Published in final edited form as:

Neurobiol Dis. 2010 October ; 40(1): 102–112. doi:10.1016/j.nbd.2010.05.008.

The *Drosophila* vesicular monoamine transporter reduces pesticide-induced loss of dopaminergic neurons

Hakeem O. Lawal¹, Hui-Yun Chang^{1,2}, Ashley N. Terrell^{1,3}, Elizabeth S. Brooks¹, Dianne Pulido¹, Anne F. Simon^{1,4}, and David E. Krantz^{1,*}

¹Department of Psychiatry and Biobehavioral Sciences, Gonda Center for Neuroscience and Genetics Research, David Geffen School of Medicine at University of California, Los Angeles, California, USA

Abstract

Dopamine is cytotoxic and may play a role in the development of Parkinson's disease. However, its interaction with environmental risk factors such as pesticides remains poorly understood. The vesicular monoamine transporter (VMAT) regulates intracellular dopamine content, and we have tested the neuroprotective effects of VMAT *in vivo* using the model organism *Drosophila melanogaster*. We find that *Drosophila* VMAT (*dVMAT*) mutants contain fewer dopaminergic neurons than wild type, consistent with a developmental effect, and that dopaminergic cell loss in the mutant is exacerbated by the pesticides rotenone and paraquat. Over-expression of *dVMAT* protein does not increase the survival of animals exposed to rotenone, but blocks the loss of dopaminergic neurons caused by this pesticide. These results are the first to demonstrate an interaction between a VMAT and pesticides *in vivo*, and provide an important model to investigate the mechanisms by which pesticides and cellular DA may interact to kill dopaminergic cells.

Introduction

The pathological hallmark of Parkinson's disease (PD) is the selective loss of dopaminergic (DA) neurons in the substantia nigra (Olanow and Tatton, 1999). Although the pathophysiology of DA cell loss in PD remains unclear, cellular dopamine (DA) itself may play an important role in this process (Guillot and Miller, 2009; Hastings et al., 1996; Michel and Hefti, 1990; Mosharov et al., 2009). Cytosolic DA and its metabolites conjugate to PD-related proteins (Conway et al., 2001; LaVoie et al., 2005; Xu et al., 2002) and more generally increase oxidative stress (Dauer and Przedborski, 2003; Giasson and Lee, 2001; Moore et al., 2005; Stokes et al., 1999). The neurotoxic properties of DA suggest that the gene products responsible for DA homeostasis have the potential to act as neuroprotectants (Miller et al., 1999). These include distinct transporters that: 1) take up DA from the extracellular space- the plasma membrane dopamine transporter or DAT (Kelada et al., 2006; Nass and Blakely, 2003; Ritz et al., 2009); and 2) package DA into synaptic vesicles- the vesicular monoamine transporter or VMAT (Chaudhry et al., 2008; Eiden et al., 2004).

© 2010 Elsevier Inc. All rights reserved.

*Corresponding Author: David E. Krantz, 310 206 9877 (fax), 310 206 8508 (phone), dkrantz@ucla.edu.

²Current Address: Department of Life Sciences, National Tsing Hua University, Hsinchu, Taiwan

³Current Address: Division of Environmental Health Sciences, University of Minnesota, Minneapolis, MN

⁴Current Address: Biology Department, York College / CUNY, Jamaica, New York, USA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Despite widespread speculation, experimental evidence for the neuroprotective effects of DAT or VMAT in animal models of DA cell death is surprisingly limited, and the effects of VMAT expression on animal models of pesticide exposure are not known.

Since DA is synthesized in the cytoplasm, VMATs are required in all aminergic neurons to actively transport neurotransmitter out of the cytoplasm and into the lumen of synaptic vesicles (Chaudhry et al., 2008; Eiden et al., 2004; Liu and Edwards, 1997). Mammals contain two VMAT genes, including VMAT2, which is expressed in all aminergic neurons in the brain (Weihe et al., 1994). Invertebrates such as *Drosophila melanogaster* (Greer et al., 2005) and *C. elegans* (Duerr et al., 1999) each contain a single VMAT gene, and provide genetically tractable models to study the effects of VMAT on neurodegenerative processes relevant to PD.

We and others have postulated that, since VMAT regulates cytosolic DA levels, it may function as a neuroprotective gene (Chang et al., 2006; Choi et al., 2005; Hanson et al., 2004; Liu and Edwards, 1997b; Mosharov et al., 2006; Park et al., 2007; Sai et al., 2008; Weingarten and Zhou, 2001). Mouse models suggest that lower levels of VMAT2 may increase the toxicity of methamphetamines and also reduce the number of DA neurons in aged mice (Caudle et al., 2007; Fumagalli et al., 1999; Guillot et al., 2008). Epidemiologic data suggest that a VMAT2 haplotype conferring high expression may be neuroprotective for PD (Glatt et al., 2006). We have previously shown that overexpression of the *Drosophila* ortholog of VMAT (DVMAT) protects against the DA cell death caused by a gene (*parkin*) implicated in a familial form of PD (Sang et al., 2007). However, it remains unclear whether VMAT may be more generally neuroprotective against other risk factors, and more specifically, whether it will interact with pesticides thought to potentiate the risk of PD.

Several pesticides have been linked to sporadic PD in epidemiologic studies, and/or shown to kill dopaminergic neurons in cellular and animal models of disease (Barbeau et al., 1987; Bocchetta and Corsini, 1986; Cicchetti et al., 2009; Hirsch et al., 2003; Hubble et al., 1993; Koller et al., 1990; Rajput et al., 1987; Seidler et al., 1996; Semchuk et al., 1992). Multiple neurotoxic mechanisms have been proposed for pesticides associated with PD and/or models of DA cell death, including inhibition of the mitochondrial respiratory chain by rotenone (Betarbet et al., 2000; Chance et al., 1963; Panov et al., 2005) and more general oxidative processes caused by paraquat (Bus and Gibson, 1984; Przedborski and Ischiropoulos, 2005). Gene-environment interactions, thought to be critical for the generation of idiopathic PD, may also vary between different classes of pesticides. However, to date, the manner in which toxins and genetic risk factors may interact in PD is poorly understood, and it remains unclear how specific risk alleles or protective genes might influence the neurotoxicity of pesticides.

By changing cytosolic DA concentrations, up- or down-regulation of VMAT has the potential to either prevent or exacerbate, respectively, the neurotoxic properties of pesticides on DA cells (Guillot and Miller, 2009; Park et al., 2007; Richardson et al., 2006; Richardson et al., 2008; Sai et al., 2008). It is also conceivable that VMAT could more broadly provide cytoprotection beyond its effects on DA cells, as recently suggested by results obtained in a VMAT2 knockdown mouse (Caudle et al., 2007). However, to our knowledge, the possibility that VMAT could mitigate the effects pesticides in DA cells or otherwise has never been investigated *in vivo*. Here, we determine how changes in *Drosophila* VMAT expression may affect the neurotoxic properties of two pesticides, rotenone and paraquat, suggested to increase DA cell death in model organisms (Chaudhuri et al., 2007; Coulom and Birman, 2004; Di Monte, 2003; Thiffault et al., 2000) and/or the risk of PD in patients (Ritz et al., 2009). We find that *dVMAT* mutant flies show a decrease in the number of DA neurons in the adult fly brain at baseline, and that this number is further reduced by either

paraquat or rotenone. Conversely, although overexpression of *dVMAT* does not appear to interact with paraquat to protect DA cells, it blocks the DA cell loss caused by rotenone. The effects of *DVMAT* overexpression on DA cells does not correlate with its potential to alter the survival of flies exposed to rotenone, thus demonstrating the limitations of *VMAT* in altering the more general, cytotoxic effects of pesticides. Our data demonstrate that *VMAT* provides a relatively specific protection for DA cells against the effects of some pesticides, and provide an important genetic model to pursue further in vivo studies on the mechanisms by which DA promotes neurotoxicity.

