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Abstract

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

Introduction

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

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

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

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

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

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

Results

PACAP protects wild-type flies from PQ-induced neurotoxicity

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

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

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

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

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

under acute PQ intoxication in the *Drosophila* CNS.

Neuronal downregulation of the PDF receptor decreases PQ susceptibility

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

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

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

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

Neuroprotection by PACAP requires PDF and the PDF receptor

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

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

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

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

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

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

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

protective effect in the VNC of *tim>Pdfr*^{RNAi} flies could specifically result from PDFR downregulation in these neurons. These lateral abdominal axons could be part of a neuronal network controlling oxidative stress susceptibility in *Drosophila*.

Discussion

The neuroprotective function of PACAP is conserved in *Drosophila*

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

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

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

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

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

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

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

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

decapitated *Drosophila*? To explore the mechanisms of action of this neuropeptide, we first used pan-neuronal RNAi downregulation to search among GPCR receptors of known fly neuropeptides for one that might be implicated in the protective effect of PACAP. Among the six GPCRs that were tested, only PDFR was identified as being involved in the modulation of PQ sensitivity and PACAP-mediated neuroprotection. Indeed, downregulating this receptor in all neurons with *elav-Gal4*, or in *tim-Gal4*-targeted cells, was sufficient to protect efficiently the flies from PQ-induced early lethality. This indicates that PDFR signaling, which acts through elevated cAMP/PKA activity (51, 69, 71, 95–99) and modulation of Ca^{2+} level and activities (51, 96, 98, 100, 101), potently contributes to PQ susceptibility in *Drosophila*. In contrast, $Pdfr^{5304}$ and Pdf^{01}null mutants did not appear more resistant to PQ than wild-type flies, which might be related to compensating spontaneous mutations accumulated in the mutant genomes over generations (67), or, alternatively, to the absence of PDFR signaling at all stages and in all cells in the mutants compared to a relative downregulation in neurons only in the RNAi experiment. Note that *Pdfr* knock down by RNAi in PDF neurons was not sufficient to decrease PQ susceptibility, indicating that enhanced PQ resistance requires targeting other *Pdfr*-expressing neurons.

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

auto-receptor activation.

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

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

PACAP neuroprotection depends on PDFR signaling in PDF and DA neurons

We observed that PACAP pre-treatment did not prolong the survival of PQ-exposed null Pdf^{01}

352 or *Pdfr*^{5304} 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$_v$s) and four small (s-LN$_v$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$_v$s and some of the l-LN$_v$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$_v$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*^{RNAi} decapitated flies are not

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

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

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

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

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 strains were used: Canton-S (CS) as wild type, $Pdfr^{5304}$ (49), Pdf^{01} (66), *elav-GAL4* (110), *repo-GAL4* (111), *TH-Gal4* (73), *TH-LexA* (112) (kindly provided by Dr. Ronald L. Davis), *tim-Gal4* (62), *Pdf-Gal4* (72), *Dot-Gal4* (65), *UAS-Pdfr* (49) (kindly provided by Dr. François Rouyer), *UAS-Kir2.1* (113) (kindly provided by Dr. Sean Sweeney), recombined *UAS-mCD8::GFP*, *UAS-nSyb::GFP* (114), and from the Bloomington *Drosophila* Stock Center (BDSC): *elav-Gal4*; *UAS-Dcr-2* (BDSC #25750); *UAS-Dcr-2*; Pin^{1}/*CyO* (BDSC #24644), *LexAop-n-syb::spGFP*$_{1-10}$, *UAS-CD4::spGFP*$_{11}$ (BDSC #64314); *UAS-n-syb::spGFP*$_{1-10}$, *LexAop-CD4::spGFP*$_{11}$ (BDSC #64315), and the TRiP RNAi strains: JF01945 (BDSC #25925), JF03208

