ resistance between 30 and 90 min of  
201 exposure (highest at 90 min: +27.7%,  $p < 0.001$ ) (Fig. 3C), indicating that PDF neuron activity is  
202 at least in part involved in PDFR-induced increase in PQ susceptibility. The rescue was not as  
203 strong as in the case of the *Pdfr<sup>RNAi</sup>* flies, which suggests that a constitutive activity of PDFR may  
204 indeed contribute to decrease oxidative stress resistance in wild-type flies. Furthermore, we  
205 observed that pre-treatment with 2  $\mu$ M (Fig. 3D), or even 20  $\mu$ M (Supplementary Material, Fig.  
206 S5), PDF had no effect on PQ resistance, suggesting that to increase PQ susceptibility in the  
207 CNS, not only PDF but also another signaling molecule is needed. This other molecule could be  
208 the unidentified classical neurotransmitter that is co-released with PDF by PDF neurons (69, 70).

### 209 **The protective action of PACAP requires PDFR expression in PDF and DA neurons**

210 PDFR is known to be expressed in the PDF-producing neurons themselves, allowing an

211 autocrine modulation of these cells by the neuropeptide they release (60, 71). We observed that  
212 silencing PDFR selectively in PDF neurons with *Pdf-Gal4* (72) did not lead to increased PQ  
213 resistance, but fully prevented PACAP neuroprotective effects (Fig. 4A). Therefore, PDFR  
214 expression in the PDF neurons appears essential for PACAP-mediated neuroprotection. A quite  
215 similar effect was observed when we restricted PDFR knock down to the Abd PDF neurons of  
216 the VNC, using the *Dot-Gal4* driver (65). PQ susceptibility did not increase while the protective  
217 effect of PACAP was lost (Fig. 4B). This suggests that the presence of PDFR in the Abd PDF  
218 neurons is required for PACAP-mediated neuroprotection. We next investigated whether other  
219 neuronal cells could be involved in the protective effect of PACAP against PQ oxidative stress.  
220 Since DA neurons are very sensitive to PQ toxicity (39), we evaluated the effect of *Pdfr*  
221 downregulation in these cells using *TH-Gal4* (73). Interestingly, this also led to loss of PACAP  
222 protective effects, without increasing PQ resistance (Fig. 4C). PDFR appears therefore required  
223 both in PDF and DA neurons for PACAP-mediated neuroprotection.

224 These results prompted us to search for potential synaptic connections between the PDF and  
225 DA neurons in the *Drosophila* VNC by using split-GFP reconstitution (74). The fusion protein n-  
226 syb::spGFP1–10, which is targeted to synaptic vesicles (75), was expressed in PDF neurons with  
227 *Pdf-Gal4* and the other membrane-associated GFP moiety, CD4::spGFP11, in DA neurons using  
228 the *TH-LexA* driver, respectively (*PDF>nSyb::GFP<sub>1-10</sub> + TH>CD4::spGFP<sub>11</sub>* flies).  
229 Reconstituted split GFP (rsGFP) fluorescence was clearly visible in prominent bilateral axonal  
230 bundles located laterally in the abdominal ganglia (Fig. 5, arrowheads). In contrast, no specific  
231 rsGFP signal could be detected in the abdominal ganglia of *TH>nSyb::GFP<sub>1-10</sub> +*  
232 *PDF>CD4::spGFP<sub>11</sub>* flies (data not shown). These observations suggest that PDF neurons  
233 synapse onto DA neurons in this region of the VNC and not the reverse. Interestingly, these  
234 lateral axons appear to be also targeted by *tim-Gal4* (Fig. S4, arrows), suggesting that the

235 protective effect in the VNC of *tim>Pdf<sup>RNAi</sup>* flies could specifically result from PDFR  
236 downregulation in these neurons. These lateral abdominal axons could be part of a neuronal  
237 network controlling oxidative stress susceptibility in *Drosophila*.

238

## 239 **Discussion**

### 240 **The neuroprotective function of PACAP is conserved in *Drosophila***

241 The neuropeptide PACAP has been widely shown to have potent antioxidant and anti-  
242 apoptotic properties in several neuronal cell types *in vitro*, including cultured rat cerebellar  
243 granule neurons and astroglial cells, and *in vivo* models of ischemia, stroke, traumatic brain  
244 injury, multiple sclerosis, and of various neurodegenerative diseases such as Huntington's chorea,  
245 Alzheimer's disease and PD (21, 22, 76–80). Its neuroprotective action against DA neuron  
246 degeneration has been demonstrated in drug-induced PD models in rodents, using pro-oxidant  
247 toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-  
248 OHDA), rotenone and PQ (22, 81). PACAP injection also prolonged the survival of pond snails  
249 (*Lymnaea stagnalis*) exposed to rotenone, and partly rescued the reduction in locomotor activity  
250 and CNS DA level triggered by the toxin in this invertebrate model (82).

251 PACAP is also a survival-promoting peptide in mammalian species, playing major and pivotal  
252 roles in immunity and inflammation, and extending thereby the survival of mice with ileitis  
253 (intestinal inflammation) (83) and rats subjected to kidney ischemia-reperfusion (84). PACAP<sup>-/-</sup>  
254 null mutant mice show a high early-mortality rate, in part related to increased susceptibility to  
255 cold stress (85), and are poorly resistant to doxorubicin-induced myocardial damage (86).  
256 Moreover, PACAP<sup>-/-</sup> mice are more sensitive to PQ-induced DA neuron depletion, suggesting  
257 that endogenous PACAP has a physiological neuroprotective action in the brain (25).

258 Here we examined the effect of PACAP in a widely used sporadic PD model induced by PQ  
259 exposure in *Drosophila* (34, 37, 39, 87). Since the fly orthologue of PACAP has not yet been  
260 identified with confidence (41), we used the amino acid sequence of PACAP-38, which is  
261 identical in all mammals, and because PACAP could not cross the intestinal barrier and remain  
262 intact, we directly applied the neuropeptide to the exposed VNC of decapitated *Drosophila*  
263 before PQ exposure, using a previously established procedure (39). We repeatedly observed that  
264 PACAP pre-treatment at 2  $\mu$ M prolonged the survival of wild-type decapitated *Drosophila*  
265 exposed to PQ and that it was also protective against neurotoxic DA concentrations. In contrast,  
266 an opposite effect was observed, that is, an increase in fly susceptibility to PQ, when the  
267 antagonist PACAP-6-38 was applied, which is structural similar to PACAP-38 except for a short  
268 deletion of 5 amino acids in the N-terminal that suppresses its activity. This indicates that the  
269 neuroprotection conferred by PACAP in flies is a genuine and specific effect that requires the  
270 same structural domain than for its protective action in vertebrate systems. In addition, the  
271 opposite effect of the PACAP antagonist may suggest that a still unidentified, most likely  
272 peptidic, endogenous molecule, binds on the same receptor as PACAP and contributes to  
273 oxidative stress resistance in the *Drosophila* nervous system. We attempted to overexpress  
274 amnesiac in neurons (*elav>amn* flies), but this had no effect on PQ resistance (data not shown),  
275 suggesting that amn is not the functional counterpart of PACAP in the flies.

276 As was shown in mammalian cultured cerebral cells, PACAP is able: *i*) to stimulate  
277 endogenous antioxidant system by increasing the level of glutathione, the major free radical  
278 scavenger in the brain, and superoxide dismutase (SOD) and catalase activity, and *ii*) to prevent  
279 inhibition of endogenous ROS defense system under oxidative stress (77, 79, 80). This suggests  
280 that the neuropeptide could block ROS overproduction and dampen oxidative stress through an  
281 upregulation of ROS-detoxifying enzymes *in vivo*. Consistent with this observation, it has been

282 shown that enhancing antioxidant SOD2 and glutathione peroxidase 1 (GPX1) activity by  
283 PACAP provided protection against oxidative damage and neuroprotection in mice with  
284 traumatic brain injury (88) and that inhibition of the endogenous antioxidant system in astrocytes  
285 suppresses the cell survival-promoting effect of PACAP under oxidative conditions (80). Here  
286 we show accordingly that PACAP efficiently alleviated the increase in ROS accumulation  
287 triggered by PQ in the fly VNC. A possible effect of PACAP on the activity of endogenous  
288 antioxidant systems in PQ-treated *Drosophila* could thus be a key player in its mechanisms of  
289 action. These results are striking because PQ is a very strong inducer of ROS production in  
290 *Drosophila* (18, 39, 40, 87, 89), and we found here that a low dose of PACAP was sufficient to  
291 largely prevent the neurotoxic effects of this herbicide.