Materials and Methods

Drosophila strains and maintenance

The *dVMAT* loss of function, P-insertion allele (*dVMAT^P*, also known as *l(2)SH0459/Cyo*) (Oh et al., 2003; Romero-Calderón et al., 2008; Simon et al., 2009) and *UAS-DVMAT* transgenes (Chang et al., 2006) have been described previously. For all of the experiments described here, *UAS-DVMAT* refers to the insertion on the third chromosome (*UAS-DVMATIII*) encoding the neuronal isoform of *DVMAT*, *DVMAT-A* (Greer et al., 2005). *UAS-DVMAT-A* contains an HA epitope tag in the luminal domain between the first and second transmembrane domains (Greer et al., 2005). The HA-tag here, and at a similar site in mammalian *VMAT2* does not affect transport activity (Krantz et al., 1997; Greer et al., 2005), trafficking (Krantz et al., 2000; Greer et al., 2005) or the ability of the transgene to genetically rescue the *dVMAT* mutant phenotype (Simon et al., 2009). A second *Drosophila* *VMAT* mRNA splice variant, *DVMAT-B*, is expressed exclusively in a small subset of glia in the optic ganglia (Greer et al., 2005; Romero-Calderón et al., 2009), and therefore is not relevant to the experiments described here. Flies expressing *Ddc-Gal4* on the second chromosome (Li et al., 2000) were a generous gift from Jay Hirsh (University of Virginia, Charlottesville).

All lines were outcrossed for five generations into either the wild-type strain Canton S (CS), obtained from the laboratory of Seymour Benzer, or *w¹¹¹⁸* CS₁₀ (*w¹¹¹⁸* outcrossed 10 times to CS). To obtain flies over-expressing one copy of *UAS-DVMAT* in dopaminergic and serotonergic neurons (*Ddc-GAL4/+;UAS-DVMAT/+*), *Ddc-Gal4* homozygotes were genetically crossed to *UAS-DVMAT* homozygotes using standard methods. An additional stable line homozygous for both *Ddc-Gal4* and *UAS-DVMAT* (*Ddc-GAL4;UAS-DVMAT*) was used for some experiments as indicated. Strains were maintained at 25°C on standard cornmeal molasses agar media in a 12 hour light dark cycle unless otherwise specified in the text.

Pesticides/Drugs

Serial dilutions of drugs were made in either dimethyl sulfoxide (DMSO, Sigma, MO, USA), for rotenone, benomyl and ziram (Sigma, MO, USA) or in distilled water for paraquat (Sigma). Unless otherwise specified, stocks or vehicle were added with vigorous mixing to molten cornmeal molasses agar media to a final concentration of 0.25% DMSO and the indicated concentration of toxin.

Survival assays

Lifespan measurements of adult flies were performed as described previously (Li et al., 2000; Simon et al., 2009) with minor modifications. For chronic dosing, twenty male flies, 3-7 days post-eclosion were placed on food containing the indicated concentration of pesticide, then scored every three days for the number of flies that had died, until at least 90% of control flies were dead. Flies were transferred to fresh vials containing food+drug (or food alone) every 2-3 days. We note that experiments testing the effects of each toxin

were performed independently and at different times, and the absolute lifespan of flies across panels A-C are not directly comparable.

For acute (0-72 hours) dosing of high concentration paraquat, flies were starved before treatment (females for 6 hours, males for 4 hours) then administered 10-20 mM paraquat in a solution containing 5% sucrose, 1% red food dye. The solution (500 µl) was spotted onto filter paper (20 mm) resting on ~2 mm of cotton padding to prevent pooling of excess liquid. Flies transferred to the test vials (without additional food) two hours before the lights-off during the standard 12-hour light/dark cycle. The solution and disk were changed every 12 hours for the duration of the experiment. For some paraquat experiments using exposure times longer than 24 hours but less than 48 hours, the sucrose, dye and drug were mixed into 1.5% agar to prevent dessication.

Climbing assay

Climbing assays were performed as described previously (Simon et al., 2009). Briefly, flies were placed in vials mounted on a T-maze and allowed to habituate for 1 min. Flies were forced to the bottom of the lower chamber by mechanical agitation and the flies were allowed 30 sec to climb to the upper chamber. The number of flies in the upper chamber relative to the lower chamber was quantified as a performance index (PI).

Immunolabeling

For immunolabeling experiments involving rotenone, adults 3-7 days post eclosion were exposed to toxin or vehicle continuously for 10 days or exposed for 10 days followed by 6 week recovery period on standard food as indicated. For immunolabeling experiments with paraquat, 3-7 day post eclosion animals were exposed to paraquat for 16 hours. Immunohistochemical staining was performed using whole, dissected adult brains as described previously (Chaudhuri et al., 2007; Sang et al., 2007). Briefly, dissected brains were fixed in 4% paraformaldehyde at room temperature for 30 minutes, washed extensively in 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4 (PBS) then incubated in PBS containing 2% v/v TX 100 (PBST) with 5% normal goat serum (blocking solution) overnight at 4°C followed by incubation in primary antibody diluted in blocking solution. Primary antibodies included rabbit anti-*Drosophila* TH antibody (1:8,000), a generous gift of Wendy Neckameyer (St. Louis University School of Medicine), Mouse anti-rat TH (Immunostar, Hudson, PA, 1:50) and rat anti-5HT (Sigma, 1:300 dilution). Secondary antibodies included donkey anti-mouse and anti-rabbit conjugated to either Alexafluor 488 or 555 (Invitrogen Molecular Probes, Oregon, USA, 1:1000). Labelled brains were mounted in Aquamount (Polysciences, Inc, Warrington, WI). Whole mounts of dissected adult brains were examined for the of number of dopaminergic or serotonergic neurons in defined clusters using either a Zeiss Pascal LSM 5 confocal microscope with 40x/0.75 EC Plan-Neofluar objective and stacked optical sections or using an AxioSkop Zeiss upright microscope with the same objective and manually focusing through the entire depth of the cluster.

Results

The effects of pesticide exposure on lifespan

To identify pesticides appropriate for investigating an interaction with VMAT in flies, we considered two established model pesticides, rotenone and paraquat, and two additional compounds that are neurotoxic *in vitro* (Chou et al., 2008; Wang et al., 2006a) and may also increase the risk for PD in epidemiological studies, ziram and benomyl (B. Ritz personal communication). To determine the general cytotoxic effects of ziram and benomyl exposure

on flies, we first measured survival in adult wild type (Canton S strain, or “CS”) flies exposed to increasing concentrations of each agent (Fig. 1). Ziram had no effect on lifespan under these conditions (Fig. 1A). Due to the apparent absence of any effects on the function of the organism as a whole, we did not perform additional studies of neurotoxicity. High concentrations of benomyl (250 μ M) appeared to have some effect on the health of the organism, and reduced median adult lifespan to 29 days, as compared to 35 days for flies treated with vehicle alone (Fig. 1C). In contrast, both rotenone and paraquat dramatically decrease lifespan in a dose-dependent manner (Fig. 1B, D), consistent with previous reports on their toxic effects in *Drosophila* (Chaudhuri et al., 2007; Clark et al., 2006; Coulom and Birman, 2004; Meulener et al., 2005). The effects of rotenone on lifespan are detectable at concentrations as low as 25 μ M (median lifespan reduced to 34 days versus 47 days in vehicle-only control) and in general, the decrease in survival is seen over the course of days to weeks, with the highest concentration decreasing median survival to 7 days (Fig. 1B). Paraquat appears to exert toxic effects more rapidly and at high concentrations death occurs within hours, although at lower concentrations of paraquat (0.5-1 mM), flies can survive for up to 60 days (Fig. 1D).

Survival in adult flies is not an index for DA cell death, nor can it differentiate neurotoxic effects from damage to other organ systems. Rather, it is useful to test the broad cytotoxic potential of environmental agents, and to estimate doses that might be useful for further studies. Accordingly, to further determine the neurotoxic potential of benomyl in the fly, we used a standard climbing assay and employed a benomyl dose of 200 μ M, just below that shown to decrease survival in Fig. 1C (250 μ M). As reported previously (Coulom and Birman, 2004), climbing ability is severely reduced in flies exposed for 25 days to 125 μ M rotenone (Fig. 1E). In contrast, flies treated with 200 μ M benomyl for 1, 2 or 3.5 weeks do not show significant defects in climbing relative to controls treated with vehicle alone (Fig. 1E). Furthermore, flies treated with a combination of rotenone and benomyl were not impaired to a greater degree than those fed rotenone alone (Fig. 1E). These data are consistent with a previous report on the behavioral deficits and DA cell loss seen in flies treated with rotenone (Coulom and Birman, 2004). They also suggest that benomyl as it was applied here is unlikely to cause appreciable damage to any of the neuronal systems responsible for motor behavior, including dopaminergic circuits. We therefore used rotenone or paraquat for all further experiments involving DVMAT.