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

PQ and PACAP application and survival score

PQ and PACAP treatments were performed on 9 to 11-day-old adult females by direct application of the drug and neuropeptide onto the exposed VNC of decapitated flies as previously described (39). Briefly, flies were anaesthetized on ice for 10 min and their heads were cut off with 7-mm blade spring scissors (Fine Science tools). ~30 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 (methyl viologen dichloride hydrate; Sigma-Aldrich 856177) 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 (R) for controls, was applied for 5 s with a P10 Pipetman. The same droplet was successively used for 10 flies. A concentration of 80 mM PQ was generally used that gave 30-40 % survival of wild type flies after 2 h (39). Flies were considered as dead when they laid on the side or back and did not react to a slight mechanical stimulus on the legs. Survival rate was monitored every 30 min. Each experiment was repeated independently at least twice. The same procedure was used for DA application to the VNC except that DA concentration in the droplet was 35 mM, as previously described (39). For PACAP, PACAP-6-38 and PDF pretreatments, a droplet containing the neuropeptide at 2 µM (precisely 2.2 µM, i.e. 10, 8.9 and 4.3 µg/ml, respectively), in Ringer's, or 20 µM for PDF in some experiments, was applied to the decapitated flies the same way 30 min prior to the exposure to PQ. The 38-amino acid form of PACAP (His-Ser-Asp-Gly-Ile-Phe-Thr-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-NH2) 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-NH2) 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^{2+}-free Ringer's, fixed in 4% (wt/vol)
461 paraformaldehyde in phosphate-buffered saline (PBS: 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM
462 KH_2PO_4), 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

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

Immunohistochemistry and split-GFP reconstitution

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

Fluorescence confocal microscopy

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

Statistical analysis

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

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Conflict of interest statement

The authors have no conflict of interest to declare.

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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 μM 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 μM 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 μm. (**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 μM 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 μm. (**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*^{RNAi}, *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>Pdfr*) did not alter *Drosophila* PQ susceptibility, as compared to the heterozygous driver (*elav*/+) and effector (*Pdfr*/+) controls. (**C**) *Pdfr* downregulation in all clock cells with the *tim-Gal4* driver (*tim>Pdfr*^{RNAi}, *Dcr-2*) significantly protected flies against PQ compared to the survival rate of *Pdfr*^{RNAi}/+; *Dcr-2*/+ controls (R + PQ). No further protection could be observed by PACAP pre-application on the *tim>Pdfr*^{RNAi}, *Dcr-2* flies, whereas the *Pdfr*^{RNAi}/+; *Dcr-2*/+ controls were strongly protected by PACAP (P + PQ). (**D**) Monitoring the survival of PQ-treated *elav>Pdfr*^{RNAi}, *Dcr-2* and *tim>Pdfr*^{RNAi}, *Dcr-2* flies in a parallel experiment showed similar levels of protection compared to *Pdfr*^{RNAi}/+; *Dcr-2*/+ controls when *Pdfr* 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 *Pdfr* and *Pdf* null mutants. (**A**) In the absence of PDF receptor (mutant *Pdfr*^{5304}), PQ resistance was not significantly different(ns) from that of wild-type CS flies (*Pdfr*^{5304}, R + PQ *vs* CS, R + PQ), and no protective effect of PACAP against PQ could be observed in these mutant flies (*Pdfr*^{5304}, P + PQ *vs* R + PQ). (**B**) Without expressed PDF neuropeptide (mutant *Pdf*^{01}), 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*^{RNAi}, *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*^{RNAi}, *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*^{RNAi}, *Dcr-2*) similarly prevented PACAP-induced neuroprotection compared to controls (i.e. survival of *TH>Pdfr*^{RNAi}, *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*^{RNAi}/+; *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$_{1–10}$, which is targeted to synaptic terminals, was expressed in PDF neurons with *Pdf-Gal4* (*PDF>nSyb::GFP$_{1-10}$*) and the fusion membrane protein CD4::spGFP$_{11}$was expressed in DA neurons with *TH-LexA* (*TH>CD4::spGFP$_{11}$*). 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	***elav>Dcr-2***		***elav>*R^{RNAi}, *Dcr-2***	
Treatment	**PQ**	**PACAP + PQ**	**PQ**	**PACAP + PQ**
DH31-R	45	85***	49	84***
DH44-R1	54	76**	46	62,5*
DH44-R2	34	71***	45	72**
sNPF-R	46	75***	38	71***
FMRFaR	41	65**	39	59*
Pdfr	45	90***	$77^{###}$	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^{RNAi}, *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 μM PACAP. Statistical significance: PACAP + PQ *vs* PQ, **p*< 0.05, ***p* < 0.01, ****p* < 0.001; PQ (*elav>*R^{RNAi}, *Dcr-2*)*vs*PQ (*elav>Dcr-2*), $^{###}p$ < 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 µM 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).