292 Previous work demonstrated that PACAP prevents cerebellar granule neurons from apoptotic  
293 cell death through inhibition of caspase-3 activity (90). Comparably, in the mollusk *Helix*  
294 *pomatia*, PACAP efficiently attenuated caspase-3 activation induced by cytotoxic levels of DA  
295 (91). We have thus investigated the effect of PACAP on PQ-induced activation of effector  
296 caspases in the *Drosophila* CNS with the CaspaseTracker biosensor system (59). PQ is known to  
297 cause an increase in caspase activity, both in mice and *Drosophila* (40, 92, 93). Pre-treatment of  
298 the flies with the neuropeptide PACAP significantly decreased caspase activation under PQ in the  
299 VNC, possibly resulting from oxidative stress reduction. It has been reported indeed that PACAP  
300 can regulate the expression of Bcl-2 family members to prevent apoptotic cell death provoked by  
301 oxidative insult in neuronal cells (21, 93, 94).

### 302 **PDFR signaling significantly contributes to PQ neurotoxicity in the *Drosophila* CNS**

303 How can such a tiny amount of PACAP (40,000 times less than PQ) prevent oxidative stress-  
304 induced defects in the whole VNC and thus significantly prolong the survival of PQ-intoxicated

305 decapitated *Drosophila*? To explore the mechanisms of action of this neuropeptide, we first used  
306 pan-neuronal RNAi downregulation to search among GPCR receptors of known fly  
307 neuropeptides for one that might be implicated in the protective effect of PACAP. Among the six  
308 GPCRs that were tested, only PDFR was identified as being involved in the modulation of PQ  
309 sensitivity and PACAP-mediated neuroprotection. Indeed, downregulating this receptor in all  
310 neurons with *elav-Gal4*, or in *tim-Gal4*-targeted cells, was sufficient to protect efficiently the  
311 flies from PQ-induced early lethality. This indicates that PDFR signaling, which acts through  
312 elevated cAMP/PKA activity (51, 69, 71, 95–99) and modulation of Ca<sup>2+</sup> level and activities (51,  
313 96, 98, 100, 101), potently contributes to PQ susceptibility in *Drosophila*. In contrast, *Pdfr*<sup>5304</sup>  
314 and *Pdfr*<sup>0</sup> null mutants did not appear more resistant to PQ than wild-type flies, which might be  
315 related to compensating spontaneous mutations accumulated in the mutant genomes over  
316 generations (67), or, alternatively, to the absence of PDFR signaling at all stages and in all cells  
317 in the mutants compared to a relative downregulation in neurons only in the RNAi experiment.  
318 Note that *Pdfr* knock down by RNAi in PDF neurons was not sufficient to decrease PQ  
319 susceptibility, indicating that enhanced PQ resistance requires targeting other *Pdfr*-expressing  
320 neurons.

321 The fact that PDF release is involved in PDFR-induced increase in PQ susceptibility was  
322 suggested by the Kir2.1 experiment, in which *in vivo* silencing of PDF neurons with this  
323 potassium channel significantly enhanced PQ resistance of the flies. However, we show here that  
324 direct PDF application to the VNC of decapitated flies had no effect on PQ susceptibility, and we  
325 also observed that pan-neuronal expression of membrane-tethered PDF (t-PDF) (99) similarly did  
326 not affect PQ sensitivity of the flies (data not shown). This suggests that an additional molecule is  
327 required, potentially the classical transmitter that is co-released with PDF by PDF neurons (69).  
328 Indeed, these last authors showed the release of this co-transmitter is also increased by PDFR

329 auto-receptor activation.

330 By which mechanisms could PDFR signaling contribute prominently to PQ neurotoxicity is a  
331 matter of speculation. It has been demonstrated that PDF can convey signals that lead to  
332 activation of caspases and enhanced neurodegeneration in target cells in several parts of the  
333 *Drosophila* brain (102). This occurred in young flies with circadian dysfunction and in older  
334 wild-type flies. These effects correlated with enhanced tauopathy in a fly model of Alzheimer's  
335 disease (102). We have also recently reported that a mutation in the circadian gene *Clock* (*Clk*<sup>AR</sup>),  
336 or RNAi-induced *Clk* knock down in PDF neurons, led to accelerated age-related locomotor  
337 decline and apoptosis-related loss of DA neurons in the PPL1 cluster, all effects which were  
338 found to be strictly dependent on PDFR signaling (103). These and other findings suggest the  
339 implication of the circadian system and PDF/PDFR signaling in age-related loss of physical or  
340 cognitive abilities and enhanced neurodegeneration in *Drosophila* (104–106).

341 The ROS-related disturbance induced by PQ exposure could trigger the depolarization of PDF  
342 neurons, thus promoting caspase activation and apoptosis in *Pdfr*-expressing target cells, and the  
343 increased resistance to PQ when *Pdfr* is downregulated in *tim*-expressing neurons might be the  
344 result of the negative regulation of these molecular events. PDF neuron overactivation could  
345 directly or indirectly lead to similar defects in DA neurons, which would result in abnormally  
346 high level of DA release, inducing cytotoxic and excitotoxic effects through overstimulation of  
347 the D<sub>1/5</sub>-like DA receptor DAMB, which, as we have previously reported, can provoke calcium  
348 release in the cytosol of glutamatergic neurons through the ryanodine receptor and finally  
349 contribute to nervous system failure in PQ-intoxicated flies (39).

### 350 **PACAP neuroprotection depends on PDFR signaling in PDF and DA neurons**

351 We observed that PACAP pre-treatment did not prolong the survival of PQ-exposed null *Pdfr*<sup>01</sup>



352 or *Pdfr*<sup>5304</sup> mutants that are deficient for PDF and PDFR, respectively, at variance with the potent  
353 protective effect of this neuropeptide on wild-type flies. No effect of PACAP could be detected  
354 after *Pdfr* downregulation in all neurons or in all clock cells, but this could be because PQ  
355 resistance was already much increased in these flies. In contrast, targeting *Pdfr* RNAi in PDF  
356 neurons selectively did not increase PQ resistance and instead fully prevented PACAP protective  
357 effects. Furthermore, restricting *Pdfr* RNAi knock down in the VNC Abd PDF neurons also  
358 suppressed the beneficial effect of PACAP pre-treatment for fly survival under acute oxidative  
359 stress. Although PDFR has binding affinity for PACAP *in vitro* (51), PACAP is apparently not an  
360 agonist of this receptor since it has been shown that its application to dissected brain did not  
361 significantly alter the FRET signal of a cAMP sensor in the PDF neurons that express PDFR  
362 (71).

363 In adult *Drosophila*, there are only ~24 PDF-expressing neurons in the CNS: four or five large  
364 (l-LN<sub>v</sub>s) and four small (s-LN<sub>v</sub>s) ventral lateral neurons per hemisphere in the brain, and four  
365 large (l-Abd) and four small (s-Abd) abdominal neurons in the VNC (98, 107, 108). It is known  
366 that brain PDF neurons (the s-LN<sub>v</sub>s and some of the l-LN<sub>v</sub>s) express PDFR autoreceptors (60,  
367 69). The l-Abd and s-Abd PDF neurons do not express the molecular clock and the PDF  
368 neuropeptides released from these neurons are not required for locomotor rhythms (65).  
369 Nevertheless, both the brain LN<sub>v</sub>s and VNC Abd PDF neurons contribute to the normal phasing  
370 of the molecular clock in the oenocytes, which are pheromone-producing peripheral clock cells  
371 (61, 98). Furthermore, only the PDF released from the Abd PDF neurons is necessary to  
372 maintain wild-type expression levels of male sex pheromones (61).