Survival of DVMAT overexpressers exposed to rotenone and paraquat

To test potential gene-environment interactions involving pesticides and DVMAT overexpression, we used the well-characterized GAL4/UAS system (Duffy, 2002). We employed a previously described *UAS-DVMAT* transgene (Chang et al., 2006) and the *Dopa decarboxylase* (*Ddc*) promoter in the *Ddc-Gal4* transgene to drive expression of *UAS-DVMAT* in both DA and 5HT neurons (Chang et al., 2006; Li et al., 2000). We have previously shown that these transgenes increase the expression of DVMAT protein *in vivo* (Chang et al, 2006, see also Supplemental Fig. 1).

Although some fly models of PD-related genes and toxins have specifically focused on their effects in DA cells (e.g. Auluck et al., 2002; Chaudhuri et al., 2007; Coulom and Birman, 2004; Feany and Bender, 2000; Whitworth et al., 2005; Sang et al., 2007), others have demonstrated cytotoxicity in non-neural tissues or more general effects on the survival of the animal (e.g. Clark et al., 2006; Meulener et al., 2005). To allow a comparison to these studies, and determine whether DVMAT might reduce the general toxicity of pesticides in the fly, we tested the effect of DVMAT overexpression on survival (Fig. 2). Although the median survival of rotenone-treated animals over-expressing DVMAT (*Ddc-Gal4/+;UAS-DVMAT/+*) was higher than the control lines CS and *Ddc-Gal4/+* (19 days) (Fig. 2A) it did not differ from that of the *UAS-DVMAT/+* transgene alone. The difference between the

survival of *UAS-DVMAT* and CS could be due to “leaky” expression of the transgene in cells other than DA neurons (see Supplemental Fig. 1), or an insertional effect unrelated to DVMAT expression. Flies overexpressing DVMAT (*Ddc-Gal4/+; UAS-DVMAT/+*) may be somewhat resistant to paraquat as compared to flies containing one copy of *Ddc-Gal4* or *UAS-DVMAT* alone, or wild type controls (Fig. 2C), but the effect size is relatively small. Together, these data indicate that, in contrast to other broadly expressed, cytoprotective genes such as DJ-1 and pink (Clark et al., 2006; Meulener et al., 2005), DVMAT overexpression in DA and 5HT neurons does not have a major effect on the survival of flies exposed to pesticides.

Immunolabeling of DA neurons in flies exposed to rotenone and paraquat

To determine whether DVMAT over-expression could more specifically provide neuroprotection to dopaminergic neurons exposed to pesticides, we immunolabeled DA neurons using an antibody to tyrosine hydroxylase as previously described (Sang et al., 2007). This assay has been used previously by a number of laboratories to assess the neurotoxic effects of both pesticides and genes associated with familial forms of PD (Auluck et al., 2002; Chaudhuri et al., 2007; Coulom and Birman, 2004; Feany and Bender, 2000; Whitworth et al., 2005). Such studies include a previous report showing that treatment of flies with rotenone for one week caused a loss of DA cells in the PAL and PPL1 cell clusters (Coulom and Birman, 2004) (see Fig. 3A for diagram of DA neuron clusters). Since the reported decrease in DA neurons in previous studies was relatively modest (Coulom and Birman, 2004) and in some cases difficult to observe (Meulener et al., 2005), we sought to modify the assay to better detect the neurotoxic effects of rotenone. Continuous, long-term exposure to high-dose rotenone dramatically reduces lifespan (see Fig 1A), thus limiting our ability to measure the neurodegenerative effects of high-dose rotenone on flies aged for relatively long periods of time. We therefore modified our protocol to include a recovery period (6 weeks) on standard food after an initial exposure of 250 μ M rotenone for 10 days. Flies treated with rotenone and aged for an additional 6 weeks showed a 50% loss of DA neurons compared to vehicle (DMSO) treated flies, ($p < 0.01$) in the PAL cluster (Fig. 3K, see also Fig. 3A-I) and a 33% reduction in DA neurons in the PPL1 cluster (Fig. 3J, see also Fig. 3A-I) as compared to vehicle controls. By comparison, flies treated with rotenone for 10 days then immediately sacrificed showed a lesser, 16.5% loss in DA neurons in the PAL cluster (Fig. 3K) and a 16.9% in the PPL1 cluster (Fig. 3J). DA neuron loss was significantly greater (two-way ANOVA $p < 0.0001$, Bonferroni post test $**p < 0.01$ as indicated) in flies allowed to age after exposure to rotenone, as compared to flies immediately sacrificed after exposure both the PPL1 (Fig. 3J) and PAL clusters (Fig. 3K).

To test the specificity of our modified, rotenone-exposure paradigm, we immunolabeled neurons for 5HT following toxin exposure and aging. In each of the neuronal clusters we assayed (SP1, SP2 and LP2, see Fig. 3A for diagram of 5HT neuron clusters), the number of 5HT neurons was essentially identical in flies treated with rotenone (Fig. 3L) versus vehicle alone (DMSO). These data indicate that our modified exposure paradigm is not toxic to 5HT neurons, but rather, is relatively selective for DA neurons. The increase in neurotoxicity to DA neurons we observed in our modified protocol as well as its specificity indicated that it would be useful for further experiments testing potential interactions of pesticides with DVMAT overexpression.

Over-expression of DVMAT is neuroprotective against rotenone-induced neurotoxicity

Flies overexpressing DVMAT are less sensitive to the neurotoxic effects of mutant *parkin* overexpression, and show improvements in motor behavior when tested at 1 or 2 weeks post-eclosion (Sang et al., 2007). Our extended rotenone paradigm (1 week exposure plus 6 weeks recovery) uses very old flies, which perform worse than younger flies on most motor

assays regardless of genetic or environmental insults (Simon et al, 2006). We therefore tested motor behavior in younger flies exposed to rotenone for 10 days. Wild type flies exposed to 250 μ M rotenone for ten days showed significant deficits in climbing relative to matched cultures exposed to vehicle alone, and the behavior of additional controls containing either *Ddc-Gal4/+* or *UAS-DVMAT/+* alone was similar to the wild type (Supplemental Fig. 2). In contrast, the behavior of DVMAT overexpressing flies exposed to rotenone did not differ from those exposed to vehicle alone (Supplemental Fig. 2). These data are consistent with the notion the DVMAT might provide neuroprotection to DA neurons, but motor behavior, like survival, is dependent on multiple circuits, and more specific tests for DA cell death are needed to address this question.

To directly test the hypothesis that DVMAT could decrease the neurotoxic effects of rotenone on DA cells, we counted the number of DA neurons in flies exposed to rotenone with and without DVMAT over-expression. To maximize our ability to detect changes in DA cell death, we first used exposure for 10 days followed by recovery for 6 weeks on normal food. As controls, we treated wild type flies in parallel with the same concentration of rotenone. In control flies, we observed a ~35% loss in DA neurons in the PPL1 cluster (Fig. 4I, two-way ANOVA, Bonferonni post-test, *** $p < 0.001$, see also Fig. 4A-H) and a 50% loss in the PAL cluster (Fig. 4J, two-way ANOVA, Bonferonni post-test, *** $p < 0.001$). Similar losses were seen in *UAS-DVMAT/+* controls (data not shown). In striking contrast, flies treated with rotenone and over-expressing DVMAT, (*Ddc-Gal4/+; UAS-DVMAT/+*) showed no detectable loss of DA neurons in either the PPL1 (Fig. 4I) or the PAL cluster (Fig. 4J) as compared to flies over-expressing DVMAT and exposed to vehicle alone (DMSO, 0.25%). These data strongly suggest that overexpression of DVMAT protects against the neurotoxic effects exerted by rotenone on DA neurons.

To confirm these results, we also tested the effects of DVMAT overexpression in a shorter rotenone exposure paradigm: 10 days exposure to rotenone immediately followed by immunolabeling rather than further aging. Consistent with the results shown in Fig. 3, the amount of DA cell death is less pronounced in flies sacrificed after 10 days as opposed to those tested using 10 day exposure paradigm followed by additional aging (see Supplemental Fig. 3). Nonetheless, under both conditions, we found that DVMAT over-expression significantly blocked the loss of DA cells caused by rotenone (Supplemental Fig. 3). These data confirm that DVMAT can provide neuroprotection to DA cells exposed to rotenone.