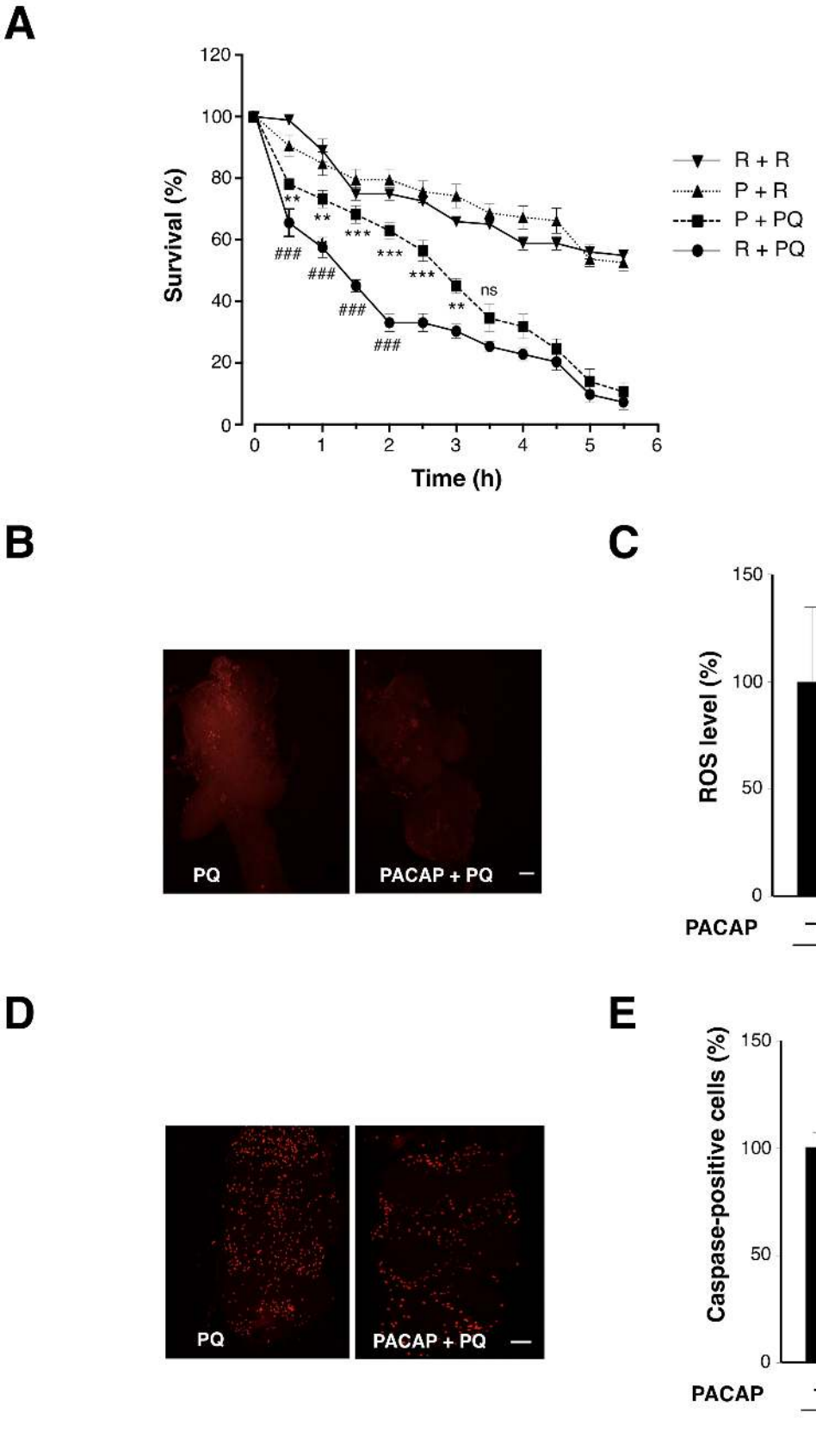
Figure S2. The PACAP receptor antagonist PACAP-6-38 increases *Drosophila* susceptibility to PQ. Pre-treatment with 2 µM PACAP-6-38 (P-6-38) for 30 min significantly accelerated the death of decapitated flies intoxicated by PQ (P-6-38 + PQ vs R + PQ, ***$p < 0.001$), compared to control flies pre-treated 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*^{RNAi}; *Dcr-2* decapitated flies was not improved compared to *Pdfr*^{RNAi}/+; *Dcr-2*/+ controls (ns: not significant). (Related to Figure 2).