373 Since we have performed our experiments on decapitated flies, it is natural to consider that the  
374 PDF neurons in which PDFR is required for PACAP neuroprotection are specifically the Abd  
375 neurons. This seems to be supported indeed by the fact that *Dot>Pdfr*<sup>RNAi</sup> decapitated flies are not

376 sensitive to PACAP. However, we cannot exclude that long-term PDFR deficiency in the brain  
377 PDF neurons might have a distant influence on other organs, including the VNC, that would  
378 contribute to PACAP-induced protection. Such an influence would have to be indirect because it  
379 is known that the brain PDF neurons do not send descending projections to the VNC (108). It is  
380 quite possible that, like for the phasing of oenocyte molecular clock, full PACAP neuroprotection  
381 requires PDFR expression in both the brain and VNC PDF neurons. Note that the same issue  
382 arises for the higher PQ resistance of decapitated flies in which *Pdfr* was downregulated with *tim-*  
383 *Gal4*. It is possible that the higher PQ resistance of *tim>Pdfr<sup>RNAi</sup>* flies results from long-term  
384 PDFR deficiency in the brain clock neurons that would make decapitated flies less susceptible to  
385 PQ neurotoxicity by a systemic mechanism. However, we show here that *tim-Gal4* expresses in  
386 subsets of neurons in the abdominal ganglia, some of which could express PDFR (the PDFR  
387 pattern has not been described in the VNC to date) and be directly involved in the control of PQ  
388 susceptibility in the CNS.

389 Finally, we observed that downregulating *Pdfr* in DA neurons also prevented PACAP-  
390 mediated neuroprotection. As in the case of PDF neurons, both the brain and VNC DA neurons  
391 could be involved in this effect, even though we used decapitated flies for the survival test. A  
392 recent report provided evidence that a subset of dopaminergic neurons respond to PDF and that  
393 the s-LN<sub>v</sub> axons and some brain DA neurons form synaptic contacts in the brain (109). As  
394 mentioned above, we have also previously shown that *Clk* downregulation in the sLN<sub>v</sub>s leads to  
395 PDFR-dependent degeneration of DA neurons (103). Here we show using split-GFP  
396 reconstitution that Abd PDF neurons also contact DA neurons in the abdominal region of the  
397 VNC, before these PDF neurons send projections out of the VNC (98). The synapses appear  
398 distributed along large bilateral axonal bundles. The fact that the silencing of PDFR in either PDF  
399 or DA neurons has no effect on the sensitivity of the flies to PQ, may suggest that PDFR has to

400 be knocked down in both cell types and probably in other clock neurons to increase PQ  
401 resistance. Remarkably, downregulating PDFR either in PDF or DA neurons was sufficient to  
402 fully prevent PACAP neuroprotection, indicating that PACAP must act on these two neuronal  
403 subsets to protect flies from PQ.

404 In conclusion, our current findings indicate that the neuropeptide PACAP offers a potent  
405 protection against PQ neurotoxicity in *Drosophila* and that this effect can be ascribed to the  
406 modulation of PDF/PDFR signaling in specific neuronal subsets. Further studies are required to  
407 characterize the signaling pathways that trigger this neuroprotective effect and so better  
408 understand the neuronal circuits and mechanisms controlling oxidative stress susceptibility in the  
409 *Drosophila* CNS.

410

## 411 **Materials and methods**

### 412 ***Drosophila* culture and strains**

413 Fly stocks were raised at 25°C on standard cornmeal-yeast-agar medium supplemented with  
414 methyl-4-hydroxy-benzoate as a mold protector, under 12 h-12 h light-dark cycle and 70%  
415 humidity. The following strains were used: Canton-S (CS) as wild type, *Pdfr*<sup>5304</sup> (49), *Pdf*<sup>01</sup> (66),  
416 *elav-GAL4* (110), *repo-GAL4* (111), *TH-Gal4* (73), *TH-LexA* (112) (kindly provided by Dr.  
417 Ronald L. Davis), *tim-Gal4* (62), *Pdf-Gal4* (72), *Dot-Gal4* (65), *UAS-Pdfr* (49) (kindly provided  
418 by Dr. François Rouyer), *UAS-Kir2.1* (113) (kindly provided by Dr. Sean Sweeney), recombined  
419 *UAS-mCD8::GFP*, *UAS-nSyb::GFP* (114), and from the Bloomington *Drosophila* Stock Center  
420 (BDSC): *elav-Gal4*; *UAS-Dcr-2* (BDSC #25750); *UAS-Dcr-2*; *Pin<sup>1</sup>/CyO* (BDSC #24644),  
421 *LexAop-n-syb::spGFP<sub>1-10</sub>*, *UAS-CD4::spGFP<sub>11</sub>* (BDSC #64314); *UAS-n-syb::spGFP<sub>1-10</sub>*, *LexAop-*  
422 *CD4::spGFP<sub>11</sub>* (BDSC #64315), and the TRiP RNAi strains: JF01945 (BDSC #25925), JF03208

423 (BDSC #28780), JF03289 (BDSC #29610), JF02657 (BDSC #27507), HMJ02073 (BDSC  
424 #42508) and JF01879 (BDSC #25858), to knock down *Dh31-R*, *Dh44-R1*, *Dh44-R2*, *sNPF-R*,  
425 *Pdfr* and *FMRFaR*, respectively.

#### 426 **PQ and PACAP application and survival score**

427 PQ and PACAP treatments were performed on 9 to 11-day-old adult females by direct  
428 application of the drug and neuropeptide onto the exposed VNC of decapitated flies as previously  
429 described (39). Briefly, flies were anaesthetized on ice for 10 min and their heads were cut off  
430 with 7-mm blade spring scissors (Fine Science tools). ~30 decapitated flies per condition were  
431 transferred to a 2-inch Petri dish (10 flies per dish) and allowed to recover for a few minutes until  
432 they stood on their legs. A 5 µl droplet of PQ (methyl viologen dichloride hydrate; Sigma-  
433 Aldrich 856177) diluted in *Drosophila* Ringer's solution (in mM: 130 NaCl, 4.7 KCl, 1.8 CaCl<sub>2</sub>,  
434 0.5 Na<sub>2</sub>HPO<sub>4</sub>, 0.35 KH<sub>2</sub>PO<sub>4</sub>, pH 7.4 adjusted with 150 Na<sub>2</sub>HPO<sub>4</sub>) or Ringer's only (R) for  
435 controls, was applied for 5 s with a P10 Pipetman. The same droplet was successively used for 10  
436 flies. A concentration of 80 mM PQ was generally used that gave 30-40 % survival of wild type  
437 flies after 2 h (39). Flies were considered as dead when they laid on the side or back and did not  
438 react to a slight mechanical stimulus on the legs. Survival rate was monitored every 30 min. Each  
439 experiment was repeated independently at least twice. The same procedure was used for DA  
440 application to the VNC except that DA concentration in the droplet was 35 mM, as previously  
441 described (39). For PACAP, PACAP-6-38 and PDF pretreatments, a droplet containing the  
442 neuropeptide at 2 µM (precisely 2.2 µM, i.e. 10, 8.9 and 4.3 µg/ml, respectively), in Ringer's, or  
443 20 µM for PDF in some experiments, was applied to the decapitated flies the same way 30 min  
444 prior to the exposure to PQ. The 38-amino acid form of PACAP (His-Ser-Asp-Gly-Ile-Phe-Thr-  
445 Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Ala-Ala-Val-Leu-Gly-

446 Lys-Arg-Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys-NH<sub>2</sub>) was synthesized by solid-phase methodology  
447 as previously described (115). PACAP-6-38 was purchased from Tocris Bioscience, Bristol, UK,  
448 and *Drosophila* PDF (Asn-Ser-Glu-Leu-Ile-Asn-Ser-Leu-Leu-Ser-Leu-Pro-Lys-Asn-Met-Asn-  
449 Asp-Ala-NH<sub>2</sub>) from NovoPro Bioscience Inc., Shanghai, China.

#### 450 **Detection of caspase activity**

451 We have used the CaspaseTracker biosensor system to detect caspase activity in VNC cells of  
452 adult flies exposed to PQ. This system is composed of two genetic components: a biosensor  
453 encoding the Gal4 transcription factor sequestered in the cytoplasm by a caspase-cleavable  
454 membrane anchor (59), and the G-TRACE fluorescent protein system (116). Following caspase  
455 activation, Gal4 is released from its membrane anchor, translocates to the nucleus and drives the  
456 expression of both cytosolic red fluorescent protein (RFP), indicating recent or on-going caspase  
457 activity, and FLP recombinase, which induces permanent expression of nucleus-targeted GFP.  
458 10-11-day-old CaspaseTracker female flies were pretreated with either Ringer's alone (controls)  
459 or 10 µg/ml PACAP in the same solution for 30 min before applying PQ. After 90 min of PQ  
460 treatment, 4 or 5 VNCs per condition were dissected in Ca<sup>2+</sup>-free Ringer's, fixed in 4% (wt/vol)  
461 paraformaldehyde in phosphate-buffered saline (PBS: 130 mM NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM  
462 KH<sub>2</sub>PO<sub>4</sub>), washed 3 times in PBS and then mounted for confocal microscopy examination.

