Neurobiology of Disease

A *Drosophila* Model of Mutant Human Parkin-Induced Toxicity Demonstrates Selective Loss of Dopaminergic Neurons and Dependence on Cellular Dopamine

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Mutations in human parkin have been identified in familial Parkinson's disease and in some sporadic cases. Here, we report that expression of mutant but not wild-type human parkin in *Drosophila* causes age-dependent, selective degeneration of dopaminergic (DA) neurons accompanied by a progressive motor impairment. Overexpression or knockdown of the *Drosophila* vesicular monoamine transporter, which regulates cytosolic DA homeostasis, partially rescues or exacerbates, respectively, the degenerative phenotypes caused by mutant human parkin. These results support a model in which the vulnerability of DA neurons to parkin-induced neurotoxicity results from the interaction of mutant parkin with cytoplasmic dopamine.

Key words: **dopaminergic; *Drosophila*; monoamine; aging (ageing); neuroprotection; neuronal death**

Introduction

The clinical features of Parkinson's disease (PD), including bradykinesia, rigidity, resting tremor, and postural impairment, are primarily resulting from the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) (Fahn, 2003). Although the vast majority of PD cases are sporadic, insights from studies of rare inherited forms have provided opportunities to investigate the pathogenesis of idiopathic disease. Mutations in parkin are thought to be the second most common genetic cause of sporadic PD, after LRRK2/dardarin (Kitada et al., 1998; Foroud et al., 2003; Klein et al., 2003; Lincoln et al., 2003; Hedrich et al., 2004; Gilks et al., 2005; Hernandez et al., 2005). Parkin mutations originally were identified in families with autosomal recessive juvenile parkinsonism (AR-JP) (Kitada et al., 1998).

Although neuropathology is not limited to the nigrostriatal system, ablation of DA neurons in the SNc remains the hallmark of sporadic PD; yet, the reason that DA neurons are selectively vulnerable is unresolved. Dopamine is a highly reactive amine, and its oxidation generates neurotoxic quinones and reactive oxygen species (Stokes et al., 1999; Sulzer and Zecca, 2000). DA quinone spontaneously reacts with reduced sulfhydryl groups of cysteine, and recent results indicate that covalent modification of parkin by DA quinone reduces its E3 ligase activity and also decreases solubility (LaVoie et al., 2005). Cell culture models also suggest that dopamine and other potential oxidants modify parkin solubility and may reduce its neuroprotective effects (Sriram et al., 2005; Wang et al., 2005a,b). These data support the longstanding suspicion that dopamine itself may be the basis for the regional vulnerability seen in some forms of PD (Cookson, 2005). Mouse and *Drosophila* models used to test this hypothesis show variable degrees of pathology in DA neurons (Bonifati et al., 2003; Goldberg et al., 2003, 2005; Von Coelln et al., 2004; Chen et al., 2005; Menzies et al., 2005; Meulener et al., 2005; Perez and Palmiter, 2005).

Here, we use *Drosophila* to explore whether parkin mutations identified in familial PD can exert cell-specific toxic effects *in vivo*, suspending conventional notions regarding the genetics of parkin-linked AR-JP. Using histological and behavioral analysis, we demonstrate that expression of mutant but not wild-type human parkin in *Drosophila* induces progressive, age-dependent degeneration of DA neurons as well as motor dysfunction. Manipulation of dopamine storage using the *Drosophila* vesicular monoamine transporter (DVMAT) has profound effects on mutant parkin-induced toxicity. These *in vivo* data support a neurotoxic mechanism in which interaction of mutant parkin with dopamine underlies selective vulnerability of DA neurons in parkin-associated familial PD.

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Materials and Methods

Molecular biology. Plasmid cDNAs encoding wild-type human parkin, including an N-terminal Myc tag and two different FLAG-tagged mutant forms (Q311Stop and T240R) (Shimura et al., 2000), were subcloned as *Pme*I fragments into the *Stu*I site using Exelixis (San Francisco, CA) modification of the *Drosophila* upstream activation sequence (UAS) expression vector (pEx-UAS) (Ollmann et al., 2000). The identity of all constructs was confirmed by sequencing. UAS-DVMAT has been described previously (Chang et al., 2006). To generate the DVMAT interference (DVMATi) construct, a modified hairpin-loop RNA interference (RNAi) procedure (Kalidas and Smith, 2002) was used. Two PCR products were ligated in head-to-tail (genomic DNA) and tail-to-head (cDNA) configurations. The genomic DVMAT fragment corresponded to exon 2–intron 2–exon 3–intron 3–exon 4–intron 4 (forward primer, 5′-tgcccatcatacccgagttcctg-3′; reverse primer, 5′-ctaagaaaaggttatgggttcaa-3′), whereas the cDNA used exons 2–4 (forward primer, 5′-tctaga-tgcccatcatacccgagttc-3′, underline indicates an *Xba*I site; reverse primer, 5′-ttatcgttgagaggaacatatcac-3′). These were engineered using PCR and subcloned into the pCRII-topoisomerase I (TOPO) vector (Invitrogen, San Diego, CA). The pCRII-TOPO–VMAT cDNA–exon 2–4 construct was then cut with *Xba*I and *Not*I and inserted into the pCRII-TOPO–VMAT genomic construct. The orientation and junctions of the inserts were confirmed by sequencing. A partial digest using *Eco*RI and *Xba*I yielded the complete VMATi construct, which was then subcloned into pEx-UAS.

Drosophila *genetics*. Germline transformation followed standard methods (Rubin and Spradling, 1982; Spradling and Rubin, 1982), and multiple transgenic lines for each construct were obtained. Both immunohistochemical and immunoblotting studies confirmed increased expression using UAS-DVMAT (Chang et al., 2006) as well as decreased expression of DVMAT using DVMATi (supplemental Fig. 7, available at www.jneurosci.org as supplemental material). *DOPA decarboxylase* (*ddc*)-GAL4 was generously provided by Jay Hirsh (University of Virginia, Charlottesville, VA). UAS–green fluorescent protein (GFP)-based Ca^{2+} sensor (G-CaMP) was a gift from Richard Axel (Columbia University, New York, NY). UAS-mCD8-GFP and UAS–tetanus neurotoxin light chain (TNT) stocks were obtained from Larry Zipursky (University of California, Los Angeles, CA). Leo Pallanck (University of Washington, Seattle, WA) furnished the *dpark* mutants. *appl*-GAL4 was a kind gift from Kalpana White (Brandeis University, Waltham, MA). The UAS-Q108 stock was from Leslie Michels Thompson and Larry Marsh (University of California, Irvine, CA). Serge Birman (Universite de la Mediterranee, Marseille, France) generously provided the tyrosine hydroxylase (TH)-GAL4 line. The recombined *choline acetyltransferase* (*chat*)-GAL4, UAS-GFP chromosome, as well as 24B-GAL4, were from the Bloomington *Drosophila* Stock Center (Bloomington, IN). All experiments were performed at room temperature (~24°C).