DVMAT overexpression does not appear to provide neuroprotection against paraquat

We next tested whether DVMAT overexpression would modify the neurotoxic effects of paraquat on DA neurons. We initially tested the effects of 2 mM paraquat, which decreased the flies' lifespan but allowed survival for up to 30 days (Supplemental Fig. 4). Despite the toxicity of this dose to the organism as a whole, we did not detect any change in the number of DA neurons in the surviving flies (Supplemental Fig. 4). We therefore tested the effects of high dose, short-term exposure, a paradigm previously reported to cause DA cell death in flies (Chaudhuri et al., 2007) focusing on PPL1 because of its relatively high sensitivity to toxic insults (Chaudhuri et al., 2007; Coulom and Birman, 2004). Consistent with an earlier report (Chaudhuri et al., 2007) we observed a 25% loss of DA neurons in the PPL1 cluster in flies incubated for 16 hours in 10mM paraquat (Fig. 5, one-way ANOVA $p < 0.001$, Bonferroni post test ** $p < 0.01$). However, in contrast to our results using rotenone, DA cell loss caused by paraquat was not significantly reduced by DVMAT overexpression (Fig. 5). These data suggest that, at least under the conditions used for these experiments, rotenone and paraquat may interact differently with DVMAT and possibly cytosolic DA, or alternatively, that the rate of DA cell death can influence the neuroprotective effects of DVMAT (see Discussion).

dVMAT loss of function mutants show fewer DA neurons

To further explore the potential neuroprotective effects of DVMAT, we tested the effects of a *dVMAT* loss of function mutation (Simon et al., 2009). We have previously shown that the *dVMAT* mutant does not express detectable levels of DVMAT protein on Western blots (Romero-Calderón et al, 2008; Simon et al, 2009). VMAT2 knockout and knockdown mice (Fon et al., 1997; Mooslehner et al., 2001; Takahashi et al., 1997; Wang et al., 1997) as well as *dVMAT* mutants (Simon et al., 2009) show profound changes in behavior, some of which are likely to represent functional adaptations to reduced signaling. Here, we sought to determine whether decreased levels of *dVMAT* would also alter the survival of DA neurons and their response to pesticide. Since *dVMAT* mutants show a reduced lifespan of ~3 weeks (Simon et al., 2009), we focused exclusively on DA cell loss over relatively short periods of time.

Previous work in VMAT2 loss of function mice suggests that reduced VMAT activity may compromise DA neurons in aged mice even under baseline conditions (Simon et al., 2009). Therefore, before testing the effects of pesticides, we determined the number of DA neurons in flies raised on normal food (Fig. 6). We were surprised to find that the number of DA neurons in *dVMAT* mutant was reduced by ~25% in both the PAL and the PPL1 clusters relative to wild type flies (Fig. 6A, B, Student's t-test, * $p < 0.05$, ** $p < 0.01$ as indicated). To rule out spurious effects due to other potential mutations on the chromosome containing the *dVMAT* mutation, we counted the number of DA neurons in another line in which the P element responsible for the *dVMAT* mutation had been precisely excised. The number of DA neurons in the PPL1 and PAL clusters did not differ from wild type (Supplemental Fig. 5), supporting the idea that the *dVMAT* mutation itself was responsible for the loss of DA cells. We have also performed genetic rescue experiments, using *UAS-DVMAT* with a *da-Gal4* driver as described previously (Simon et al. 2009). We see a partial rescue of DA neuron loss, demonstrating that mutation of *dVMAT* contributes to this phenotype (Supplemental Fig. 6).

To test the time course of neuronal loss in the *dVMAT* mutant, we compared the number of DA neurons in freshly eclosed adults versus aged adult flies. *dVMAT* loss of function flies showed the same reduction in DA neurons whether they were assayed immediately after eclosion (day 1) or after aging for an additional 2 weeks (Fig. 6C, D, two-way ANOVA $p < 0.0001$, Bonferroni post test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). These results indicate that the reduced number of DA neurons seen in the *dVMAT* mutant may represent a developmental rather than neurodegenerative effect. In contrast, the loss of DA neurons seen in VMAT2 knockdown mice is seen in aged but not young animals, more consistent with a neurodegenerative effect (Caudle et al., 2007).

DA neurons in dVMAT loss of function mutants are sensitive to rotenone and paraquat

We next determined the effect of chronic rotenone exposure on DA neurons in the *dVMAT* mutant. Because *dVMAT* mutants die within 3 weeks post eclosion (Simon et al., 2009), we limited our rotenone neurotoxicity assays to a 10-day exposure followed immediately by immunohistochemical analysis. Consistent with the data shown in Fig. 6, both control and *dVMAT* mutant flies treated with rotenone contained fewer DA neurons compared to flies of the same genotype treated with vehicle alone (Fig. 7 A, B, two-way ANOVA with Bonferroni post test, significance as indicated). However, the *dVMAT* flies treated with rotenone contained significantly fewer DA neurons than wild type flies for both the PAL (Fig. 7A) and PPL1 (Fig. 7B) clusters (two-way ANOVA with Bonferroni post test $p < 0.001$, see black arrows).

To determine how the loss of *dVMAT* might affect the neurotoxicity of paraquat, we exposed *dVMAT* mutant flies to 10mM paraquat for 16 hours as described above. Both CS and *dVMAT* mutant flies treated with paraquat showed DA neuron loss in the PAL (Fig. 7C) and PPL1 cluster (Fig. 7D). However, as with rotenone, *dVMAT* mutant flies treated with paraquat contained significantly fewer DA neurons than wild type flies in both clusters (Fig. 7C, D, two-way ANOVA with Bonferroni post test $p < 0.001$, see black arrows). In summary, the *dVMAT* loss of function mutants are sensitive to both rotenone and paraquat and, more importantly, the overall number of DA neurons in *dVMAT* mutants treated with either pesticide is less than the number of cells remaining in identically treated wild type flies.

Discussion

A large number of environmental toxins are neurotoxic, and may increase the risk of PD (Chou et al., 2008; Costello et al., 2009; Di Monte et al., 2002). The cytotoxic effects of DA may also play a role in the survival of DA neurons and the pathophysiology of PD (Caudle et al., 2007; Graham, 1978; Kuhn et al., 1999; Stokes et al., 1999; Ritz et al., 2009). However, despite much speculation on this topic, the actual interplay between pesticides and genes such as DAT and VMAT that regulate cellular DA remains poorly understood, and the notion that VMAT might alter the neurotoxic effects of pesticides in an animal model had not been investigated. We have used the genetic model organism *Drosophila melanogaster* and *DVMAT* to directly test this hypothesis.

Dopamine homeostasis is controlled by VMAT in both mammals (Fon et al., 1997; Liu et al., 1992; Mooslehner et al., 2001; Takahashi et al., 1997; Wang et al., 1997) and the fly (Chang et al., 2006; Greer et al., 2005). We have previously shown that overexpression of *DVMAT* protein in flies can decrease DA cell death caused by the overexpression of a human mutant form of *parkin* (Sang et al., 2007). In addition, RNAi directed against *dVMAT* modestly exacerbated the *parkin* overexpression phenotype (Sang et al., 2007). We have recently characterized a null *dVMAT* loss of function mutation, which provides an even more potent method than RNAi for reducing *dVMAT* expression (Simon et al., 2009). Here we have explored the influence of *dVMAT* on the neurotoxic effects of pesticides, using both the loss of function *dVMAT* mutant and *DVMAT* overexpression.

We find that *dVMAT* mutants contain fewer DA neurons than wildtype at eclosion (the onset of the adult phase) in the absence of toxin exposure. Interestingly, this appeared to be the case from the earliest adult time point, more consistent with a developmental rather than a degenerative effect. In contrast, VMAT2 knockdown mice retaining ~20% of wild type VMAT2 levels show a decrease in DA neurons with age, but wild type numbers during development (Caudle et al., 2008). To our knowledge, similar stereological measurements have not been performed in VMAT2 knockouts, which die soon after birth (Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997). Our data suggest the possibility that a complete loss of VMAT2 activity in mice might decrease the number of DA in mice as it does in flies.

dVMAT mutants show a greater cell loss than wild type flies when treated with either one of two pesticides, rotenone and paraquat, both of which have been used previously in animal models of DA cell death (Betarbet et al., 2000; Chaudhuri et al., 2007; Coulom and Birman, 2004; McCormack et al., 2002). Conversely, overexpression of *DVMAT* blocks DA cell death caused by rotenone. Inhibition of a mammalian VMAT with reserpine has been shown to increase the cytotoxicity of rotenone in cultured cells (Sai et al., 2008). However, our data are the first to show that VMAT expression can influence the toxic effects of pesticides *in vivo*, and together with previous studies (Park et al., 2007; Sang et al., 2007), strongly support the idea that VMAT, and by extension the sequestration of cellular DA, can play a neuroprotective role for DA neurons in an intact organism.