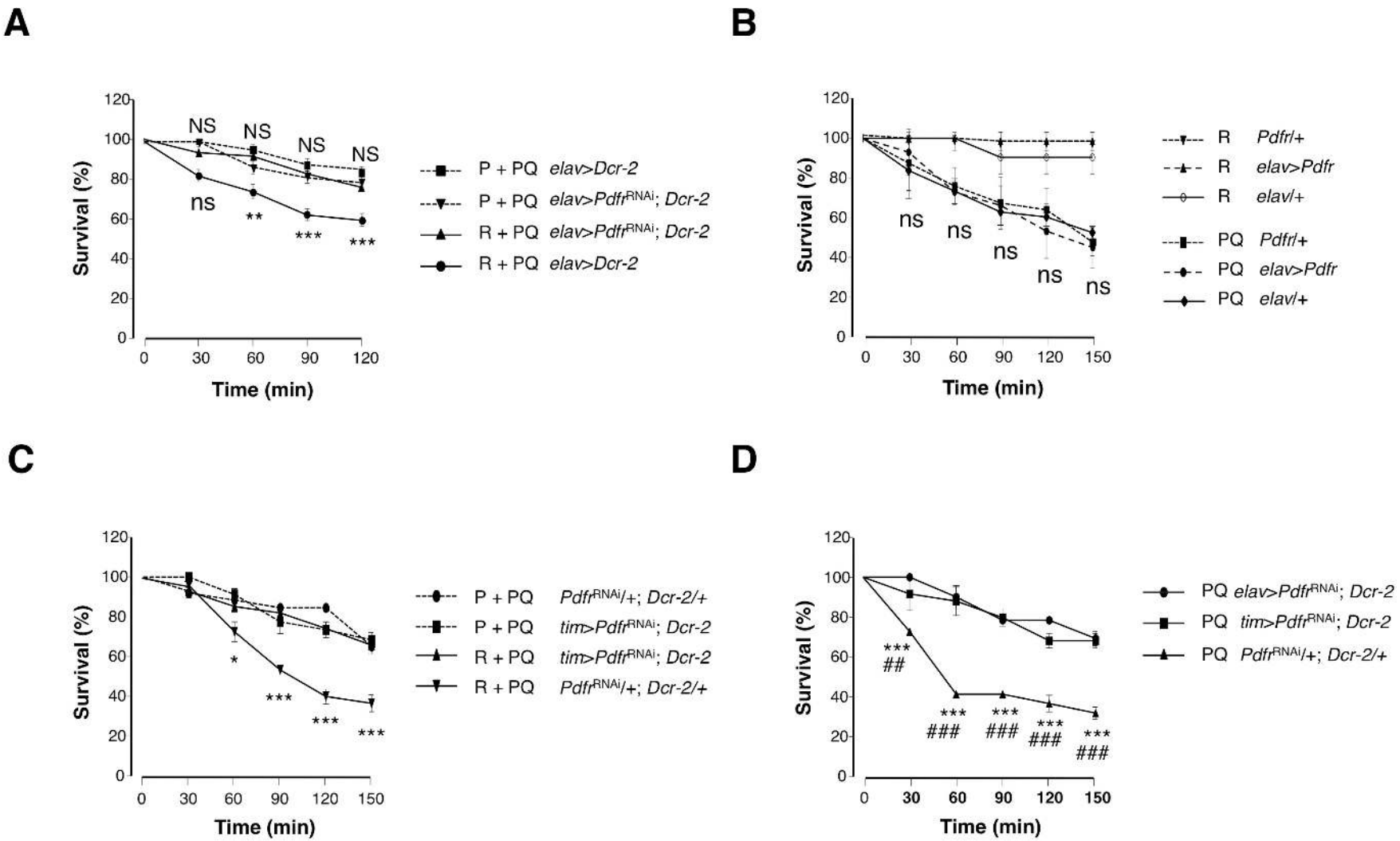
Figure S4. Expression pattern of the *tim-Gal4* driver in the abdominal ganglia. (**A, B**) Whole-mount GFP and PDF co-immunostaining in the VNC of young adult *Drosophila* expressing membrane-associated GFP under *tim-Gal4* control (*tim>mCD8::GFP, nSyb::GFP* flies). (**A**) *tim-*

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 μm. (Related to Figure 2 and 5).

Figure S5. No effect of PDF on PQ susceptibility of wild-type flies. Direct application of 20 μM 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).