Immunohistochemistry. Immunohistochemistry and TRITC (tetramethylrhodamine isothiocyanate)-phalloidin (Sigma, St. Louis, MO) staining were performed using whole-mount preparations as described previously (Greer et al., 2005; Sang et al., 2005; Chang et al., 2006). Each brain was scanned using optical sections covering 40 μm that included DA neurons of both dorsomedial (DM) and dorsolateral (DL) clusters. Each brain was examined with the examiner blinded to the genotype. The collected Z-series from each brain were then projected into a three-dimensional animation to precisely quantitate DA neuron numbers. We defined neuronal loss as a complete loss of somatic TH staining. To avoid confocal settings in different batches of samples that might artificially affect data interpretation, age-matched brains from different genotypes were examined simultaneously. Experiments in Figures 4*D* and 7, *D* and *E*, as well as supplemental Figures 5*B* and 7, *A* and *B* (available at www.jneurosci.org as supplemental material), used alignment of one genotype in a row adjacent to samples of another genotype. Primary antibodies used were mouse anti-TH (1:100; Immunostar, Hudson, WA), rabbit anti-human parkin 2132 (1:50; Cell Signaling Technology, Danvers, MA), or mouse anti-parkin PRK8 (1:100; Abcam, Cambridge, MA), rat anti-5-hydroxytryptamine (5-HT; 1:50; Millipore, Bedford, MA), chicken anti-GFP (1:100; Millipore), rabbit anti-DVMAT (Chang et al., 2006), and mouse anti-FLAG M5 (1:50; Sigma). Fluorochrome-conjugated antibodies included FITC, Cy3, and Cy5 (1:100; Jackson ImmunoResearch, West Grove, PA) corresponding to the appropriate species of primary antibodies used. NIH Image was used to quantitate pixel intensity for fluorescence images. Toxicity of mutant compared with wild-type forms of parkin was confirmed using blinded analysis, and at least two independently transgenic insertions for each transgene were studied to avoid potential confounds resulting from positional effects (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material).

Behavior. Flies used for behavioral analysis were precisely aged and selected randomly. Control groups were included in each test. We used the startle-induced negative geotactic response and a countercurrent apparatus (Benzer, 1967) to test the climbing ability of the flies. For each time point, at least five cohorts of 16–18 flies from each genotype were scored. After transfer of flies to 14 ml Falcon plastic tubes, each tube was inserted into the countercurrent apparatus. Flies were gently knocked down to the bottom of tubes and allowed 10 s to climb up to the top receiver tubes. Flies failing to climb to the receiver tubes were trapped in the bottom tubes; flies were counted, and each test was repeated three times to deduce the percentage of flies that were able to reach the top. For the righting reflex assay, flies were transferred individually to small chambers [5 mm (diameter) × 2 mm (height)] and digitally recorded for 2 min after a 2 h recovery from CO_2 anesthesia. Video clips documenting righting reflex episodes were then analyzed in a frame-by-frame manner. The function of the righting reflex was defined as the time between falling from the chamber roof and resumption of upright posture in 1/100 s units.

The *Drosophila* rotarod consists of six 25-mm-diameter glass cylinders that can be rotated between 3 and 15 rpm under the control of a motor, which requires flies to maintain an upside-down position while rotating. There are five infrared beams placed at 60, 120, 180, 240, and 300° around the tube that shine axially along the circumference of each cylinder. Beam breaks were automatically recorded using DAMSystem software (Trikinetics, Waltham, MA). The readout for flies that can adhere to the walls of the cylinder for a complete turn consists of five beam breaks. Flies with postural impairment generally fall before reaching the 180° beam and thus fail to break the beam at 180° and beyond. They may break 60 and 120° beams more frequently because they will encounter these beams more often during a complete rotation cycle after falls. For each test, individual flies were placed into chambers and recorded for 30 min, and the average number of beam breaks at each position was divided by the average frequency of beam breaks at all angles to obtain an arbitrary index. Data plotting and statistics were performed using Prism software (GraphPad Software, San Diego, CA).

Immunoblotting. Protein preparation from *Drosophila* heads and SDS-PAGE was performed as described previously (Sang et al., 2005). Blots were probed using rabbit anti-parkin (1:2000; Cell Signaling Technology), mouse anti-parkin (PRK8, 1:2000; Abcam) (Pawlyk et al., 2003), mouse anti-FLAG M2 (1:200), and mouse anti-β-tubulin (1:1000; Accurate Chemical and Scientific, Westbury, NY). Immunoblots for DVMAT used anti-DVMAT (Chang et al., 2006) (1:2000) or anti-*Drosophila* vesicular glutamate transporter (1:10,000; provided by Aaron DiAntonio, Washington University, St. Louis, MO). Films from immunoblots were scanned with a Powerlook 1000 transmissive flatbed scanner (UMAX, Dallas, TX). Densities of bands were measured with NIH ImageJ (http://rsb.info.nih.gov/ij). Graphical and statistical analyses were performed using SigmaPlot 9.0 and SigmaStat 3.1 (Systat, San Jose CA.).

Neurochemical analysis. Fly heads (three per sample) were manually collected and homogenized in 0.1 M perchloric acid containing 0.1% EDTA using a glass-on-glass microtissue grinder (Kimble/Kontes, Vineland, NJ). Insoluble debris were sedimented by centrifugation, and the supernatant was filtered through a Millipore MC cartridge. The filtrate was diluted 10-fold before analysis, and 5 μl of the diluted sample was analyzed using HPLC with electrochemical detection (Antec Leyden, Leiden, The Netherlands) using a mobile phase consisting of sodium acetate (75 mM), sodium dodecane sulfonate (0.75 mM), EDTA (10 mM), triethylamine (0.01%), acetonitrile (12%), methanol (12%), and tetrahydrofuran (1%), pH 5.5, pumped at a rate of 200 ml/min (model LC-10AD; Shimadzu, Columbia, MD) through a 100 × 2 mm column (3 μm,

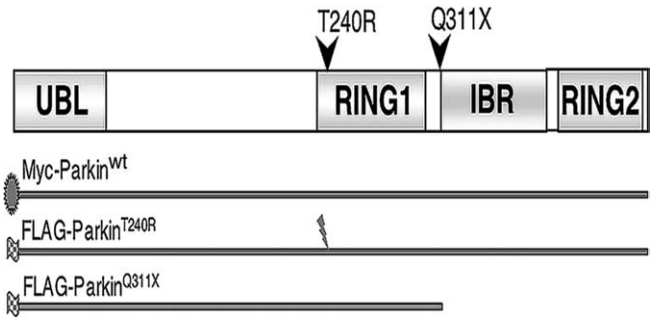

Figure 1. Domain structure of human parkin and Myc-tagged wild-type and FLAG-tagged mutant constructs used to generate transgenic flies.

Hypersil C18; Keystone Scientific, Bellefonte, PA). The system was calibrated at regular intervals and provided a limit of detection of 0.5 fmol for a 5 μl injection of sample. The data were collected and analyzed using ChromPerfect software (Justice Innovations, Mountain View, CA).

Results

Generation of human parkin transgenic *Drosophila*

To test whether the expression of PD-linked parkin alleles might exert toxicity in DA neurons, parkin cDNAs encoding wild type and two mutant forms derived from familial PD, Gln311Stop (Q311X) and Thr240Arg (T240R) (Fig. 1) (Shimura et al., 2000), were subcloned into a *Drosophila* UAS expression vector, and transgenic lines were derived. To facilitate their detection, we generated transgenes expressing wild-type parkin tagged with either the myc or FLAG epitope. FLAG- and myc-tagged lines expressing wild-type parkin were behaviorally and histologically indistinguishable from each other and from controls (see Fig. 4 and supplemental Fig. 1, available at www.jneurosci.org as supplemental material), indicating that neither tag generates a neurotoxic phenotype and thus allowing the tags to be used interchangeably in transgenes expressing human parkin.