The preservation of DA neurons in DVMAT-overexpressing flies treated with rotenone appears to represent a gene x environment interaction since the observed effects are not additive: DVMAT-overexpression alone does not increase the number of DA neurons, and its effects on DA cell number are only observed when rotenone is administered. In contrast, it is not yet clear whether the interaction of the *dVMAT* loss of function allele is additive or synergistic. Since the baseline number of DA cells in the mutant is lower, the percentage of cells that are lost in mutant flies is consistently greater than wild type. In contrast, if we measure the absolute number of cells that are lost, then the mutant's cell loss in response to pesticide exposure is generally similar to wild type (however, see Fig. 7C). Additional experiments will be needed to determine whether the mutants are truly more sensitive than the wild type to pesticides, or alternatively, whether DVMAT expression might compromise the health of DA neurons via two independent mechanisms.

It is conceivable that DVMAT provides neuroprotection against rotenone by sequestering it into synaptic vesicles, thereby preventing its access to mitochondria. However, rotenone is structurally quite distinct from biogenic amines or other known VMAT substrates, such as the toxin MPP⁺ or amphetamines. Rather, the simplest interpretation of our results is that cytosolic DA can interact with rotenone to increase its cytotoxic potential, and that VMAT functions as a neuroprotectant by decreasing cytosolic DA concentrations. Rotenone inhibits complex I of the mitochondrial respiratory chain (Betarbet et al., 2000; Chance et al., 1963; Panov et al., 2005), but neurotoxicity may also be influenced by its ability to disrupt microtubules (Feng, 2006), and at least one study has questioned the importance of complex I for the neurotoxic effects of rotenone in DA cells (Choi et al., 2008). Although it remains unclear which of these mechanisms is correct, one common outcome may be an increase in oxidative stress on the cell, or if both complex I and microtubules are impaired, a more specific increase in the cell body (Feng, 2006; Ren et al., 2009). Cytosolic DA would be expected to potentiate this effect (Guillot and Miller, 2009; Hastings et al., 1996; Michel and Hefti, 1990; Mosharov et al., 2009), and the convergence of these two weak oxidative stressors might explain the relative specificity of rotenone to kill DA neurons.

The developmental loss of DA neurons due to loss of VMAT activity has not been reported previously, and as noted above may not be due to same mechanism by which DVMAT influences the animal's response to pesticides. It is possible that an unknown exogenous toxin interacts with DA during development. Alternatively, the ability of DA cells to protect themselves against oxidative stress (or other effects of DA) during development may be relatively weak, such that additional toxins are not needed. Finally, it is conceivable that DA cell loss in the mutant is a non-cell autonomous effect caused indirectly by the loss of serotonergic or octopaminergic signaling, a hypothesis that can be tested using genetic rescue in specific aminergic cell types. Regardless of the precise mechanism, our findings raise the possibility that *dVMAT* mutant flies might be used to further study how the health of DA cells early in life can influence their function at later time points. Importantly, for PD patients, it is not known how the premorbid function of DA neurons may influence the pathogenesis of PD later in life.

The death of DA neurons in DVMAT overexpression flies exposed to paraquat demonstrates the limitations of VMAT's neuroprotective effects, and may also reflect differences between the neurotoxic mechanisms of paraquat versus rotenone. If DA functions primarily as an oxidant, it would suggest that the neuroprotective effects of DVMAT might be overwhelmed by toxins with a greater oxidative potential than rotenone. Paraquat is thought to cause a massive increase in oxidative stress (Bus and Gibson, 1984; Przedborski and Ischiropoulos, 2005) and it is likely that the cellular target(s) of paraquat and rotenone are different (Ramachandiran et al., 2007). The high oxidative potential of paraquat may supersede whatever contribution cytosolic DA might have on the overall oxidative stress experienced

by a DA cell. Thus, at least in the overexpression experiments we show here, reducing the weak oxidative stress caused by dopamine may be inconsequential in face of the more potent effects of paraquat.

However, it is also possible that the rapid administration of paraquat was the most important factor in its inability to interact with DVMAT overexpression. Ideally, rotenone and paraquat would be administered similarly, and both assayed for relatively long-term (days to weeks) rather than acute (hours to days) neurodegenerative effects. In our hands, low dose (2 mM) paraquat exposure for four weeks did not cause a detectable loss of DA neurons (Supplemental Fig. 4). We therefore used acute exposure to high dose paraquat as previously reported (Chaudhuri et al., 2007) with the resultant loss of DA cells occurring over the course of hours. Thus, it is possible that over-expression of DVMAT failed to provide neuroprotection against paraquat because cell death occurred too rapidly, rather than because of a higher oxidative potential than rotenone. Further experiments will be needed to determine how the rate of cell death, versus the intrinsic activities of paraquat and rotenone affect the ability of DVMAT to provide neuroprotection.

In particular, it will be important to determine how DVMAT may influence the oxidative stress in DA neurons caused by each agent, and the time course of these effects. At least one recent study testing the effects of expressing PD-related gene in DA cells used immunolabeling to measure oxidative changes in these cells (Imai et al., 2008). Ingestion of pesticides is likely to cause a general increase in oxidative stress throughout the brain, rather than specifically in DA neurons. Under these conditions, the detection of oxidative changes in DA neurons may be difficult, and require the use of other biochemical methods.

Regardless of the precise mechanisms by which rotenone and paraquat kill DA cells, the difference we observe between the effects of *dVMAT* on paraquat versus rotenone underscore the important idea that its potential for neuroprotection must be considered within the context of both the specific type of toxin and the manner of exposure. This idea may be relevant to future gene-environment interaction studies in mammalian systems, and for epidemiological data on the potential effects of polymorphisms in the human VMAT2 gene (Glatt et al., 2006). If we can extrapolate from our data generated in flies, it would suggest that polymorphisms that increase the expression of VMAT2 might confer neuroprotection only in people exposed to specific pesticides, or alternatively only to relatively low-dose, chronic exposures rather than higher-dose, acute exposures. To test this hypothesis, epidemiological studies of PD may be used to correlate the exposure to specific pesticides and expression of particular VMAT2 haplotypes. One potentially important comparison could be the high-dose exposure of farm workers who periodically handle pesticides directly, versus others chronically exposed to lower levels of pesticides.

We have explored the possibility of using two other pesticides (ziram and benomyl) to model DA cell death in the fly. Both may increase the risk of PD in exposed populations (Ritz personal communication) and show cytotoxicity *in vitro* (Chou et al., 2008; Wang et al., 2006b). However, feeding high-dose ziram has no effect on fly survival. We therefore believe that it is unlikely to be useful for more specific studies of neurotoxicity in the fly, at least using our current exposure paradigm. Benomyl shows a weak effect at high dose suggesting that it is capable of at least some toxic effects in flies, but does not affect climbing ability, limiting the likelihood that it could be used as a model for neurotoxicity. It is possible that limited absorption or other pharmacokinetic parameters can reduce the toxicity of these agents. Alternatively, their toxicity may be revealed in *Drosophila* only with additional genetic risk factors. Here we did not pursue either toxin for more specific studies on DA cell death.

To facilitate our neurotoxicity studies, we have optimized the rotenone exposure paradigm, starting with methods published by others using a relatively short exposure (Coulom and Birman, 2004). We have modified the paradigm such that flies are exposed to rotenone for ten days, then cultured on normal food for six weeks. Using this protocol, we observe an increase in DA cell death relative to a more acute (ten day) exposure paradigm. The more robust loss of DA cells has helped to demonstrate the neuroprotective effects of DVMAT, and may be useful for other studies of neurodegeneration in the fly.

In addition to directly counting DA neurons, we have used survival and behavioral tests following exposure of flies to pesticides. DVMAT overexpression may slightly increase survival in flies exposed to paraquat, but does not increase the survival of flies exposed to rotenone as compared to the *UAS-DVMAT* transgene alone. These data indicate that DVMAT and its expression in dopaminergic cells are limited in their ability to mitigate effects of pesticides in the organism as a whole. In contrast, other genes such as DJ1 and pink may have cytoprotective effects in a variety of cell types, and their absence can markedly decrease survival in animals exposed to pesticides (Clark et al., 2006; Meulener et al., 2005). Studies in *Drosophila* of these, more widely expressed genes are likely to enhance our understanding of basic cellular mechanisms of neurodegeneration relevant to PD. In contrast, future mechanistic studies of DVMAT will help determine how DA contributes to the remarkable sensitivity of dopaminergic cells to environmental as well as genetic insults. In addition, if VMAT is indeed able to protect DA cells against some pesticides, we speculate that genetic or drug screens in *Drosophila* may be useful to develop strategies to increase VMAT activity and thus reduce toxin-induced DA cell death.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Beate Ritz and Marie-Francoise Chesselet for helpful suggestions on the manuscript and Marie-Francoise Chesselet for her leadership as principle investigator on the program project grant from the National Institute of Environmental Health and Safety [ES016732] listed above.