Hajji *et al.* Figure 1



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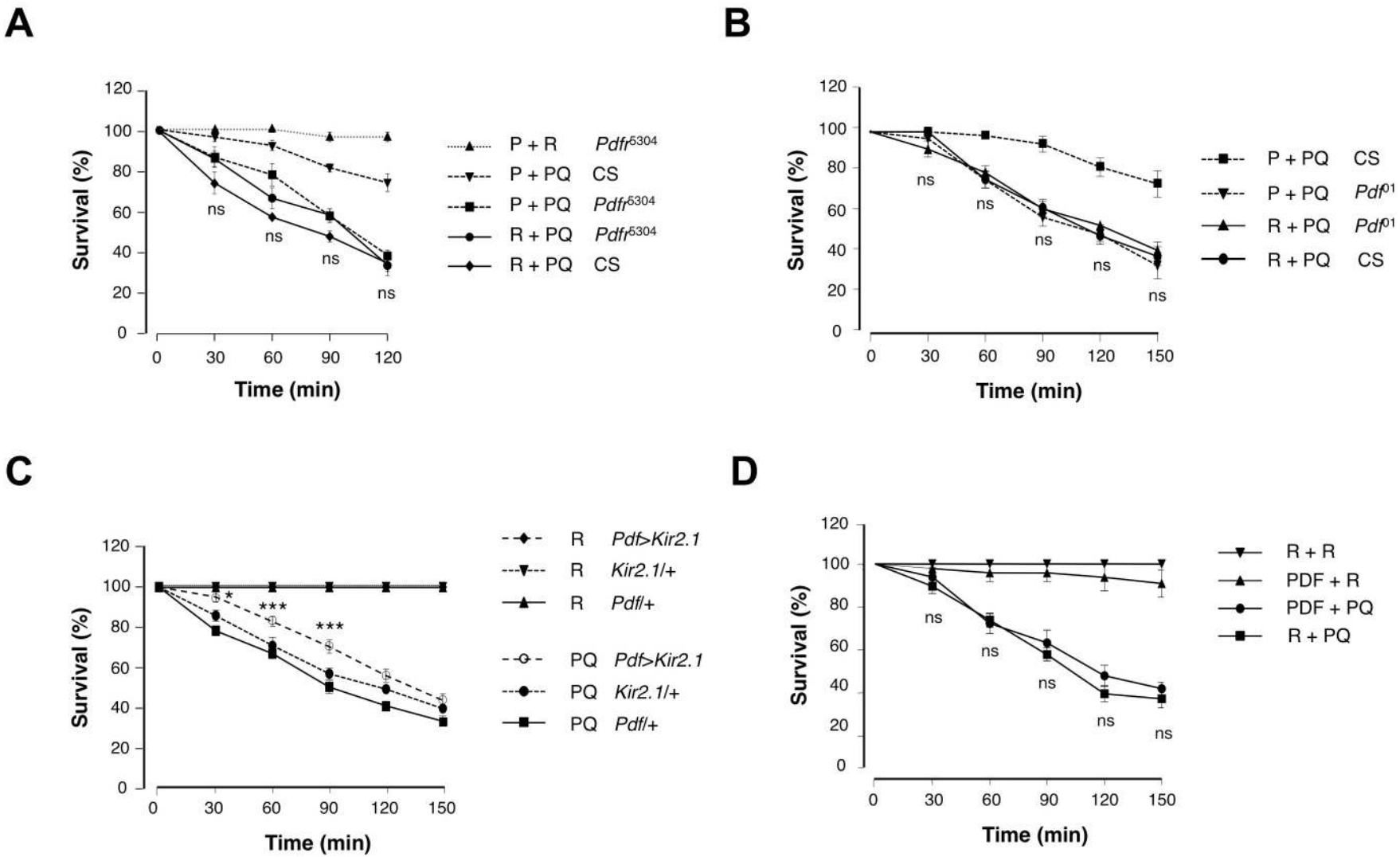