#### 463 **ROS measurement**

464 ROS detection in the *Drosophila* CNS was performed using the dihydroethidium (DHE) dye  
465 (Life technologies) following a previously described procedure (117, 118). 10-day-old  
466 decapitated female flies were exposed to PQ for 90 min with or without (control) 30-min  
467 pretreatment with PACAP. Their VNCs were dissected in Schneider's Insect Medium, and  
468 incubated in 30 µM DHE in the same medium for 5 min in the dark. After 5 min of fixation in

469 7% formaldehyde in PBS (pH 7, at room temperature), the VNCs were immediately imaged on a  
470 confocal microscope, as indicated below. Relative ROS levels were measured by quantification  
471 of the average intensity of the dye fluorescence using the Fiji software (119).

#### 472 **Immunohistochemistry and split-GFP reconstitution**

473 Whole-mount VNC immunostaining was performed as previously described (39, 120). The  
474 primary antibodies were mouse monoclonal anti-GFP (Sigma-Aldrich G6539, 1:200 or 1:250),  
475 rabbit polyclonal anti-PDF (kindly provided by Dr. François Rouyer, 1:100) and rabbit  
476 polyclonal anti-TH (Novus Biologicals NB300-109, 1:1000). The secondary antibodies were goat  
477 anti-mouse and anti-rabbit conjugated to Alexa Fluor 488 or 555 (Invitrogen Molecular Probes,  
478 1:250 or 1:1000). For the visualization of potential synaptic connectivity by split-GFP  
479 reconstitution (GRASP method) (74, 75), the *Drosophila* lines *LexAop-n-syb::spGFP<sub>1-10</sub>*, *UAS-*  
480 *CD4::spGFP<sub>11</sub>* and *UAS-n-syb::spGFP<sub>1-10</sub>*, *LexAop-CD4::spGFP<sub>11</sub>* were crossed to the  
481 recombined driver line *Pdf-Gal4; TH-LexA*. The VNCs of 7-10-day-old female flies were  
482 dissected and processed for TH and GFP co-immunostaining.

#### 483 **Fluorescence confocal microscopy**

484 VNCs were mounted on slides using as antifade reagent, either ProLong Gold Antifade  
485 reagent (ThermoFisher Scientific) for CaspaseTracker staining, or Vectashield (Vector  
486 Laboratories) for ROS measurements. Images were acquired with a Nikon A1R confocal  
487 microscope. A minimum of 4 or 5 VNCs were scored over at least 3 trials. Laser, filter and gain  
488 settings remained constant within each experiment, and channels were scanned sequentially.  
489 Confocal Z-stacks were analyzed and processed using the Fiji software.

#### 490 **Statistical analysis**

491 Statistical analyses were performed with Prism 6 (GraphPad Software, La Jolla, CA, USA),  
492 using either one-way or two-way ANOVA with Tukey's *post-hoc* multiple comparison test, or  
493 Student's *t* test. Errors bars represent standard errors of the mean (SEM) of 3 or 4 independent  
494 determinations. Statistical significance in all figures: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

495

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507

## 508 **Conflict of interest statement**

509

510 The authors have no conflict of interest to declare.

511

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513

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## Figures Legend

**Figure 1.** PACAP decreases PQ-induced oxidative stress and caspase activation in the *Drosophila* CNS. **(A)** Application of 80 mM PQ to the VNC decreased fly survival compared to controls treated with Ringer's (R) solution only (R + PQ vs R + R, ### $p < 0.001$ ). Pretreatment with 2  $\mu$ M PACAP (P) during 30 min had no effect by itself (P + R vs R + R) but markedly delayed PQ-induced lethality. The protective action of PACAP was significant until 3 hours after PQ application (P + PQ vs R + PQ, \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). Two-way ANOVA with Tukey's *post-hoc* test. **(B, C)** Evaluation of ROS levels in the VNC after 90 min of PQ exposure. **(B)** Flies pretreated with 2  $\mu$ M PACAP (*right panel*) showed reduced DHE fluorescent staining indicating lower ROS in the VNC than flies exposed to PQ only (*left panel*). Scale bar: 50  $\mu$ m. **(C)** Quantification of the effect of PACAP on PQ-induced ROS accumulation in the VNC. Student's t test, \*\* $p < 0.01$ . **(D, E)** Effect of PACAP on caspase activation induced by PQ exposure after 90 min, as detected by the CaspaseTracker biosensor system. **(D)** Pre-application of 2  $\mu$ M PACAP significantly decreased the number of activated caspase-positive VNC cells in decapitated flies treated with PQ (*right panel*) compared to flies exposed to PQ only (*left panel*). Scale bar: 50  $\mu$ m. **(E)** Quantification of the effect of PACAP on PQ-induced caspase activation in VNC cells. Student's t test, \*\* $p < 0.01$ .

**Figure 2.** *Pdfr* knock down in all neurons or all clock cells increases PQ resistance. **(A)** Survival kinetics of PQ-intoxicated *elav>Pdfr<sup>RNAi</sup>, Dcr-2* flies compared to *elav>Dcr-2* controls, pre-treated (P + PQ) or not (R + PQ) with PACAP. *Pdfr* downregulation in all neurons strongly mitigated PQ-induced lethality (as also shown in Table 1) and the survival of these flies was not further increased by PACAP, in contrast to the *elav>Dcr-2* control flies. **(B)** Overexpression of *Pdfr* in all

neurons (*elav>Pdf*) did not alter *Drosophila* PQ susceptibility, as compared to the heterozygous driver (*elav/+*) and effector (*Pdf/+*) controls. (C) *Pdf* downregulation in all clock cells with the *tim-Gal4* driver (*tim>Pdf<sup>RNAi</sup>*, *Dcr-2*) significantly protected flies against PQ compared to the survival rate of *Pdf<sup>RNAi/+</sup>*; *Dcr-2/+* controls (R + PQ). No further protection could be observed by PACAP pre-application on the *tim>Pdf<sup>RNAi</sup>*, *Dcr-2* flies, whereas the *Pdf<sup>RNAi/+</sup>*; *Dcr-2/+* controls were strongly protected by PACAP (P + PQ). (D) Monitoring the survival of PQ-treated *elav>Pdf<sup>RNAi</sup>*, *Dcr-2* and *tim>Pdf<sup>RNAi</sup>*, *Dcr-2* flies in a parallel experiment showed similar levels of protection compared to *Pdf<sup>RNAi/+</sup>*; *Dcr-2/+* controls when *Pdf* was down-regulated in neurons (\*) or in clock cells (#). Two-way ANOVA with Tukey's *post-hoc* test: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, ##*p* < 0.001, ###*p* < 0.001, ns: not significant.

**Figure 3.** Lack of PACAP-induced neuroprotection in *Pdf* and *Pdf* null mutants. (A) In the absence of PDF receptor (mutant *Pdf<sup>5304</sup>*), PQ resistance was not significantly different(ns) from that of wild-type CS flies (*Pdf<sup>5304</sup>*, R + PQ vs CS, R + PQ), and no protective effect of PACAP against PQ could be observed in these mutant flies (*Pdf<sup>5304</sup>*, P + PQ vs R + PQ). (B) Without expressed PDF neuropeptide (mutant *Pdf<sup>01</sup>*), flies were also not more resistant to PQ than wild type, and again PACAP did not protect against PQ (ns). (C) The hyperpolarization of PDF-releasing neurons (*Pdf>Kir2.1* flies) had no effect in normal conditions (Ringer, R) but increased PQ resistance at 60 and 90 min of exposure compared to driver (PDF/+) and effector (Kir2.1/+) controls. (D) Direct pre-application of 2 μM PDF neuropeptide on the VNC of decapitated flies did not affect their survival rate under control (PDF + R versus R +R) or PQ-intoxicated (PDF + PQ versus R + PQ) conditions (ns). Two-way ANOVA with Tukey's *post-hoc* test: \**p* < 0.05, \*\*\**p* < 0.001, ns: not significant.