To drive expression in both DA and 5-HT neurons, we expressed parkin under the control of a *ddc*-GAL4 driver (Li et al., 2000) to generate *ddc*::parkin progeny. Importantly, 5-HT neurons in *ddc*::parkin flies provide an internal control for the neurochemical specificity of potential neurotoxic effects. Immunoblotting demonstrated expression for multiple lines of both wild-type (*ddc*::parkin^{wt}) and mutant parkin (*ddc*::parkin^{Q311X} and *ddc*::parkin^{T240R}) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). The expression level of the lines used was higher for parkin^{T240R} than parkin^{wt} for the majority of the experiments performed. Additional experiments using different lines with similar expression also obtained toxicity with T240R but no effect with wild-type parkin. *ddc*::parkin^{wt}-41 (supplemental Fig. 2, lane 7, available at www.jneurosci.org as supplemental material) was expressed at levels comparable to *ddc*::parkin^{T240R}-147 (supplemental Fig. 2, lane 10, available at www.jneurosci.org as supplemental material); however, this wild-type line was nontoxic, whereas the comparably expressed T20R line induced loss of DA neurons (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material).

Expression of mutant but not wild-type human parkin in aminergic neurons causes age-dependent motor deficits

Motor behavior has been used as an assay for DA cell function in other *Drosophila* models of neurodegeneration (Feany and Bender, 2000; Auluck and Bonini, 2002; Whitworth et al., 2005). Therefore, to assess potentially neurotoxic effects of parkin, we first subjected the parkin-expressing lines to a battery of three behavioral tests: (1) negative geotaxis to test climbing ability, (2) a righting reflex assay to test coordination, and (3) a novel *Drosophila* rotarod test to further quantitate motor function. As a positive control, we used *ddc*-GAL4 to express TNT to block aminergic neurotransmission; as expected, this caused severe motor phenotypes in multiple behavioral tests (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Using negative geotaxis, flies expressing wild-type and mutant parkin showed comparable climbing ability immediately after eclosion. However, between 3 and 6 weeks after eclosion, flies expressing parkin^{T240R} or parkin^{Q311X} showed dramatic declines in climbing ability relative to control *ddc* flies or flies expressing parkin^{wt} (Fig. 2*A*).

We next tested motor behavior using the righting reflex, which measures the time between a spontaneous fall from the testing chamber lid and successful attainment of a normal posture (Leal and Neckameyer, 2002). Consistent with the climbing assay, aged *ddc*::parkin^{T240R} and *ddc*::parkin^{Q311X} flies showed greater difficulty in postural control and coordination compared with control and *ddc*::parkin^{wt} flies (Fig. 2*B*). As an automated assay for postural impairment, we designed a *Drosophila* rotarod (supplemental Fig. 4, available at www.jneurosci.org as supplemental material) (see Materials and Methods). Briefly, the *Drosophila* rotarod comprises six small cylinders rotated at 10 rpm in which the flies stand on the internal surface. Flies that successfully adhere to the chamber wall for a complete turn break five successive infrared beams, generating a readout consisting of a flat line at the arbitrary index of 1.0. Flies that fall can either break the beams prematurely or fail to break them; both generate a readout that deviates from 1.0 at the angle (shown on the *x*-axis; see Materials and Methods for additional details). At 2 weeks, the frequency of beam breaks at different angles was identical, indicating that young flies expressing both wild-type and mutant parkin performed equivalently in the rotarod assay (Fig. 2*C*). However, by 4 weeks the behavior of *ddc*::parkin^{T240R} and *ddc*::parkin^{Q311X} was markedly impaired relative to *ddc*::parkin^{wt} (Fig. 2*D*). These data indicate that mutant but not wild-type parkin causes a defect in motor behavior when expressed in aminergic cells. Moreover, this deficit was not manifest until a minimum of 2–3 weeks after eclosion, suggesting a degenerative rather than a developmentally induced defect.

To determine more specifically whether these behavioral deficits were caused by changes in the function of aminergic cells, we first assayed cell function at early time points, before the manifestation of behavioral deficits. In PD, it is likely that neuronal dysfunction precedes cell loss, but it has been difficult to assess this possibility experimentally. The fluorescent calcium probe G-CaMP has been used to monitor neuronal activity in the *Drosophila* olfactory system *in vivo* (Wang et al., 2003). We therefore used this technique to investigate the function of aminergic neurons in flies expressing mutant parkin. We observed G-CaMP fluorescence in 1-week-old brains expressing G-CaMP with either parkin^{T240R} or parkin^{wt}. Compared with the *ddc*::G-CaMP control (Fig. 3*A*), *ddc*::parkin^{T240R}::G-CaMP brains showed decreased fluorescence, indicating reduced neuronal activity (Fig. 3*B*,*D*). In contrast, the signal in *ddc*::parkin^{wt}::G-CaMP brains was indistinguishable from controls (Fig. 3*A*,*D*). These data confirm behavioral tests showing that wild-type parkin does not adversely affect the function of aminergic neurons in the fly. More importantly, they indicate that mutant parkin causes dysfunction of these cells, consistent with the behavioral deficits we observe.

The hallmark of PD and the gold standard for most animal

models of PD is the degeneration of DA neurons. To assay for DA cell death in flies expressing wild-type and mutant parkin, we used whole-mount staining of adult brains in conjunction with confocal analysis and three-dimensional reconstructions. We focused on DA neurons in the DM and DL clusters, which are more easily counted than other more numerous or widely dispersed clusters, and have been used extensively in other *Drosophila* models of PD (Feany and Bender, 2000; Auluck et al., 2002; Yang et al., 2003; Chen and Feany, 2005; Meulener et al., 2005; Whitworth et al., 2005). Using an anti-GFP antibody to recognize mCD8-GFP (Lee and Luo, 2001), we found that *ddc*-GAL4 drives expression in all DA neurons in both DM and DL clusters (Fig. 4*A*), indicating that all of these cells have the potential to be affected by mutant parkin.

We monitored the viability of DA neurons in brains expressing mutant and wild-type parkin driven by *ddc*-GAL4 in three ways: (1) TH staining, (2) coexpression of mCD8-GFP, and (3) staining with an antibody to human parkin. The collected Z-series from brains were projected into a single plane image (Fig. 4*Aa*), and additional three-dimensional animations were conducted to precisely quantify DA neuron numbers. Consistent with our behavioral results, TH immunoreactivity was similar in flies expressing wild-type and mutant parkin at eclosion, albeit with a modest but significant decrease in DA cell counts in flies expressing parkin^{T240R}. In contrast, beginning 3 weeks after eclosion and increasing with age, we observed robust and consistent declines in TH staining in brains expressing mutant parkin compared with parkin^{wt} transgenics or *ddc*-GAL4 controls (Fig. 4*Ba–Bd*,*C*) (supplemental Fig. 5*A*, available at www.jneurosci.org as supplemental material). Similarly, using the TH-GAL4 driver, which is expressed solely in DA neurons (Friggi-Grelin et al., 2003), we observed a decline in TH-staining flies expressing parkin^{T240R} but not parkin^{wt} (supplemental Fig. 5*B*, available at www.jneurosci.org as supplemental material). Consistent with the relative severity of their behavioral effects, parkin^{T240R} (using either *ddc*- or TH-GAL4) caused more pronounced DA neuronal loss than parkin^{Q311X}. We also observed a loss of DA neurons in clusters other than DM and DL (data not shown). However, the relatively tight apposition of the cells in these clusters limited our ability to precisely quantitate cell loss.