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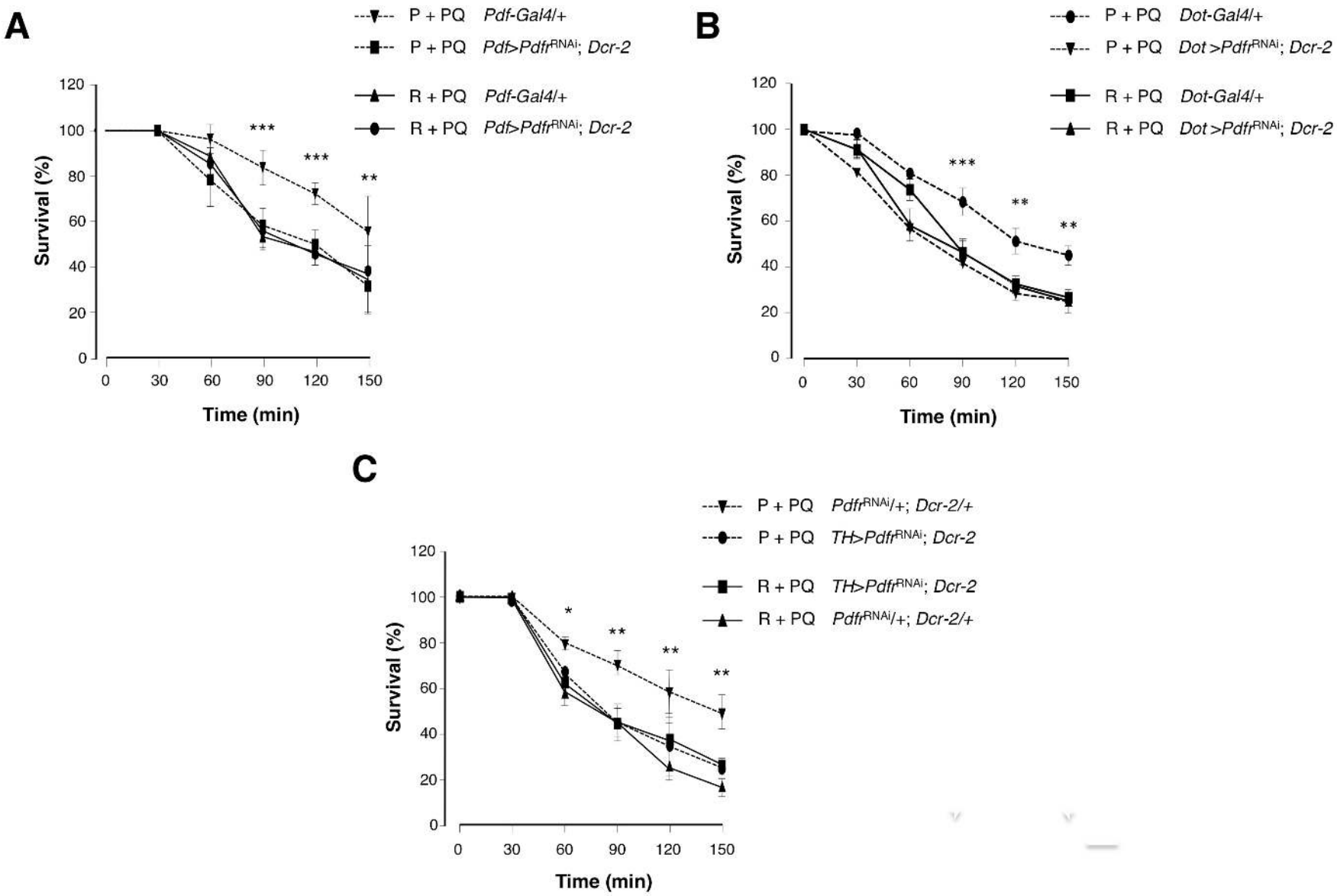