Funding

This work was supported by the National Institute of Mental Health [MH076900]; and the National Institute of Environmental Health and Safety [ES015747, ES016732].

References

- Auluck PK, et al. Chaperone suppression of alpha-synuclein toxicity in a *Drosophila* model for Parkinson's disease. *Science* 2002;295:865–8. [PubMed: 11823645]
- Barbeau A, et al. Environmental and genetic factors in the etiology of Parkinson's disease. *Adv Neurol* 1987;45:299–306. [PubMed: 3493629]
- Betarbet R, et al. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* 2000;3:1301–1306. [PubMed: 11100151]
- Bocchetta A, Corsini GU. Parkinson's disease and pesticides. *Lancet* 1986;2:1163. [PubMed: 2877313]
- Botella JA, Bayersdorfer F, Schneuwly S. Superoxide dismutase overexpression protects dopaminergic neurons in a *Drosophila* model of Parkinson's disease. *Neurobiol. Dis* 2008;30:65–73. [PubMed: 18243716]
- Bus JS, Gibson JE. Paraquat: model for oxidant-initiated toxicity. *Environ Health Perspect* 1984;55:37–46. [PubMed: 6329674]

- Caudle WM, et al. Altered vesicular dopamine storage in Parkinson's disease: a premature demise. *Trends in Neurosciences* 2008;31:303–308. [PubMed: 18471904]
- Caudle WM, et al. Reduced vesicular storage of dopamine causes progressive nigrostriatal neurodegeneration. *J Neurosci* 2007;27:8138–48. [PubMed: 17652604]
- Chance B, et al. Inhibition of electron and energy transfer in mitochondria. I. Effects of Amytal, thiopental, rotenone, progesterone, and methylene glycol. *J Biol Chem* 1963;238:418–31. [PubMed: 14020001]
- Chang HY, et al. Overexpression of the *Drosophila* vesicular monoamine transporter increases motor activity and courtship but decreases the behavioral response to cocaine. *Mol Psychiatry* 2006;11:99–113. [PubMed: 16189511]
- Chaudhry FA, et al. Pharmacology of neurotransmitter transport into secretory vesicles. *Handb Exp Pharmacol* 2008;184:77–106. [PubMed: 18064412]
- Chaudhuri A, et al. Interaction of genetic and environmental factors in a *Drosophila* parkinsonism model. *J Neurosci* 2007;27:2457–67. [PubMed: 17344383]
- Choi HJ, et al. Inhibition of vesicular monoamine transporter enhances vulnerability of dopaminergic cells: relevance to Parkinson's disease. *Neurochem Int* 2005;46:329–35. [PubMed: 15707697]
- Choi WS, et al. Mitochondrial complex I inhibition is not required for dopaminergic neuron death induced by rotenone, MPP+, or paraquat. *Proc Natl Acad Sci U S A* 2008;105:15136–41. [PubMed: 18812510]
- Chou AP, et al. Ziram causes dopaminergic cell damage by inhibiting E1 ligase of the proteasome. *J Biol Chem* 2008;283:34696–703. [PubMed: 18818210]
- Cicchetti F, et al. Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? *Trends Pharmacol Sci* 2009;30:475–83. [PubMed: 19729209]
- Clark IE, et al. *Drosophila* pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature* 2006;441:1162–6. [PubMed: 16672981]
- Conway KA, et al. Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. *Science* 2001;294:1346–9. [PubMed: 11701929]
- Costello S, et al. Parkinson's disease and residential exposure to maneb and paraquat from agricultural applications in the central valley of California. *Am J Epidemiol* 2009;169:919–26. [PubMed: 19270050]
- Coulom H, Birman S. Chronic exposure to rotenone models sporadic Parkinson's disease in *Drosophila melanogaster*. *J Neurosci* 2004;24:10993–8. [PubMed: 15574749]
- Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. *Neuron* 2003;39:889–909. [PubMed: 12971891]
- Di Monte DA. The environment and Parkinson's disease: is the nigrostriatal system preferentially targeted by neurotoxins? *Lancet Neurol* 2003;2:531–8. [PubMed: 12941575]
- Di Monte DA, et al. Environmental factors in Parkinson's disease. *Neurotoxicology* 2002;23:487–502. [PubMed: 12428721]
- Duerr JS, et al. The cat-1 gene of *Caenorhabditis elegans* encodes a vesicular monoamine transporter required for specific monoamine-dependent behaviors. *J Neurosci* 1999;19:72–84. [PubMed: 9870940]
- Duffy JB. GAL4 system in *Drosophila*: a fly geneticist's Swiss army knife. *Genesis* 2002;34:1–15. [PubMed: 12324939]
- Eiden LE, et al. The vesicular amine transporter family (SLC18): amine/proton antiporters required for vesicular accumulation and regulated exocytotic secretion of monoamines and acetylcholine. *Pflügers Arch* 2004;447:636–40.
- Feany MB, Bender WW. A *Drosophila* model of Parkinson's disease. *Nature* 2000;404:394–8. [PubMed: 10746727]
- Feng J. Microtubule: a common target for parkin and Parkinson's disease toxins. *Neuroscientist* 2006;12:469–76. [PubMed: 17079513]
- Fon EA, et al. Vesicular transport regulates monoamine storage and release but is not essential for amphetamine action. *Neuron* 1997;19:1271–1283. [PubMed: 9427250]

- Fumagalli F, et al. Increased methamphetamine neurotoxicity in heterozygous vesicular monoamine transporter 2 knock-out mice. *J Neurosci* 1999;19:2424–31. [PubMed: 10087057]
- Giasson BI, Lee VM. Parkin and the molecular pathways of parkinson's disease. *Neuron* 2001;31:885–888. [PubMed: 11580890]
- Glatt CE, et al. Gain-of-function haplotypes in the vesicular monoamine transporter promoter are protective for Parkinson disease in women. *Hum. Mol. Genet* 2006;15:299–305. [PubMed: 16339215]
- Graham DG. Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Mol Pharmacol* 1978;14:633–43. [PubMed: 98706]
- Greer CL, et al. A splice variant of the *Drosophila* vesicular monoamine transporter contains a conserved trafficking domain and functions in the storage of dopamine, serotonin, and octopamine. *J Neurobiol* 2005;64:239–58. [PubMed: 15849736]
- Guillot TS, et al. Reduced vesicular storage of dopamine exacerbates methamphetamine-induced neurodegeneration and astrogliosis. *Journal of Neurochemistry* 2008;106:2205–17. [PubMed: 18643795]
- Guillot TS, Miller GW. Protective actions of the vesicular monoamine transporter 2 (VMAT2) in monoaminergic neurons. *Mol Neurobiol* 2009;39:149–70. [PubMed: 19259829]
- Hanson GR, et al. Psychostimulants and vesicle trafficking: a novel mechanism and therapeutic implications. *Ann N Y Acad Sci* 2004;1025:146–50. [PubMed: 15542712]
- Hastings TG, et al. Reactive dopamine metabolites and neurotoxicity: implications for Parkinson's disease. *Adv. Exp. Med. Biol* 1996;387:97–106. [PubMed: 8794199]
- Hirsch EC, et al. Animal models of Parkinson's disease in rodents induced by toxins: an update. *J Neural Transm Suppl* 2003;89–100. [PubMed: 12946051]
- Hubble JP, et al. Risk factors for Parkinson's disease. *Neurology* 1993;43:1693–7. [PubMed: 8414014]
- Imai Y, et al. Phosphorylation of 4-BP by LRRK2 affects the maintenance of dopaminergic neurons in *Drosophila*. *EMBO J* 2008;27:2432–43. [PubMed: 18701920]
- Kelada SN, et al. 5' and 3' region variability in the dopamine transporter gene (SLC6A3), pesticide exposure and Parkinson's disease risk: a hypothesis-generating study. *Hum Mol Genet* 2006;15:3055–62. [PubMed: 16963468]
- Koller W, et al. Environmental risk factors in Parkinson's disease. *Neurology* 1990;40:1218–21. [PubMed: 2381528]
- Krantz DE, et al. Phosphorylation of a vesicular monoamine transporter by casein kinase II. *J. Biol. Chem* 1997;272:6752–59. [PubMed: 9045708]
- Kuhn DM, et al. Tyrosine hydroxylase is inactivated by catechol-quinones and converted to a redox-cycling quinoprotein: possible relevance to Parkinson's disease. *J Neurochem* 1999;73:1309–17. [PubMed: 10461926]
- LaVoie MJ, et al. Dopamine covalently modifies and functionally inactivates parkin. *Nat Med* 2005;11:1214–21. [PubMed: 16227987]
- Li H, et al. Ectopic G-protein expression in dopamine and serotonin neurons blocks cocaine sensitization in *Drosophila melanogaster*. *Curr. Biol* 2000;10:211–214. [PubMed: 10704417]
- Liu Y, Edwards RH. The role of vesicular transport proteins in synaptic transmission and neural degeneration. *Annu Rev Neurosci* 1997;20:125–156. [PubMed: 9056710]
- Liu Y, et al. A cDNA that suppresses MPP⁺ toxicity encodes a vesicular amine transporter. *Cell* 1992;70:539–51. [PubMed: 1505023]
- McCormack AL, et al. Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. *Neurobiol Dis* 2002;10:119–27. [PubMed: 12127150]
- Meulener M, et al. *Drosophila* DJ-1 mutants are selectively sensitive to environmental toxins associated with Parkinson's disease. *Curr Biol* 2005;15:1572–7. [PubMed: 16139213]
- Michel PP, Hefti F. Toxicity of 6-hydroxydopamine and dopamine for dopaminergic neurons in culture. *J. Neurosci. Res* 1990;26:428–435. [PubMed: 1977925]
- Miller GW, et al. Dopamine transporters and neuronal injury. *Trends Pharmacol Sci* 1999;20:424–9. [PubMed: 10498956]