The loss of TH immunoreactivity is commonly interpreted as an indication of cell loss. However, TH is subject to extensive post-translational regulation (Flatmark et al., 2002). Therefore, to determine whether the decrease in TH staining that we observed was the result of cell loss or TH downregulation, we used an independent label for DA neurons. We expressed mCD8-GFP in *ddc*::parkin^{wt} and *ddc*::parkin^{T240R} flies, and brains were examined side by side on the same slide. Quantification of fluorescent pixels revealed a significant decrease in GFP signal by 4 weeks in parkin^{T240R}-expressing DA neurons compared with those expressing parkin^{wt} (Fig. 4*Da–Dc*), indicating that the decrease in TH staining was indeed the result of cell loss. We also quantitated the number of mCD8-GFP-expressing neurons in the DM cluster and observed significant cell loss for *ddc*::parkin^{T240R} compared with *ddc*::parkin^{wt}; results obtained using cell counts correlated well with those that used pixel intensity (Fig. 4*Dc*). Finally, we found that *ddc*::parkin^{T240R} but not *ddc*::parkin^{wt} flies showed an age-dependent reduction in anti-parkin staining in DA neurons 4 weeks after eclosion (Fig. 4*Dd–Df*). Together, these data suggest that expressing mutant human parkin in *Drosophila* causes DA cells to lose function and eventually degenerate.

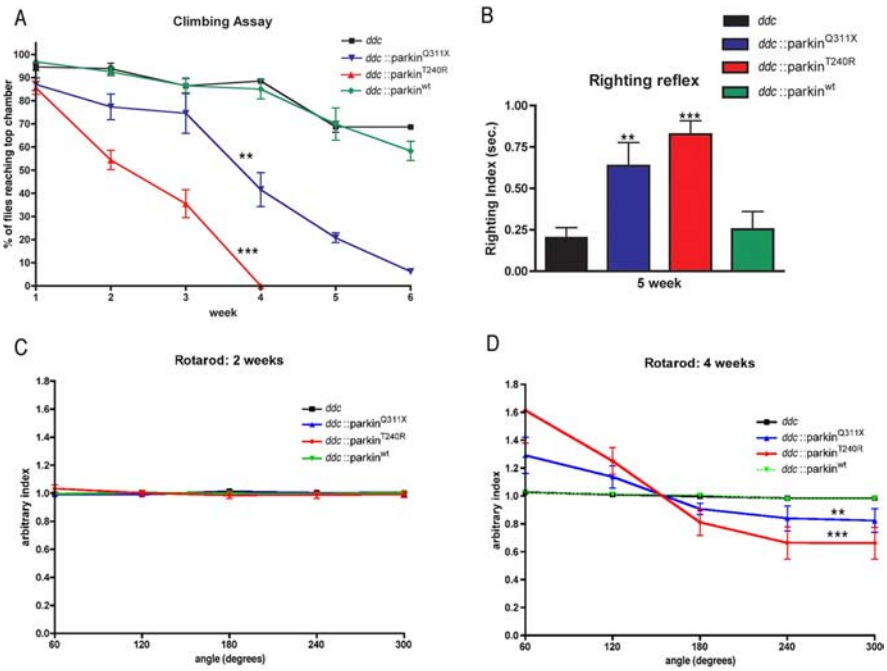

Figure 2. Mutant parkin produces age-dependent deficits in motor performance. Three independent assays were used to analyze motor behavior. ***A***, Using the *ddc*-GAL4 driver, parkin^{Q311X} and parkin^{T240R} produce age-dependent impairments in climbing performance compared with control and parkin^{wt}. For each genotype at each time point, at least five cohorts consisting of 16–18 flies each were tested. ***B***, At 5 weeks, the righting reflex test demonstrates postural instability of parkin^{Q311X} and parkin^{T240R} flies compared with parkin^{wt} or control. Twenty flies of each genotype were analyzed. ***C***, Rotarod performance demonstrates normal postural stability for all genotypes at 2 weeks. ***D***, By 4 weeks, postural instability is apparent in parkin^{Q311X} and parkin^{T240R} compared with parkin^{wt} and control flies; the <1 score for the arbitrary index at higher angles indicates flies that fall more frequently. For each genotype at each time point, $\geq$12 flies were tested. Values shown represent mean $\pm$ SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ relative to control *ddc* [two-way ANOVA with Bonferroni's multiple comparison test (***A***, ***C***, ***D***) or unpaired *t* test (***B***)].

DA neurons are selectively vulnerable to mutant human parkin

Having established that mutant parkin causes degeneration of DA neurons, we next determined whether this model might also show selective vulnerability as seen in PD. Because *ddc*-GAL4 drives expression of the parkin transgenes in 5-HT as well as DA neurons, we first examined whether mutant parkin might also affect 5-HT neurons. Brains staged 5 weeks after eclosion and expressing either wild-type or mutant parkin were stained with an anti-5-HT antibody. Small declines in 5-HT immunoreactivity were observed in brains expressing mutant parkin, with some reductions in the staining intensity of neuritic processes (Fig. 4*E*). However, in contrast to the profound degeneration of DA neuronal somata observed at this stage (Fig. 4*B*), the somata of 5-HT neurons in brains expressing mutant parkin resembled controls. Thus, 5-HT neurons are somewhat susceptible to mutant parkin, but to a lesser extent than DA neurons.

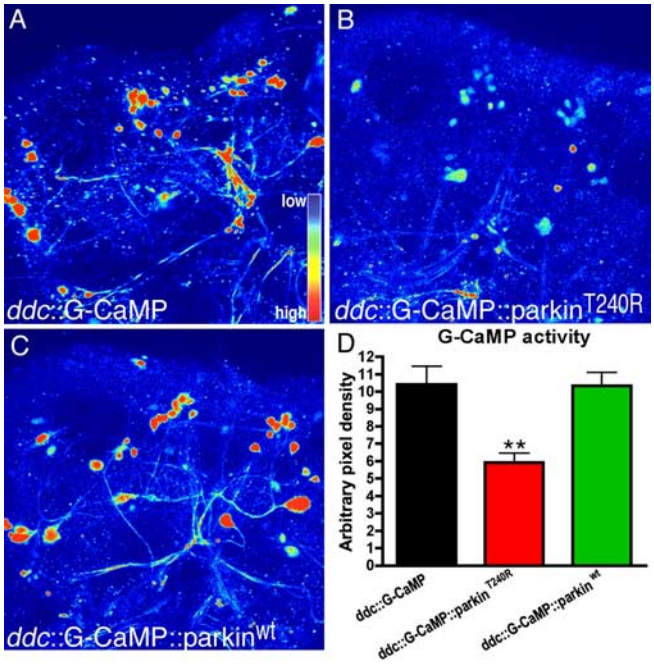

Figure 3. Analysis of activity-dependent G-CaMP signal in living brains at 1 week after eclosion. GFP signal intensity was converted into thermally coded color images. ***A***, In the control *ddc*::G-CaMP brain, intense GFP signal was detected. ***B***, parkin^{T240R} expressed in *ddc*::G-CaMP flies resulted in markedly reduced GFP signal. ***C***, Expression with parkin^{wt} resembled the control. ***D***, Analysis of GFP pixel density of at least five brains for each genotypes showed decreased G-CaMP activity in the parkin^{T240R} brain (one-way ANOVA with Bonferroni's multiple comparison test; $n \geq 5$; $^{**}p < 0.01$). The images are represented using a pseudocolor scale as indicated in ***A***, where blue is low intensity and yellow–red is high intensity. Scale bar, 40 μm.