**Figure 4.** Selective *Pdfr* knock down in PDF or DA neurons prevents PACAP-mediated neuroprotection. **(A)** *Pdfr* downregulation targeted to PDF neurons with the *Pdf-Gal4* driver did not protect against PQ but efficiently suppressed PACAP-induced increase in PQ resistance, i.e. the survival of *Pdf>Pdfr<sup>RNAi</sup>, Dcr-2* flies was not different in P + PQ and R + PQ conditions. The control flies (*Pdf-Gal4/+*) were in contrast normally protected by a PACAP pre-treatment (P + PQ vs R + PQ: \*\**p* < 0.01, \*\*\**p* < 0.001). **(B)** *Pdfr* knock down selectively in the Abd PDF neurons using *Dot-Gal4* also suppressed the protective effect of PACAP and did not increase PQ resistance. The survival of *Dot>Pdfr<sup>RNAi</sup>, Dcr-2* flies was similar in P+PQ and R+PQ conditions, whereas PACAP pre-treatment fully protected the control flies (*Dot-Gal4/+*) from PQ toxicity (P+PQ vs R+PQ: \*\**p* < 0.01, \*\*\**p* < 0.001). **(C)** PDFR downregulation in DA neurons with *TH-Gal4* (*TH>Pdfr<sup>RNAi</sup>, Dcr-2*) similarly prevented PACAP-induced neuroprotection compared to controls (i.e. survival of *TH>Pdfr<sup>RNAi</sup>, Dcr-2* flies was not significantly different in P + PQ and R + PQ conditions at all times) and also did not change PQ susceptibility, whereas the effector control flies (*Pdfr<sup>RNAi</sup>/+; Dcr-2/+*) were well protected by PACAP pre-treatment (P + PQ vs R + PQ: \**p* < 0.05, \*\**p* < 0.01). Two-way ANOVA with Tukey's *post-hoc* test.

**Figure 5.** Identification of potential synaptic connections between PDF and DA neurons in the *Drosophila* VNC by split-GFP reconstitution. The fusion protein n-Syb::spGFP<sub>1-10</sub>, which is targeted to synaptic terminals, was expressed in PDF neurons with *Pdf-Gal4* (*PDF>nSyb::GFP<sub>1-10</sub>*) and the fusion membrane protein CD4::spGFP<sub>11</sub> was expressed in DA neurons with *TH-LexA* (*TH>CD4::spGFP<sub>11</sub>*). TH (magenta) and GFP (green) co-immunostaining. **(A)** TH immunostaining. **(B)** GFP immunostaining. **(C)** Merge. Reconstituted split GFP (rsGFP) fluorescence specifically labelled bilateral axon bundles of DA neurons localized in the abdominal ganglia of the VNC (arrowheads in **B** and **C**). Scale bar: 100 μm.

**Table 1.** Effect of neuropeptide receptor downregulation on *Drosophila* PQ susceptibility

Fly genotype	<i>elav&gt;Dcr-2</i>		<i>elav&gt;R<sup>RNAi</sup>, Dcr-2</i>	
	PQ	PACAP + PQ	PQ	PACAP + PQ
<i>DH31-R</i>	45	85***	49	84***
<i>DH44-R1</i>	54	76**	46	62,5*
<i>DH44-R2</i>	34	71***	45	72**
<i>sNPF-R</i>	46	75***	38	71***
<i>FMRFaR</i>	41	65**	39	59*
<i>Pdfr</i>	45	90***	77 <sup>###</sup>	82

Genes encoding various neuropeptide GPCRs were knocked down by co-expressing specific interfering double-stranded RNAs ( $R^{RNAi}$ ) and the RNAi booster *Dcr-2* in all neurons with the driver *elav-Gal4*(*elav>R<sup>RNAi</sup>, Dcr-2*flies) or *Dcr-2* only for controls (*elav>Dcr-2*flies). The numbers indicate the percent of surviving flies 90 min after application of PQ without (PQ) or with (PACAP + PQ) pre-treatment with 2  $\mu$ M PACAP. Statistical significance: PACAP + PQ vs PQ, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; PQ (*elav>R<sup>RNAi</sup>, Dcr-2*)vsPQ (*elav>Dcr-2*), <sup>###</sup> $p < 0.001$ . One-way ANOVA with Tukey's post-hoc multiple comparison test.

## **Abbreviations used**

Clk, Clock; CNS, central nervous system; CS, Canton-S; DA, dopamine; DA neurons, dopaminergic neurons; DHE, dihydroethidium; GFP: green fluorescent protein; GPCR, G protein-coupled receptor; GPX1, glutathione peroxidase 1; GRASP, GFP reconstitution across synaptic partners; GSH, glutathione; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ns: not significant; PACAP, pituitary adenylate cyclase-activating polypeptide; PBS, phosphate-buffered saline; PD, Parkinson's disease; PDF, Pigment dispersing factor; PDFR, PDF receptor; PKA, protein kinase A; PQ, paraquat/1,1'-dimethyl-4,4'-bipyridinium; RFP, red fluorescent protein; RNAi, RNA interference; ROS, reactive oxygen species; rsGFP: reconstituted split GFP; SNpc, substantia nigra pars compacta; SOD, superoxide dismutase; TH, tyrosine hydroxylase; VIP, vasoactive intestinal peptide; VNC, ventral nerve cord; vs: versus.

## Supplementary figure legends

**Figure S1.** PACAP protects *Drosophila* against DA neurotoxicity. Application of 35 mM DA to the exposed VNC of decapitated flies significantly decreased fly survival compared to controls (R + DA vs R + R, # $p < 0.05$ , ## $p < 0.01$ ). A 30-min pretreatment with 2  $\mu$ M PACAP prevented DA-induced fly death (P + DA vs R + DA, \* $p < 0.05$ , \*\*\* $p < 0.001$ ). Two-way ANOVA with Tukey's *post-hoc* test. (Related to Figure 1).

**Figure S2.** The PACAP receptor antagonist PACAP-6-38 increases *Drosophila* susceptibility to PQ. Pre-treatment with 2  $\mu$ M PACAP-6-38 (P-6-38) for 30 min significantly accelerated the death of decapitated flies intoxicated by PQ (P-6-38 + PQ vs R + PQ, \*\*\* $p < 0.001$ ), compared to control flies pre-treated with Ringer's only (R + PQ vs R + R, ### $p < 0.001$ ), indicating that the antagonist makes *Drosophila* more sensitive to oxidative stress. PACAP-6-38 had no effect by itself on fly survival in the absence of PQ (P-6-38+R vs R+ R). Two-way ANOVA with Tukey's *post-hoc* test. (Related to Figure 1).

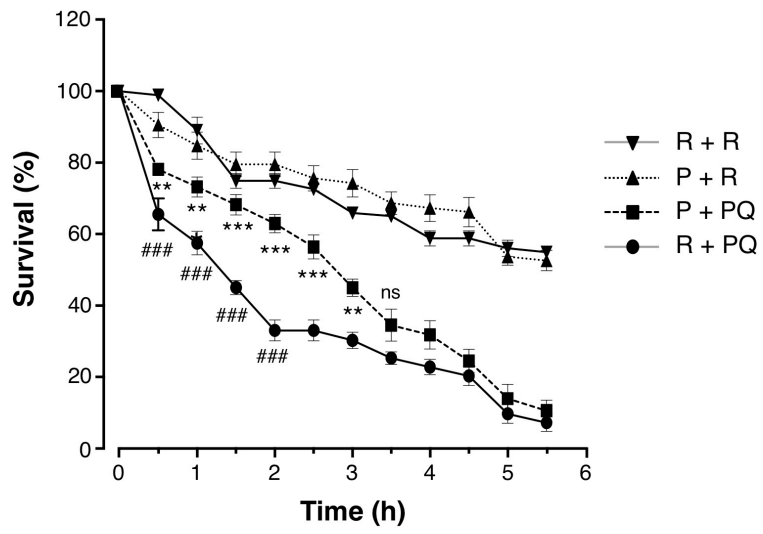
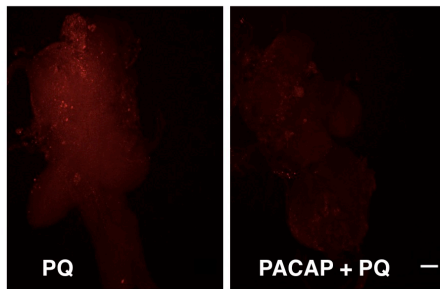
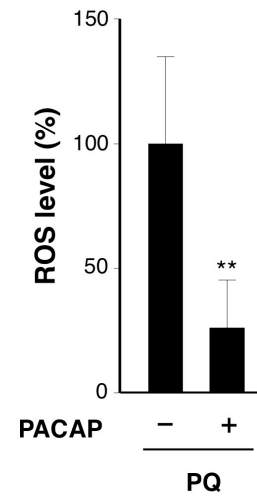
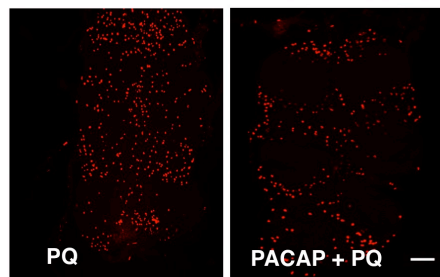
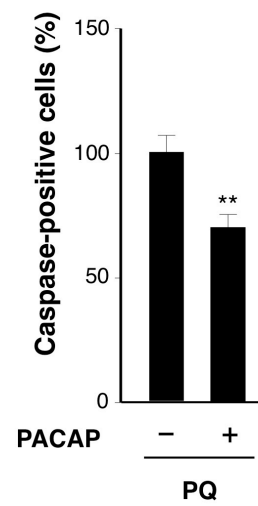
**Figure S3.** *Pdfr* downregulation in glial cells does not increase fly PQ resistance. The survival rate of PQ-treated *repo>Pdfr<sup>RNAi</sup>*; *Dcr-2* decapitated flies was not improved compared to *Pdfr<sup>RNAi/+</sup>*; *Dcr-2/+* controls (ns: not significant). (Related to Figure 2).