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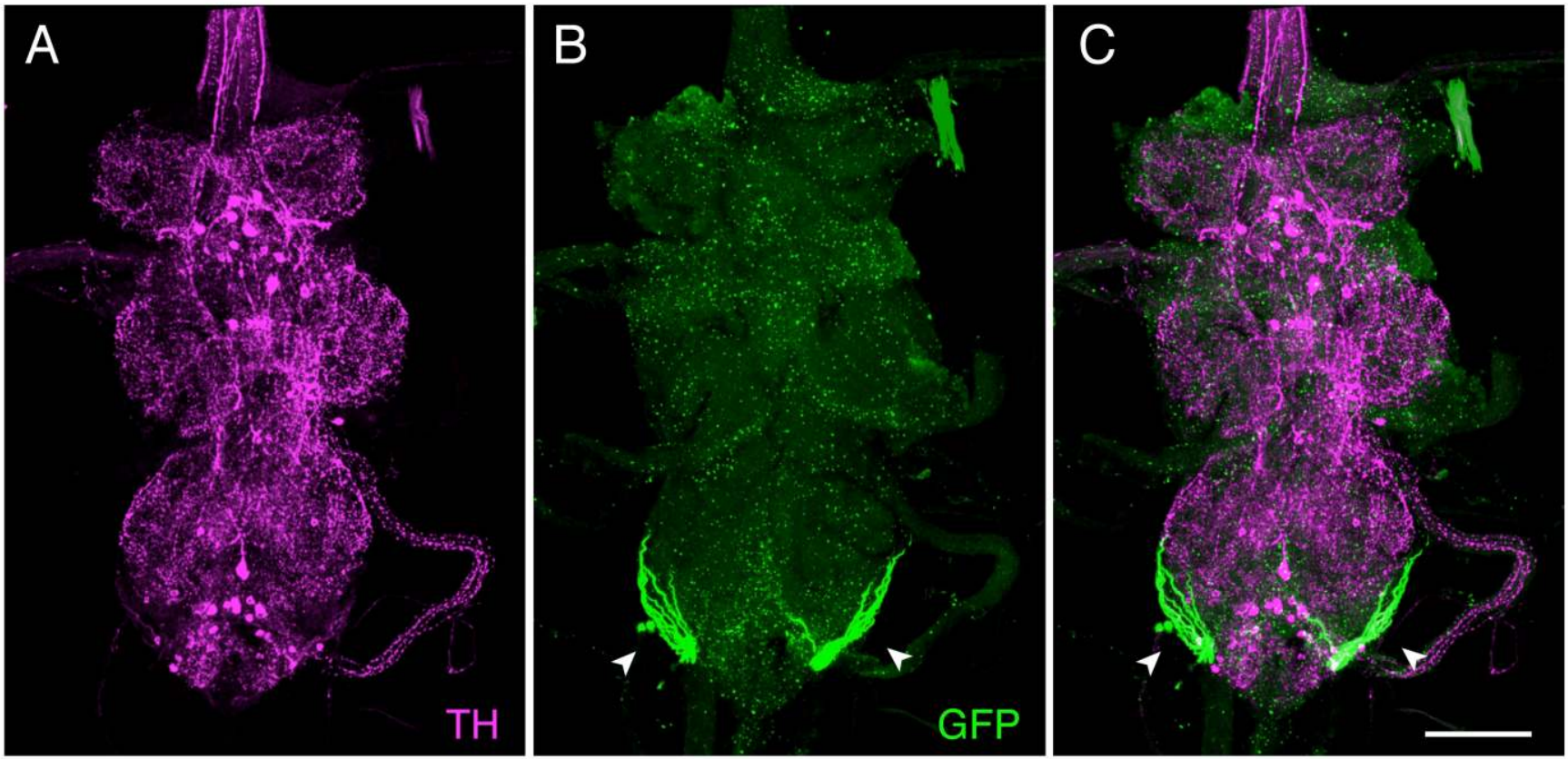
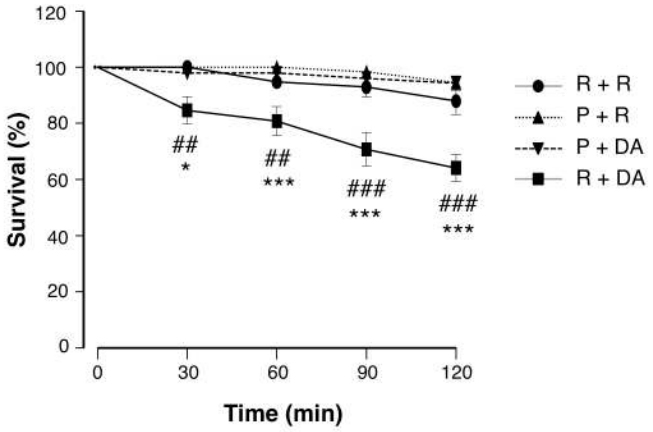