We also studied expression of parkin using the pan-neuronal driver *appl*-GAL4 (Torroja et al., 1999). Flies expressing either wild-type or mutant parkin under control of *appl*-GAL4 behaved normally at eclosion. However, by 5 weeks, the rotarod assay showed significant motor impairment of *appl*::parkin^{T240R} flies, with more modest changes in *appl*::parkin^{Q311X} flies compared with *appl*::parkin^{wt} and controls (Fig. 5*A*). Analysis of brains at this stage revealed reductions in TH immunoreactivity for *appl*::parkin^{T240R} in both DM and DL clusters compared with *appl*::parkin^{wt} and control brains (Fig. 5*Ba–Bd*). No obvious pathology such as vacuolization, which is commonly observed in fly models of neurodegenerative disease (Jackson, 2000; Wittmann et al., 2001; Jackson et al., 2002), was observed in brains when parkin^{T240R} was expressed using this pan-neural driver. Changes in TH staining in *appl*::parkin^{Q311X} could not be detected, consistent with the lower level of toxicity seen with this mutant using the *ddc*-GAL4 driver. Given that *appl*-GAL4 also drives expression in photoreceptor neurons, retinal morphology was examined at 5 weeks, but degenerative changes were not detected (Fig. 5*Be–Bh*), suggesting that these histaminergic photoreceptor neurons (Stuart, 1999) are resistant to mutant parkin^{T240R} compared with DA neurons in the central brain.

Also, to test whether DA neurons were selectively susceptible to degeneration, we used *chat*-GAL4 to drive transgene expression in cholinergic cells (Yasuyama and Salvaterra, 1999). As a positive control for neurodegeneration, flies expressing an expanded polyglutamine peptide, Q108 (Marsh et al., 2000) were examined in parallel. *chat*::Q108 flies died at a late pupal stage, and brains showed dramatically decreased GFP-labeled cells (Fig. 6*Ae–Af*). In contrast, *chat*::parkin^{Q311X} and *chat*::parkin^{T240R} flies showed normal behavior at eclosion, similar to flies expressing parkin^{wt}. Furthermore, as late as 6 weeks after eclosion, brains expressing either mutant or wild-type parkin showed comparable GFP-labeled cholinergic neurons compared with the *chat*::GFP control (Fig. 6*Aa–Ad*). Similarly, using GMR-GAL4 (Freeman, 1996), we observed degeneration of neuronal, histaminergic photoreceptor cells expressing Q108 (Marsh et al., 2000) but no effects in histaminergic photoreceptors (Stuart, 1999) expressing either mutant or wild-type parkin (data not shown). The lack of mutant parkin-induced degeneration using *chat*-GAL4 or GMR-GAL4 drivers was not attributable to lower expression, because quantitative immunoblots demonstrate higher expression of parkin^{T240R} in histaminergic and cholinergic neurons compared with the *ddc* driver (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). These data indicate that the toxicity of mutant parkin is either limited to DA neurons or significantly more potent in these cells.

Despite the cell-type specificity we observe, one potential mechanism for the degenerative phenotypes caused by mutant parkin is transdominant inhibition of the endogenous fly homolog. To test this hypothesis, we used the driver *24B*-GAL4 (Brand and Perrimon, 1993) to express both mutant parkin^{T240R} (*24B*::parkin^{T240R}) and parkin^{Q311X} (*24B*::parkin^{Q311X}) in muscle cells. We verified robust expression of both wild-type and mutant transgenes in third instar larval muscle (Fig. 6*Ba–Bc*). Unlike *dpark*$^{-/-}$ adult escapers, which were unable to fly, *24B*::parkin^{T240R} and *24B*::parkin^{Q311X} flies showed normal flight behavior (data not shown). Furthermore, as opposed to the abnormal morphology of indirect flight muscle from *dpark* mutants (Fig. 6*Cc*), *24B*::parkin^{T240R} muscle showed normal myofibril organization (Fig. 6*Cb*), indicating that misexpression of mutant human parkin in muscle does not interfere with function of endogenous fly parkin. Interestingly, in contrast to a report showing that expression of wild-type *Drosophila* parkin using *24B*-GAL4 could rescue the *dpark* null phenotype (Greene et al., 2003; Pesah et al., 2004), we found that wild-type human parkin did not rescue either the muscle phenotype (Fig. 6*Cd*) or flightless behavior of *dpark* null flies (data not shown). These data suggest potentially important functional differences between fly and human parkin.

Modulation of VMAT expression affects mutant parkin-induced neurodegeneration and motor behaviors

Given the selective vulnerability of DA neurons to mutant parkin, we hypothesized that mutant human parkin-induced toxicity may depend on dopamine or its metabolites and that alterations in DA homeostasis could modulate parkin-induced phenotypes. VMAT is known to play a crucial role in regulating the amount of dopamine that is stored in synaptic vesicles or remains in the cytosol to participate in oxidative and potentially neurotoxic processes (Fumagalli et al., 1999; Pothos et al., 2000; Sulzer et al., 2000; Weingarten and Zhou, 2001; Hansen et al., 2002; Choi et al., 2005). Similar to mammalian orthologs, DVMAT is responsible for transporting dopamine and other monoamines into synaptic vesicles (Greer et al., 2005; Chang et al., 2006). Furthermore, overexpression of DVMAT causes an increase in DVMAT levels and behavioral phenotypes consistent with an increase in dopamine release (Chang et al., 2006). To complement the UAS-DVMAT lines, we made a DVMAT RNAi construct and confirmed the efficacy of *ddc*::DVMAT-RNAi in specifically reducing endogenous DVMAT expression (supplemental Fig. 7, available at www.jneurosci.org as supplemental material). *ddc*::DVMAT also produced a modest but significant decrease in total dopamine concentrations as assessed using HPLC, whereas