- Moore DJ, et al. Molecular pathophysiology of Parkinson's disease. *Annu Rev Neurosci* 2005;28:57–87. [PubMed: 16022590]
- Mooslehner KA, et al. Mice with very low expression of the vesicular monoamine transporter 2 gene survive into adulthood: potential mouse model for parkinsonism. *Mol Cell Biol* 2001;21:5321–31. [PubMed: 11463816]
- Mosharov EV, et al. Interplay between cytosolic dopamine, calcium, and alpha-synuclein causes selective death of substantia nigra neurons. *Neuron* 2009;62:218–29. [PubMed: 19409267]
- Mosharov EV, et al. Alpha-synuclein overexpression increases cytosolic catecholamine concentration. *J Neurosci* 2006;26:9304–11. [PubMed: 16957086]
- Nass R, Blakely RD. The *Caenorhabditis elegans* dopaminergic system: opportunities for insights into dopamine transport and neurodegeneration. *Annu Rev Pharmacol Toxicol* 2003;43:521–44. [PubMed: 12415122]
- Norris EH, et al. Reversible inhibition of alpha-synuclein fibrillization by dopaminochrome-mediated conformational alterations. *J Biol Chem* 2005;280:21212–9. [PubMed: 15817478]
- Oh SW, et al. A P-element insertion screen identified mutations in 455 novel essential genes in *Drosophila*. *Genetics* 2003;163:195–201. [PubMed: 12586707]
- Olanow CW, Tatton WG. Etiology and pathogenesis of Parkinson's disease. *Annu Rev Neurosci* 1999;22:123–44. [PubMed: 10202534]
- Panov A, et al. Rotenone model of Parkinson disease: multiple brain mitochondria dysfunctions after short term systemic rotenone intoxication. *J Biol Chem* 2005;280:42026–35. [PubMed: 16243845]
- Park SS, et al. Disruption of dopamine homeostasis underlies selective neurodegeneration mediated by alpha-synuclein. *Eur J Neurosci* 2007;26:3104–12. [PubMed: 18005066]
- Przedborski S, Ischiropoulos H. Reactive oxygen and nitrogen species: weapons of neuronal destruction in models of Parkinson's disease. *Antioxid Redox Signal* 2005;7:685–93. [PubMed: 15890013]
- Rajput AH, et al. Geography, drinking water chemistry, pesticides and herbicides and the etiology of Parkinson's disease. *Can J Neurol Sci* 1987;14:414–8. [PubMed: 3676917]
- Ramachandiran S, et al. Divergent mechanisms of paraquat, MPP⁺, and rotenone toxicity: oxidation of thioredoxin and caspase-3 activation. *Toxicol. Sci* 2007;95:163–71. [PubMed: 17018646]
- Ren Y, et al. Parkin protects dopaminergic neurons against microtubule-depolymerizing toxins by attenuating microtubule-associated protein kinase activation. *J Biol Chem* 2009;284:4009–17. [PubMed: 19074146]
- Richardson JR, et al. Developmental exposure to the pesticide dieldrin alters the dopamine system and increases neurotoxicity in an animal model of Parkinson's disease. *FASEB J* 2006;20:1695–7. [PubMed: 16809432]
- Richardson JR, et al. Developmental heptachlor exposure increases susceptibility of dopamine neurons to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in a gender-specific manner. *Neurotoxicology* 2008;29:855–63. [PubMed: 18577399]
- Ritz BR, et al. Dopamine transporter genetic variants and pesticides in Parkinson's disease. *Environ Health Perspect* 2009;117:964–9. [PubMed: 19590691]
- Romero-Calderón R, et al. A glial variant of the vesicular monoamine transporter is required to store histamine in the *Drosophila* visual system. *PLoS Genet* 2008;4:e1000245. [PubMed: 18989452]
- Sai Y, et al. Rotenone-induced PC12 cell toxicity is caused by oxidative stress resulting from altered dopamine metabolism. *Toxicol In Vitro* 2008;22:1461–8. [PubMed: 18579341]
- Sang TK, et al. A *Drosophila* model of mutant human parkin-induced toxicity demonstrates selective loss of dopaminergic neurons and dependence on cellular dopamine. *J Neurosci* 2007;27:981–92. [PubMed: 17267552]
- Seidler A, et al. Possible environmental, occupational, and other etiologic factors for Parkinson's disease: a case-control study in Germany. *Neurology* 1996;46:1275–84. [PubMed: 8628466]
- Semchuk KM, et al. Parkinson's disease and exposure to agricultural work and pesticide chemicals. *Neurology* 1992;42:1328–35. [PubMed: 1620342]
- Simon AF, et al. Differential decline in behavioral performance of *Drosophila melanogaster* with age. *Mech. Age. Devel* 2006;127:647–51.

- Simon AF, et al. *Drosophila* vesicular monoamine transporter mutants can adapt to reduced or eliminated vesicular stores of dopamine and serotonin. *Genetics* 2009;181:525–41. [PubMed: 19033154]
- Sriram SR, et al. Familial-associated mutations differentially disrupt the solubility, localization, binding and ubiquitination properties of parkin. *Hum Mol Genet* 2005;14:2571–86. [PubMed: 16049031]
- Stokes AH, et al. Cytotoxic and genotoxic potential of dopamine. *J Neurosci Res* 1999;55:659–65. [PubMed: 10220107]
- Sulzer D, et al. Neuromelanin biosynthesis is driven by excess cytosolic catecholamines not accumulated by synaptic vesicles. *Proc Natl Acad Sci U S A* 2000;97:11869–74. [PubMed: 11050221]
- Takahashi N, et al. VMAT2 knockout mice: Heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity. *Proc. Natl. Acad. Sci. USA* 1997;94:9938–9943. [PubMed: 9275230]
- Taylor TN, et al. Nonmotor symptoms of Parkinson's disease revealed in an animal model with reduced monoamine storage capacity. *J Neurosci* 2009;29:8103–13. [PubMed: 19553450]
- Thiffault C, et al. Increased striatal dopamine turnover following acute administration of rotenone to mice. *Brain Res* 2000;885:283–8. [PubMed: 11102582]
- Wang C, et al. Stress-induced alterations in parkin solubility promote parkin aggregation and compromise parkin's protective function. *Hum Mol Genet* 2005;14:3885–97. [PubMed: 16278233]
- Wang D, et al. From the Cover: Antioxidants protect PINK1-dependent dopaminergic neurons in *Drosophila*. *Proc Natl Acad Sci U S A* 2006a;103:13520–5. [PubMed: 16938835]
- Wang XF, et al. Inhibitory effects of pesticides on proteasome activity: implication in Parkinson's disease. *Neurobiol Dis* 2006b;23:198–205. [PubMed: 16626962]
- Wang YM, et al. Knockout of the vesicular monoamine transporter 2 gene results in neonatal death and supersensitivity to cocaine and amphetamine. *Neuron* 1997;19:1285–96. [PubMed: 9427251]
- Weihe E, et al. Localization of vesicular monoamine transporter isoforms (VMAT1 and VMAT2) to endocrine cells and neurons in rat. *J Mol Neurosci* 1994;5:149–64. [PubMed: 7654518]
- Weingarten P, Zhou QY. Protection of intracellular dopamine cytotoxicity by dopamine disposition and metabolism factors. *J Neurochem* 2001;77:776–85. [PubMed: 11331406]
- Whitworth AJ, et al. Increased glutathione S-transferase activity rescues dopaminergic neuron loss in a *Drosophila* model of Parkinson's disease. *Proc Natl Acad Sci U S A* 2005;102:8024–9. [PubMed: 15911761]
- Xu J, et al. Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. *Nat Med* 2002;8:600–6. [PubMed: 12042811]

Figure 1.