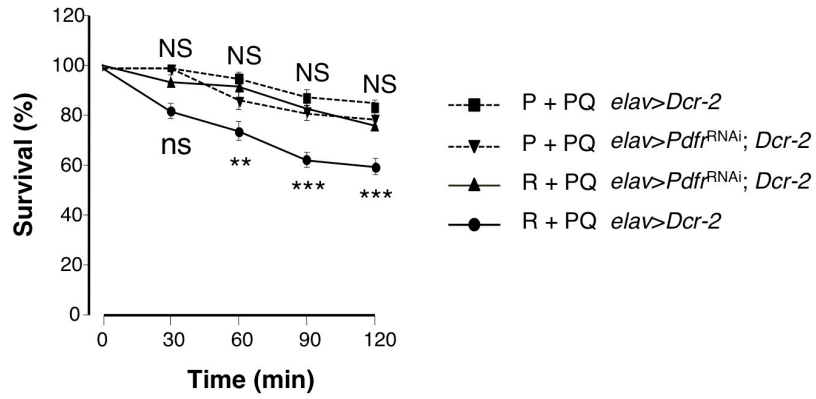
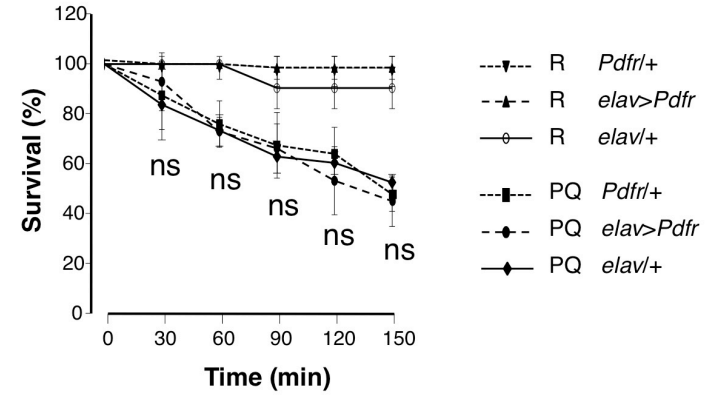
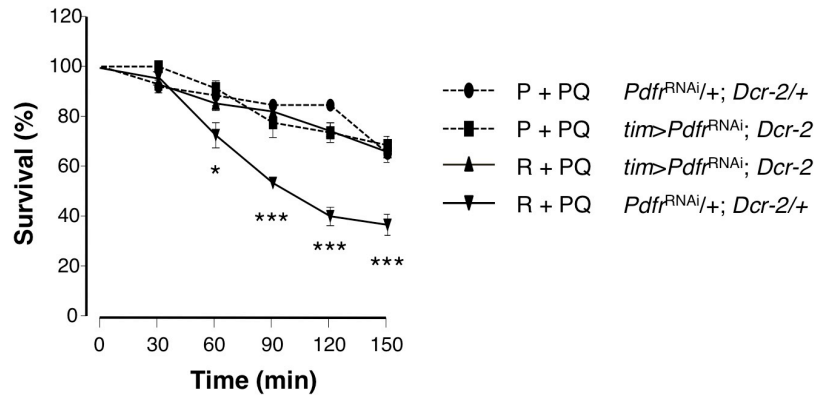
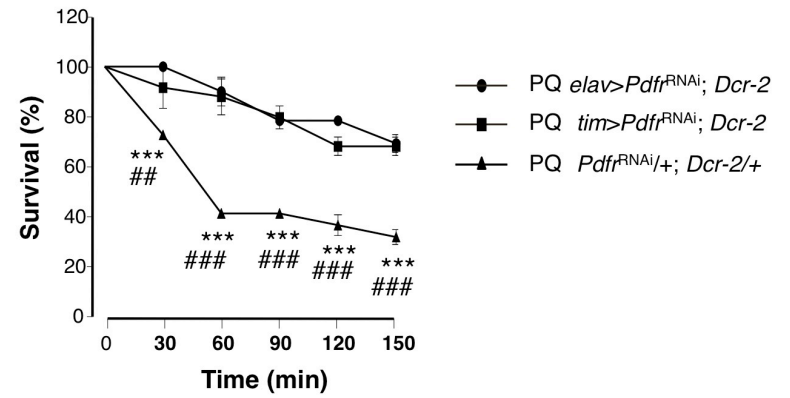
**Figure S4.** Expression pattern of the *tim-Gal4* driver in the abdominal ganglia. (A, B) Whole-mount GFP and PDF co-immunostaining in the VNC of young adult *Drosophila* expressing membrane-associated GFP under *tim-Gal4* control (*tim>mCD8::GFP*, *nSyb::GFP* flies). (A) *tim-*

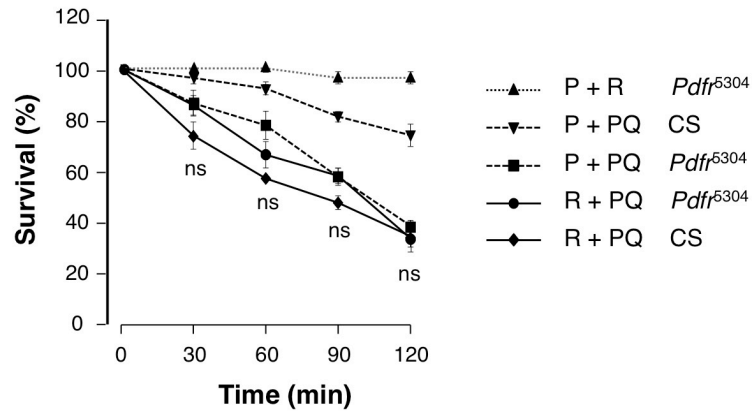
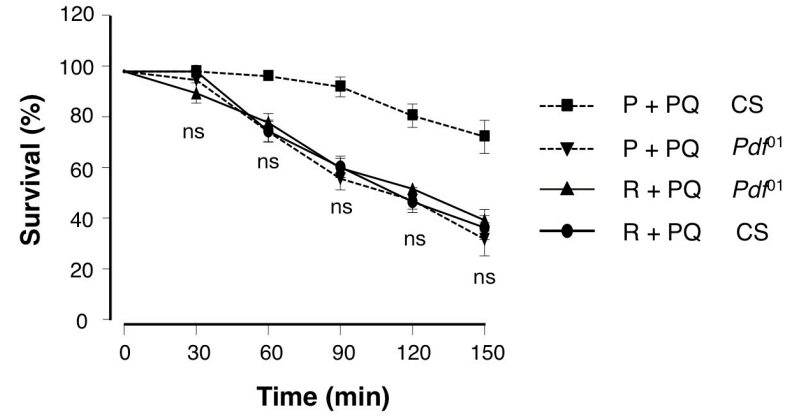
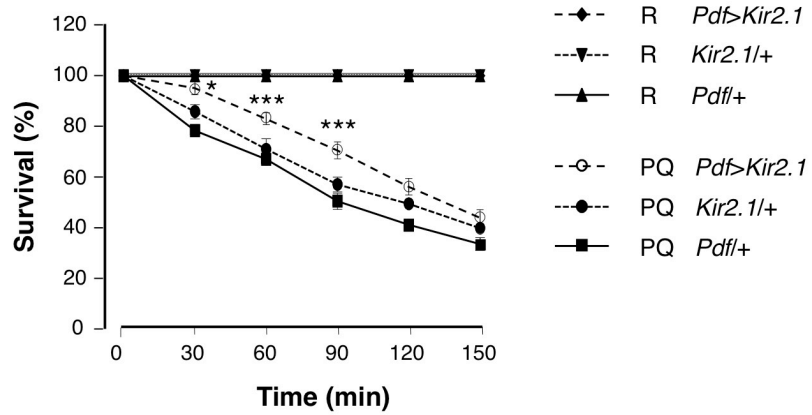
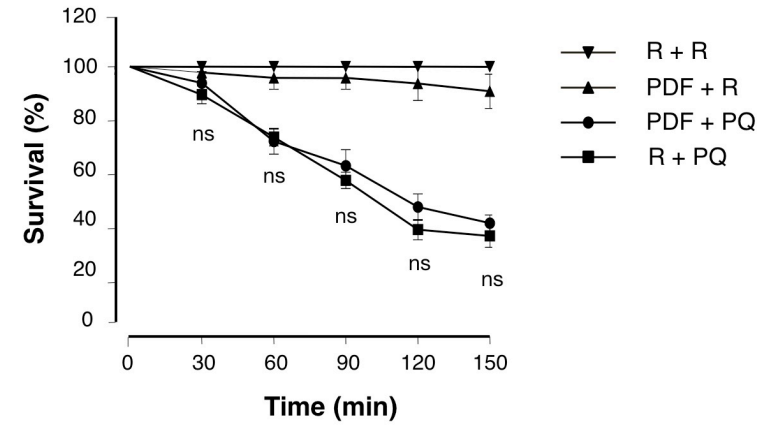
*Gal4* expresses in neuronal subsets in the abdominal ganglia and in characteristic bilateral axonal bundles (arrows) that may project from dopaminergic cell bodies (see Fig. 5). **(B)** PDF co-immunostaining shows that *tim-Gal4* does not express in the Abd PDF neurons. Scale bar: 50  $\mu\text{m}$ . (Related to Figure 2 and 5).