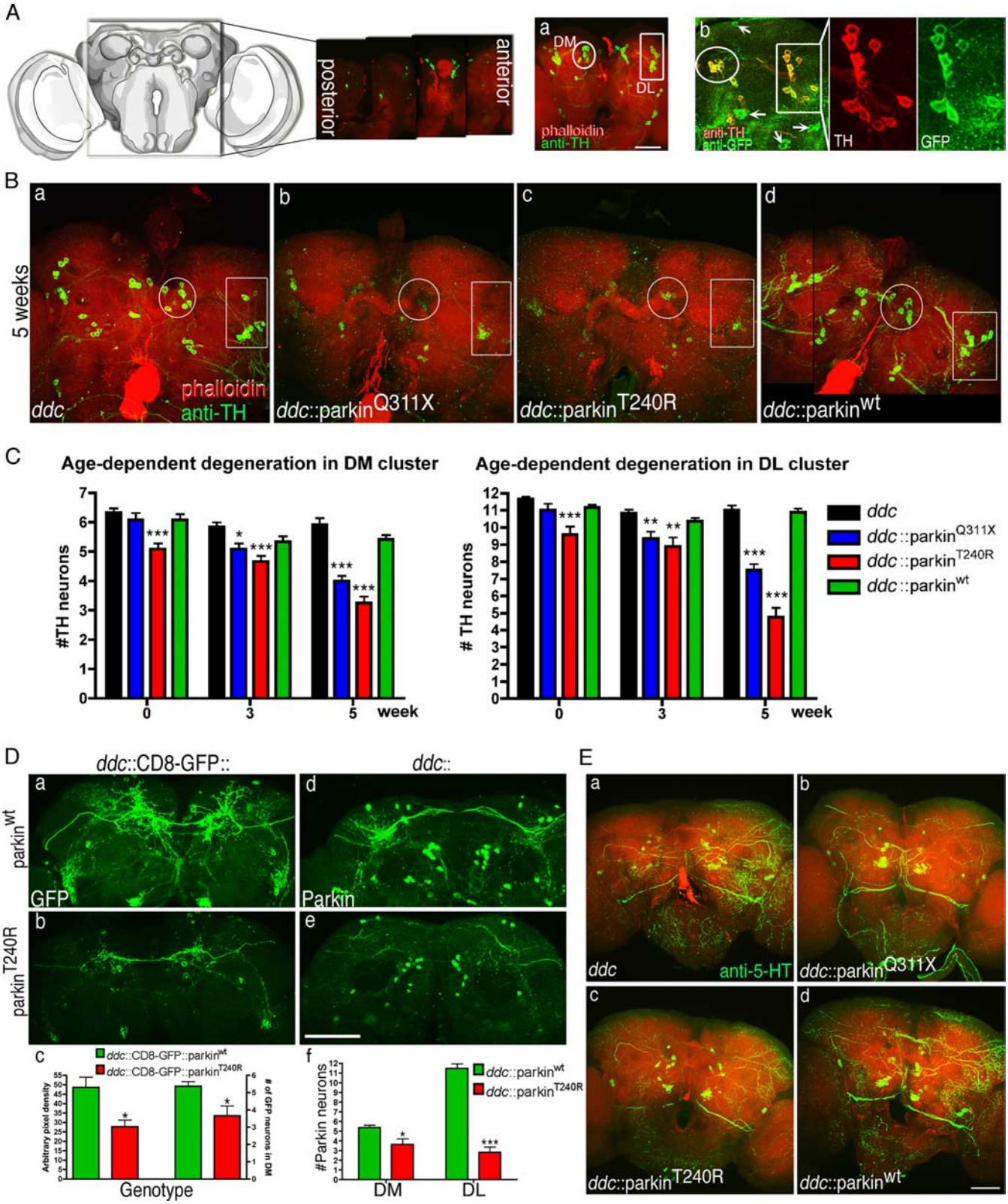

Figure 4. Expression of mutant human parkin in *Drosophila* DA neurons causes age-dependent neurodegeneration. ***A***, Schematic representation of DA neurons in the central brain of *Drosophila*. ***a***, The projected confocal image shows anti-TH and phalloidin staining spanning optical sections 40 μM in depth from posterior to anterior. Two major DA clusters, DM (circles) and DL (rectangles), are indicated. ***b***, A projected image shows anti-TH (red) and anti-GFP (green) colocalized in all DA neurons in both DM and DL (enlarged images to the right) clusters. Arrows indicate 5-HT neurons expressing GFP but not TH. ***B***, Reductions in TH immunoreactivity at 5 weeks after eclosion are apparent in *ddc*::parkin ^{Q311X} (***b***) and *ddc*::parkin ^{T240R} (***c***) but not *ddc* control (***a***) or *ddc*::parkin ^{wt} (***d***) brains. Circles, DM; rectangles, DL. ***C***, Quantification of DA neurons at 0, 3, and 5 weeks after eclosion in DM and DL clusters. Progressive loss of TH-immunoreactive neurons is induced in both clusters by *ddc*::parkin ^{Q311X} (blue bars) and *ddc*::parkin ^{T240R} (red bars) compared with the *ddc* control (black bars) and *ddc*::parkin ^{wt} (green bars). Values represent the mean $\pm$ SEM; $n = 12$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ relative to the *ddc* control. ***D***, At 4 weeks, mCD8-GFP signal (green) is reduced in parkin ^{T240R} (***b***) compared with parkin ^{wt} (***a***) brain, which is similar to changes observed in corresponding images showing reduced TH immunoreactivity. Measurement of GFP pixel density (corresponding to the left *y*-axis) and GFP cell counts in the DM cluster (corresponding to the right

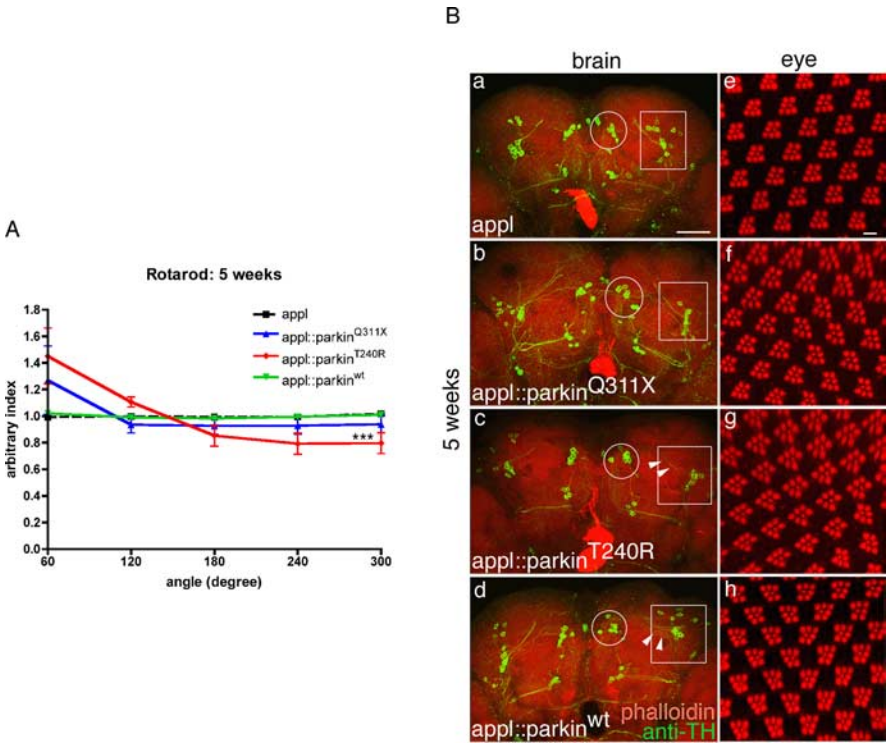

Figure 5. Pan-neuronal expression of parkin^{T240R} induces motor dysfunction and degeneration of DA neurons, whereas photoreceptor neurons are resistant. ***A***, Rotarod assays at 5 weeks demonstrate postural instability of *appl*:: parkin^{T240R} flies, which fall more frequently at higher angle positions compared with the *appl*-GAL4 control and *appl*::parkin^{wt}. *appl*:: parkin^{Q311X} flies show a modest trend toward postural instability. For each genotype at each time point, >12 flies were scored. The values shown represent mean ± SD. ***$p < 0.001$ relative to control *appl* (two-way ANOVA with Bonferroni's multiple-comparison test). ***Ba–Bd***, Confocal images of brains aged 5 weeks stained with anti-TH (green) and phalloidin (red). Circles, DM; rectangles, DL. Reductions in TH immunoreactivity are apparent in the DL cluster in appl::parkin^{T240R} brain (***c***) compared with *appl* (***a***), *appl*::parkin^{Q311X} (***b***), and *appl*::parkin^{wt} (***d***). Arrowheads indicate reduced TH-immunoreactive processes (***c***) in brains expressing mutant parkin compared with wild type (***d***). ***Be–Bh***, No differences in morphology of rhabdomeres within individual ommatidia morphology occur in *ddc* control (***e***) or flies expressing parkin^{Q311X} (***f***), parkin^{T240R} (***g***), or parkin^{wt} (***h***). Scale bar, 40 μm.

ddc::DVMATi knockdown markedly increased total dopamine in extracts of fly heads (supplemental Fig. 8, available at www.jneurosci.org as supplemental material). These and other previously published data on the phenotype of DVMAT-overexpressing flies confirm that DVMAT causes changes in dopamine storage and release (Chang et al., 2006).