Pdf>nSyb::GFP$_{1-10}$ + TH>CD4::spGFP$_{11}$

Hajji *et al.* Figure 5



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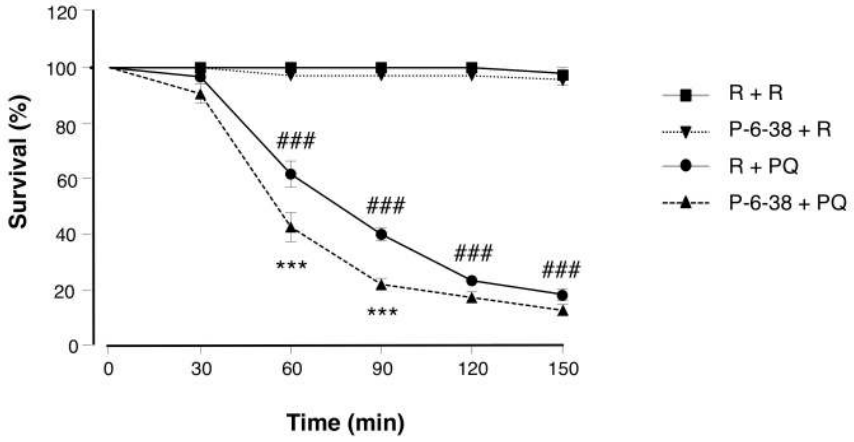
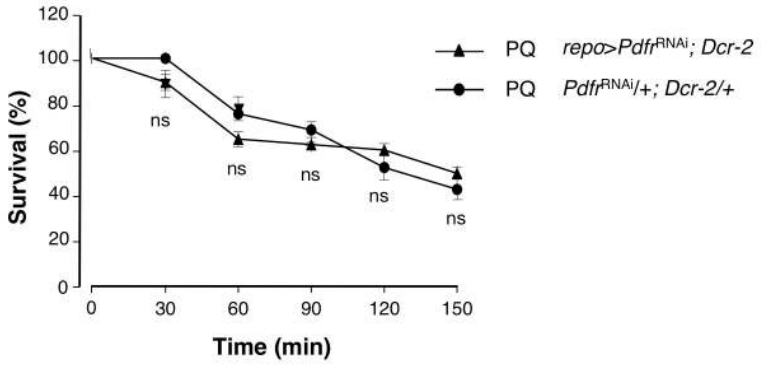
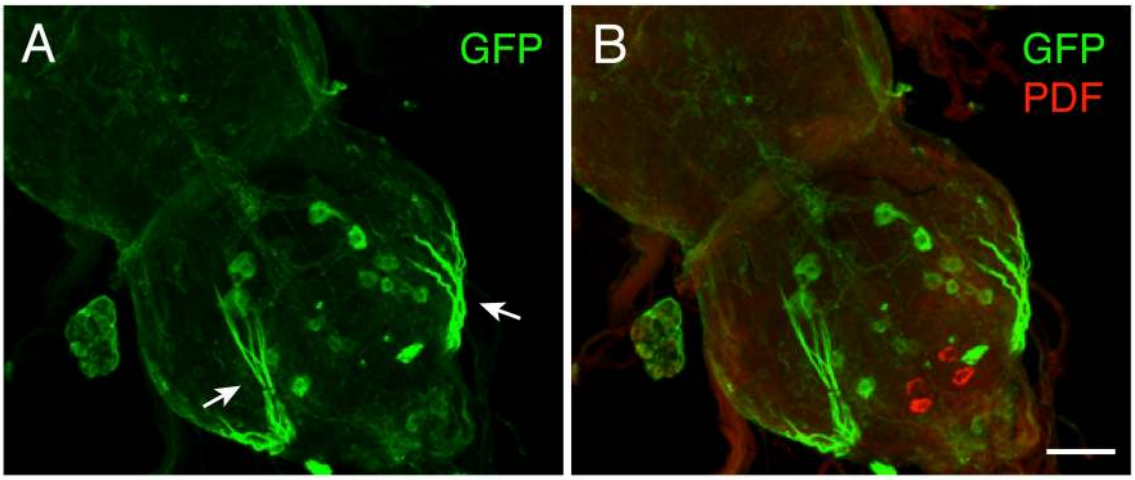


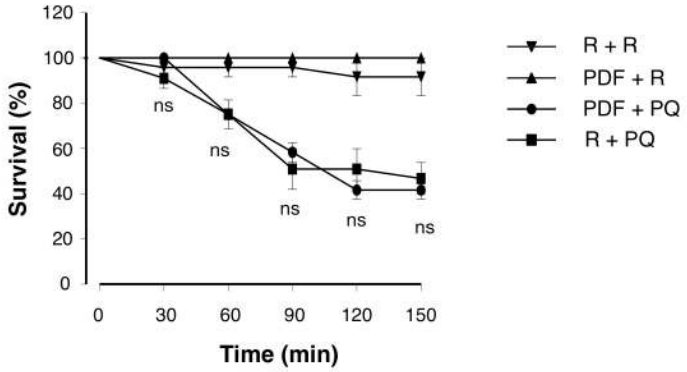
Hajji *et al*. Figure S2



Hajji *et al.* Figure S3



Hajji *et al.* Figure S4



Hajji *et al.* Figure S5