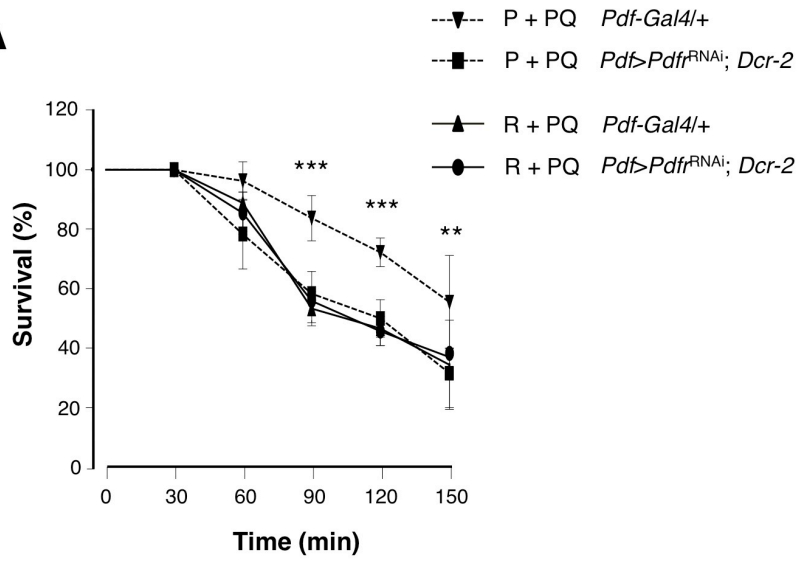
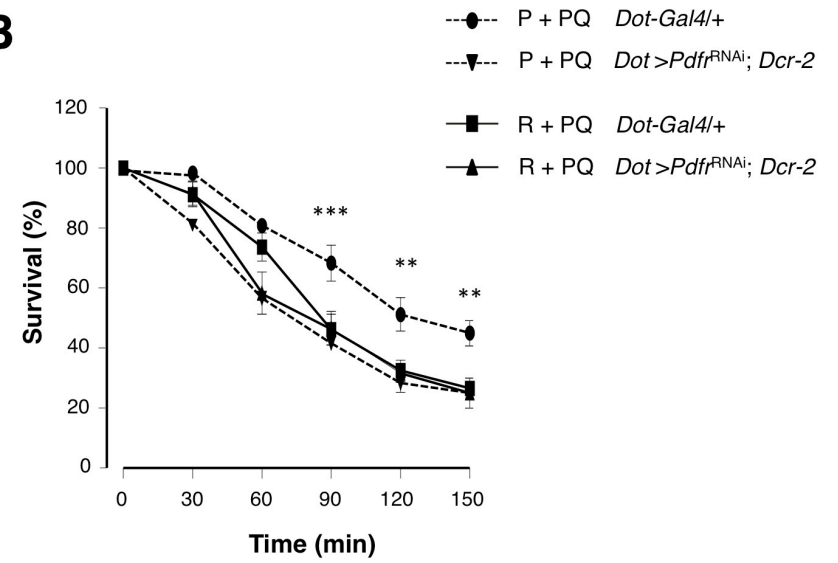
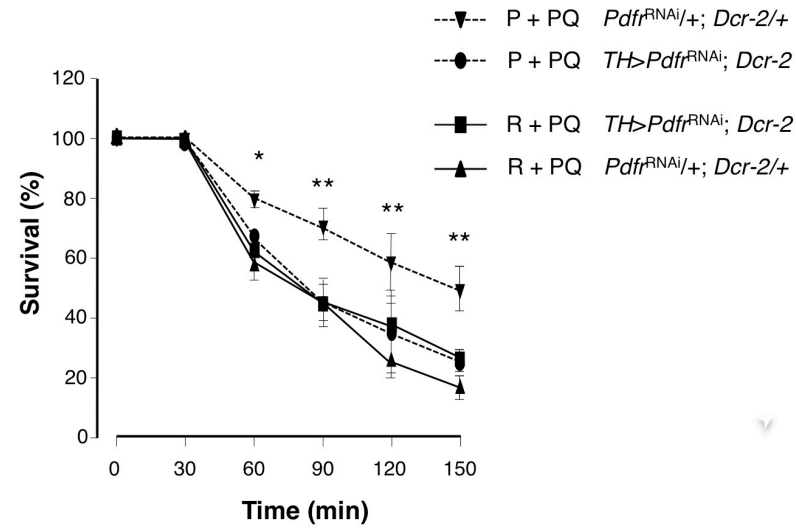
**Figure S5.** No effect of PDF on PQ susceptibility of wild-type flies. Direct application of 20  $\mu\text{M}$  PDF to the VNC of decapitated flies did not alter their survival rate under normal (PDF + R versus R +R) or PQ-intoxicated (PDF + PQ versus R + PQ) conditions (ns: not significant).(Related to Figure 3).