We first tested whether changes in DVMAT expression also modified mutant parkin behavioral phenotypes. We found that flies coexpressing DVMAT RNAi with mutant parkin (*ddc*::parkin^{T240R}::DVMATi) showed an earlier onset of postural impairment as assessed by rotarod performance (Fig. 7*A*). The righting reflex proved to be the most sensitive behavioral assay to detect modulation of mutant parkin effects by DVMAT (Fig. 7*B*). At 1 week, *ddc*::parkin^{T240R}::DVMATi flies (Fig. 7*B*, gray bar) took longer to right themselves compared with those expressing mutant parkin alone (red bar). Flies expressing DVMATi alone (pink bar) did not differ significantly from those expressing mutant parkin alone. At 2 weeks, overexpression of DVMAT provided protection against effects of mutant parkin without significantly affecting behavior on its own; the impaired righting ability of *ddc*::parkin^{T240R} flies (red bar) was suppressed in *ddc*::parkin^{T240R}::DVMAT flies (green bar). In contrast, overexpression of DVMAT alone (blue bar) did not affect performance in this assay and was similar to the rescued parkin phenotype (green bar).

Dramatic changes in aminergic function can be lethal in *Drosophila* (Neckameyer and Quinn, 1989; McClung and Hirsh, 1998; Stathakis et al., 1999). Consistent with this observation, we found that expressing high levels of parkin^{T240R} or parkin^{Q311X} using two rather than one copy of *ddc*-GAL4 causes incomplete pupal lethality. This phenotype is easily quantitated and was therefore used to further evaluate the genetic interaction between DVMAT and parkin. Importantly, overexpression or knockdown of DVMAT alone using two copies of *ddc*-GAL4 had no detectable effects on lethality (data not shown). Changes in DVMAT expression had no effect on flies expressing wild-type parkin (Fig. 7*C*, green bars). In contrast, overexpression of DVMAT significantly suppressed both parkin^{T240R}- and parkin^{Q311X}-induced pupal lethality; conversely, DVMAT knockdown enhanced parkin^{T240R}-induced lethality (Fig. 7*C*, red and blue bars). These data support a genetic interaction between DVMAT and mutant parkin and suggest that DA homeostasis might be an important factor mediating the neurotoxic effects of mutant parkin.

Others, using rotenone (Pendleton et al., 2002; Coulom and Birman, 2004) or α-synuclein to model PD in the fly, have shown that enhancing DA synthesis, and presumably release, can rescue the phenotype caused by DA cell loss without necessarily decreasing the degeneration of DA cells (Coulom and Birman, 2004). In these cases, as in the symptomatic treatment of PD, increased release by the remaining cells presumably compensates for the death of other DA neurons. Therefore, to assess more specifically the effects of DVMAT on DA cell degeneration, we performed a quantitative immunohistochemical analysis of flies coexpressing parkin and VMAT transgenes. Importantly, neither UAS-DVMAT nor DVMAT RNAi alone altered the survival of DA neurons in aged flies (data not shown). Furthermore, manipulation of DVMAT expression using either UAS-DVMAT or DVMAT RNAi had no effect on the number of DA neurons in aged

←

y-axis) from 4-week-old brains of both genotypes showed a significant decrease (unpaired *t* test; $n = 5$; *$p < 0.05$) of GFP signal in parkin^{T240R} brains compared with parkin^{wt} (***c***). The GFP signal at eclosion was indistinguishable between parkin^{T240R} and parkin^{wt} (data not shown). ***d***, ***e***, Confocal images of *ddc*::parkin^{T240R} (***e***) and *ddc*::parkin^{wt} (***d***) brains stained with an anti-parkin antibody (green) reveals reduced parkin signals in *ddc*::parkin^{T240R} compared with *ddc*::parkin^{wt} brains. ***f***, Analysis of brains at 4 weeks indicates that cell counts stained with anti-parkin for parkin^{T240R} are decreased compared with parkin^{wt} (unpaired *t* test; $n = 6$; *$p < 0.05$ for DM; ***$p < 0.001$ for DL). Parkin staining at eclosion was indistinguishable between parkin^{T240R} and parkin^{wt} (data not shown). ***a***, ***b***, ***d***, and ***e*** are confocal images of adjacent brains imaged simultaneously in the same slide but reoriented for presentation. ***E***, Anti-5-HT staining at 5 weeks after eclosion shows reduced neuritic immunoreactivity in 5-HT neurons expressing mutant parkin (***b***, ***c***) compared with those expressing wild-type parkin (***d***) or controls (***a***). Scale bar, 40 μm.

flies expressing wild-type human parkin (*ddc*::parkin^{wt} in Fig. 7*Dd–Df*). In contrast, coexpressing DVMATi and parkin^{T240R} using *ddc*-GAL4 potentiated the neurodegenerative phenotype of parkin^{T240R}; the number of TH-positive neurons was significantly reduced in *ddc*::parkin^{T240R}::DVMATi at 2 weeks compared with those without DVMATi or overexpressing DVMAT (Fig. 7*Da–Dc*). In addition, we observed that increased DVMAT expression in mutant parkin brains suppressed mutant parkin-induced DA neurodegeneration; the loss of TH-positive neurons evident in *ddc*::parkin^{T240R} brain at 4 weeks (Fig. 7*Ea*,*Ec*) was rescued by coexpression of parkin^{T240R} with UAS-DVMAT (Fig. 7*Eb*,*Ec*). Thus, modulation of VMAT activity may significantly affect mutant parkin-induced degeneration of DA neurons, and such genetic interaction might, at least in part, underlie regional vulnerability in PD.