Lifespan of flies exposed to pesticides. (A) Continuous exposure to ziram (0-500 μ M) has no effect on the survival of wild type (CS) flies. For this and all other panels, each point represents mean $\pm$ SEM, $n=3-4$ vials, 20 flies per vial. (B) Increasing concentrations of rotenone (0-500 μ M) progressively decreases lifespan, and each curve shows a statistically significant reduction in median survival in comparison to vehicle only, Gehan-Breslow-Wilcoxon test comparing survival curves, *** $p<0.0001$. (C) Benomyl (0-250 μ M) decreases survival at 250 μ M as compared to vehicle only ("0 μ M") or 125 μ M (Gehan-Breslow-Wilcoxon test, $p<0.0001$ ***). (D) Paraquat at both low (0.5-1 mM) and high concentrations (10, 20 mM) reduce lifespan albeit more dramatically at high concentrations.

Treatment with 1, 10 and 20 mM is statistically different from vehicle only (0 μ M), ** $p < 0.01$, *** $p < 0.0001$, *** $p < 0.0001$ respectively, Gehan-Breslow-Wilcoxon test. (E). Benomyl has no effect on climbing ability at 14 or 25 days and did not potentiate the effects of rotenone at 25 days. In contrast rotenone alone is sufficient to impair mobility at 25 days, one-way ANOVA $p < 0.001$, Bonferroni post-test ** $p < 0.01$, (n=3 trials per condition, 25 flies per trial, mean $\pm$ SEM).

Figure 2.

Survival of DVMAT over-expressing flies exposed to rotenone and paraquat. Each data point represents n=3-4 trials per condition, 25 flies per trial, mean $\pm$ SEM. (A) Flies exposed to rotenone and overexpressing DVMAT using one copy of both the *Ddc-Gal4* and *UAS-DVMAT* transgenes (*Ddc-Gal4*/+; *UAS-DVMAT*/+) did not survive longer than controls containing one copy of *UAS-DVMAT* alone (*UAS-DVMAT*/+), but did survive longer than wt CS flies (Gehan-Breslow-Wilcoxon test, *** p <0.0001) and flies containing one copy of *Ddc-Gal4* (*Ddc-Gal4*/+). (B) Lifespan in 10mM paraquat is modestly increased in flies over-expressing one copy of *DVMAT* (*Ddc-Gal4*/+; *UAS-DVMAT*/+) as compared to the controls

Ddc-Gal4/+ (***p*<0.0001), *UAS-DVMAT/+* (***p*<0.0001) and CS (***p*<0.01, Gehan-Breslow-Wilcoxon test).

Figure 3.

Rotenone-induced degeneration of dopaminergic neurons. (A) Dopaminergic (DA) neurons in the Protocerebral Anteriolateral (PAL) and one of the Protocerebral Posteriolateral clusters (PPL1), and serotonergic (5HT) neurons in the SP1, SP2 and LP2 clusters were analyzed. Low power (B, F) and high power (C, D, E, G, H, I) views of the central brain show the PPL1 (B-E) and PAL (F-I) clusters in wild type (CS) flies treated with rotenone (250 μ M) for 10 days (10d Rot, panels D, H), or rotenone for 10 days followed by a 6 week recovery period on standard food ("10d Rot, 6 wk rec", E, I) or DMSO ("vehicle") for 10d followed by 6 weeks on standard food (C, G). Quantitation ($n=12-14$ clusters/condition, mean $\pm$ SEM for each bar) shows that flies fed rotenone for 10 days then aged for 6 weeks lose a greater number of DA neurons than flies fed rotenone for 10 days and immediately sacrificed (two-way ANOVA $p<0.0001$, Bonferonni post test $**p<0.01$ as indicated). Flies fed vehicle alone showed similar numbers of DA neurons in the PPL1 (J) and PAL (K) clusters at 10 days versus 10 days plus 6 weeks recovery. (L) The number of 5HT neurons in the SP1, SP2 and LP2 clusters was similar in flies treated with rotenone for 10 days and aged for 6 weeks versus those exposed to vehicle alone in parallel. Scale bars B, F 50 μ m; C, G 20 μ m.

Figure 4.

Overexpression of DVMAT protects against rotenone-induced DA neurodegeneration. Flies were treated for 10 days with (+) or without (–) rotenone (“Rot”, 250 μ M) followed by recovery for 6 weeks on standard food as in Fig. 3. A pronounced loss of DA neurons can be seen in both the PPL1 cluster (B) and PAL cluster (F) in wild type (CS) flies treated with rotenone versus those exposed to vehicle alone (A, E). In flies overexpressing 1 DVMAT (*Ddc-Gal4/+; UAS-DVMAT/+*), the number of neurons in the PPL1 and PAL clusters appears similar in flies treated with rotenone (D and H respectively) versus vehicle alone (C, G). Scale bar 20 μ m. Quantitation of DA cell numbers ($n=14-16$ clusters/bar, mean $\pm$ SEM) for both the PPL1 (I) and PAL (J) confirms that rotenone-treatment (black bars) dramatically decreases DA cell counts in wild type (CS) flies, but does not decrease DA cell counts in flies overexpressing DVMAT (“DVMAT”). White bars (“vehicle”) represent parallel treatment with DMSO. For both PPL1 and PAL 1: two-way ANOVA, $p<0.001$, Bonferroni post test *** $p<0.001$ as indicated.

Figure 5.

Overexpression of DVMAT does not appear to protect DA neurons against paraquat. (A) The number of DA neurons in the PPL1 cluster of wild type (CS) flies treated with 10mM paraquat for 16hr is lower than that seen in flies treated with vehicle alone. Over-expression of one (*Ddc-Gal4/+; UAS-DVMAT/+*) or two copies of the *Ddc-Gal4* and *UAS-DVMAT* transgenes (*Ddc-Gal4; UAS-DVMAT*) did not significantly increase the number of PPL1 DA neurons compared to paraquat-treated CS controls.

Figure 6.

Developmental loss of DA neurons in the dVMAT mutant. (A-B) Quantitation of DA neurons in the PAL (panel A) and PPL1 (panel B) clusters shows fewer cells in the dVMAT mutant (“P/P”) compared to age-matched wild type (CS) controls (mean $\pm$ SEM, $n=10$ clusters/bar, Student’s t test, $*p<0.05$, $**p<0.01$ as indicated. Note that the results of panel B were part of a larger data set examining multiple other genotypes as shown in Supplemental Fig 3. (C-D) Fewer DA neurons relative to CS controls ($n=10-12$ clusters/bar) are seen in both the PAL (C) and PPL1 (D) clusters of the dVMAT mutant immediately after eclosion (Age: 1d) and in flies aged 3-14 days (two-way ANOVA $p<0.0001$, Bonferroni

post test, * $p < 0.05$, ** $p < 0.01$). The difference between genotypes does not significantly increase with age.

Figure 7. *dVMAT* mutant exposure to paraquat and rotenone. DA neuron counts (mean $\pm$ SEM, $n=10-12$ clusters/bar) for PAL (A, C) and PPL1 clusters (B, D) in *dVMAT* mutants and wild type (CS) controls exposed to 250 μ M rotenone for 10 days (A, B “Rot”, black bars) or 10 mM paraquat for 16 hours (C, D, “PQ”, black bars) versus vehicle alone (A, B, C, D white bars). In each case, the number of DA neurons in the *dVMAT* mutants was lower than CS controls (see black arrows). Two-way ANOVA shows significant effects for genotype and treatment (panels B, C, D, $p<0.0001$) or genotype alone (panel A, $p<0.0001$) with Bonferroni post test comparisons between conditions as indicated, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.