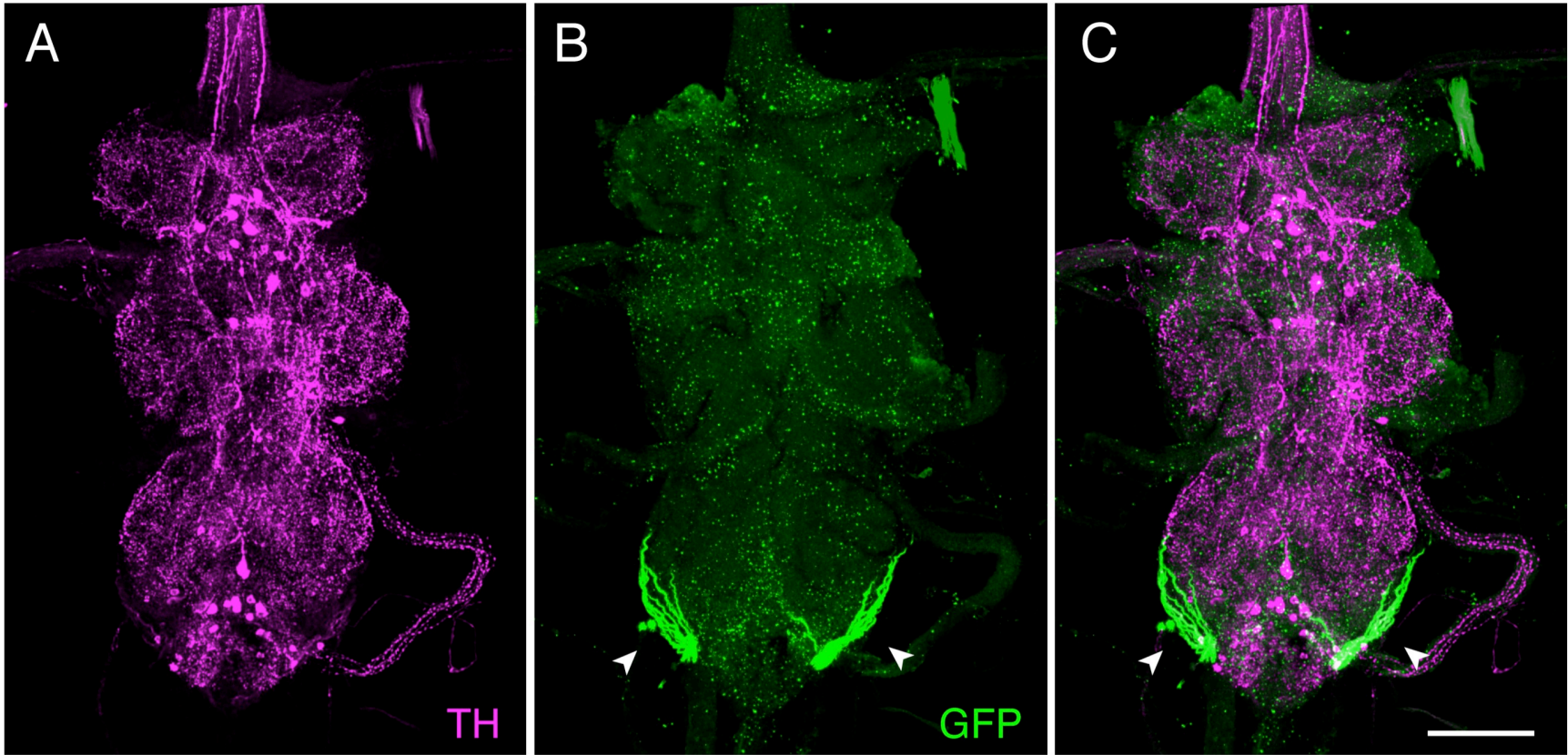


**A****B****C****D****E**

**A****B****C****D**

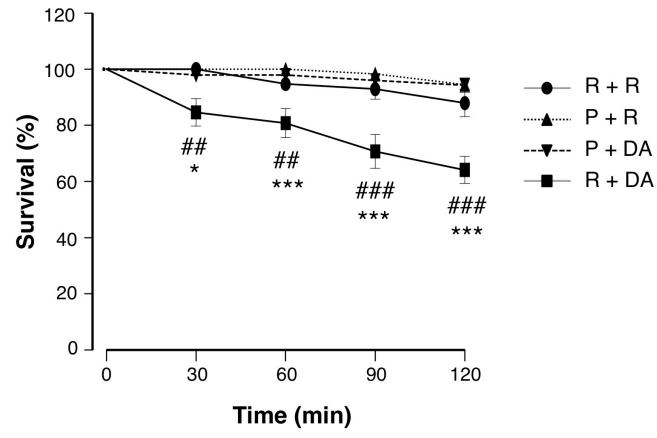
**A****B****C****D**Hajji *et al.* Figure 3

**A****B****C**

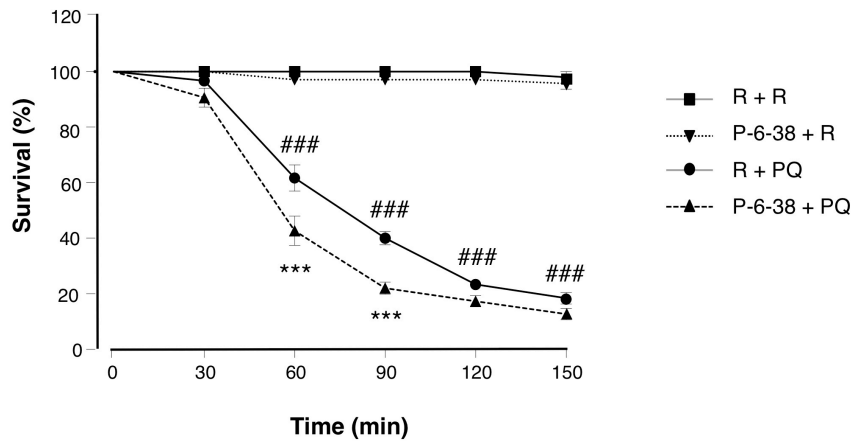


*Pdf>nSyb::GFP<sub>1-10</sub> + TH>CD4::spGFP<sub>11</sub>*

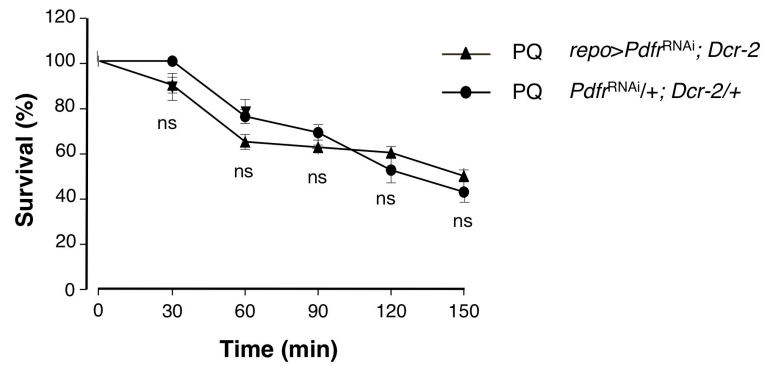
Hajji *et al.* Figure 5



Hajji *et al.* Figure S1

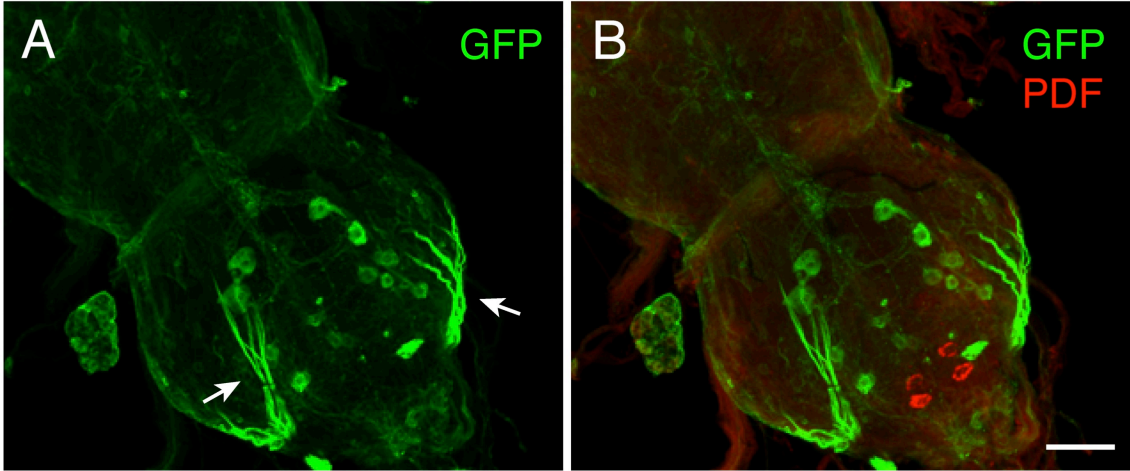


Hajji *et al.* Figure S2

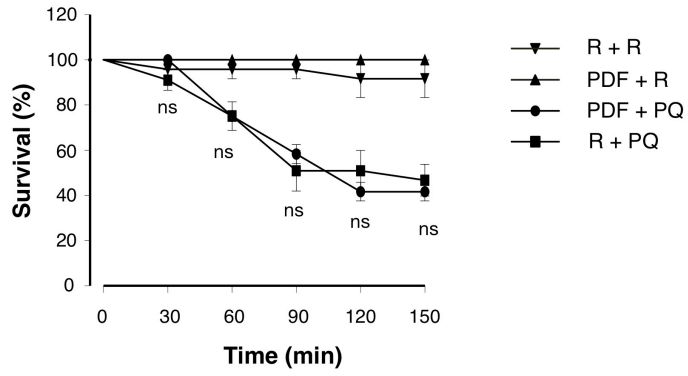


Hajji *et al.* Figure S3





Hajji *et al.* Figure S4



Hajji *et al.* Figure S5