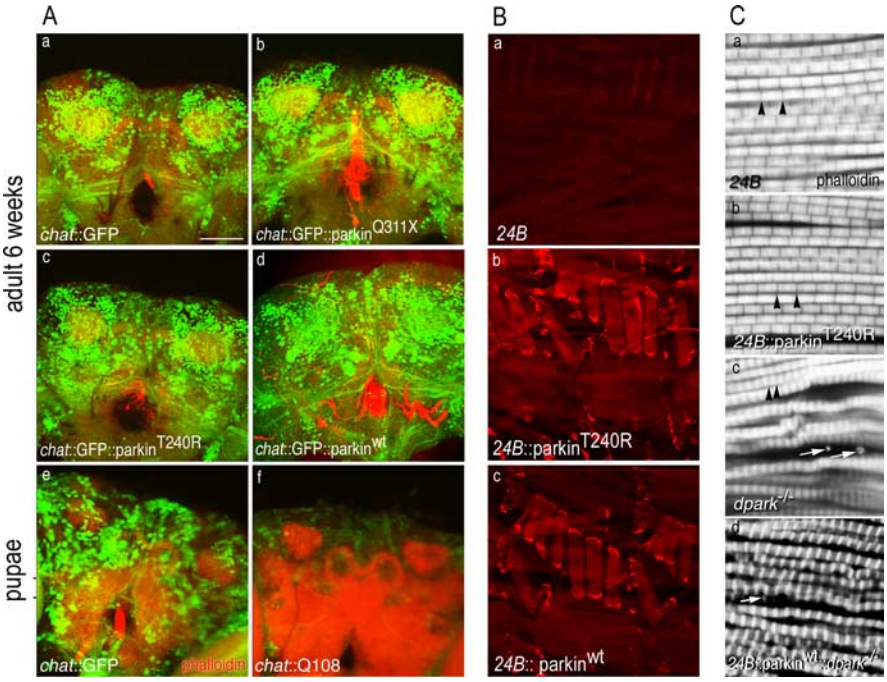

Figure 6. Cholinergic neurons and indirect flight muscle are resistant to mutant parkin. ***A***, Images show signals for TRITC (tetramethylrhodamine isothiocyanate)-phalloidin (red) and GFP (green). ***a–d***, Confocal images of *chat*::GFP brains coexpressing mutant or wild-type parkin transgenes at 6 weeks. Expression of either parkin^{Q311X} (***b***) or parkin^{T240R} (***c***) has no effect on GFP signal that is distinguishable from the expression of parkin^{wt} (***d***) or control (***a***). ***e***, ***f***, In contrast to the robust fluorescence of mutant parkin brains (***a–d***), expression of a toxic polyglutamine construct, Q108 (***f***), markedly reduces GFP signal in midpupal brains compared with controls (***e***). (Images show pupal brains for Q108 as expression of this transgene is late pupal lethal.) ***B***, Staining of larval muscle verified expression of human parkin under control of *24B*-GAL4. ***a–c***, The driver-alone control (***a***) shows only background staining with PRK8 monoclonal antibody, whereas a robust signal in larval muscle is observed for both mutant (***b***) and wild-type human parkin transgenes (***c***). ***C***, Confocal image of sarcomeres from indirect flight muscle stained with phalloidin. ***a***, ***b***, *24B*-GAL4 control (***a***) and *24B*::parkin^{T240R} (***b***) show normal organization of sarcomeres. Black arrowheads show normal Z-bands. ***c***, The control *dpark* null mutant shows abnormal deposition of actin-containing debris in indirect fly muscle (white arrows) with irregularly organized sarcomeres. ***d***, *24B*-GAL4::parkin^{wt} fails to rescue the muscle phenotype of the *dpark* null mutant. The white arrow shows abnormal debris. Scale bar, 40 μm.

Discussion

Here, we report that expression of mutant but not wild-type human parkin in *Drosophila* results in progressive, age-dependent dysfunction and degeneration of DA neurons. Degeneration is less marked in 5-HT neurons and does not occur in histaminergic or cholinergic neurons expressing mutant parkin. The selective vulnerability of DA neurons to mutant parkin is likely to be associated with dopamine itself and/or its metabolites, because genetic manipulation of VMAT affects mutant parkin-induced neurodegeneration and motor dysfunction.

Relationship between parkin mutations and cell death

The discovery of monogenic forms of PD has opened new avenues toward understanding disease pathogenesis. The linkage of parkin to PD was originally described in families with inherited AR-JP (Kitada et al., 1998). Although the vast majority of parkin-related PD cases are inherited recessively, an increasing number of reports identify a proportion of PD patients in which only a single copy is mutated (Leroy et al., 1998; Maruyama et al., 2000; Farrer et al., 2001; Lucking et al., 2001; West et al., 2002; Foroud et al., 2003; Tan et al., 2003; Mata et al., 2004). Given the complexity of analyzing the 1.3 Mb parkin genomic locus, it is possible that a proportion of these apparent dominant cases represent a failure to ascertain a second site mutation. Nonetheless, several reports have identified abnormalities in Fluoro-DOPA positron emission tomography (Hilker et al., 2001; Khan et al., 2002, 2005; Scherfler et al., 2004) and 99mTc-TRODAT-1 single-photon emission computed tomography scans (Shyu et al., 2005) in heterozygous parkin mutant "carriers." Intriguingly, functional magnetic resonance imaging has also identified motor reorganization in otherwise asymptomatic parkin mutation carriers (Buhmann et al., 2005). One plausible explanation for such findings is that heterozygosity for parkin mutations increases the susceptibility of DA neurons to other environmental or genetic factors; however, this remains to be proven. Nonetheless, it is clear that heterozygous parkin mutations are an important determinant of age of onset (Sun et al., 2006). The observation of affected PD patients carrying a combination of both normal and mutant parkin alleles suggests that haploinsufficiency might serve as a risk factor for disease. An alternative explanation for single-copy parkin mutations that give rise to symptomatic PD is that certain mutations confer dominant-negative or toxic gain of function properties.

Cell culture and *in vitro* studies indicate that parkin functions as an E3 ligase (Giasson and Lee, 2001, 2003; Hattori and Mizuno, 2004; Moore et al., 2005), although additional roles in microtubule-based transport (Ren et al., 2003) and regulation of dopamine transporter activity have been suggested (Jiang et al., 2004). It is generally thought that the loss of E3 ligase activity is involved in the pathogenesis of parkin-linked PD. Recently, Brice and colleagues (Lesage et al., 2007) identified a family with compound heterozygous and homozygous deletions of the parkin promoter, resulting in a complete absence of parkin transcripts; these patients are clinically indistinguishable from those bearing other severe mutations in the parkin gene. Clearly, DA cell death in humans can result from a complete absence of parkin activity. However, additional mechanisms may contribute to at least some neurotoxic processes associated with mutant forms of parkin. It is interesting to note that, apart from the neurodegeneration in locus ceruleus identified in parkin exon 7 knock-out mice reported by Dawson and coworkers (Von Coelln et al., 2004), a

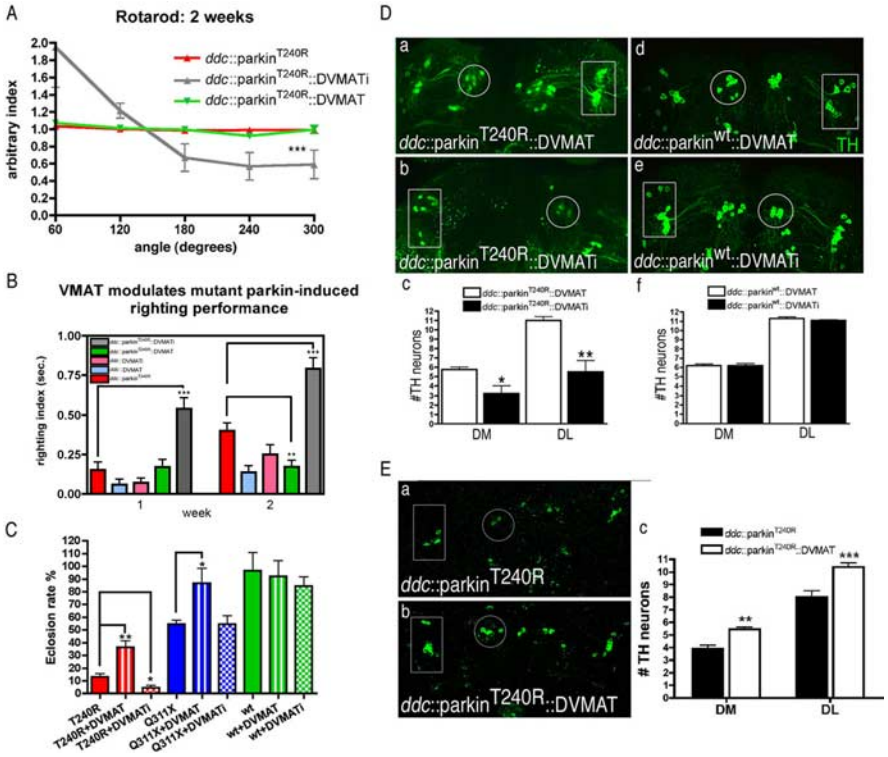

Figure 7. DVMAT modulates mutant parkin-induced degeneration and motor phenotypes. ***A***, Rotarod performance reveals early onset of postural instability in *ddc*::parkin ^{T240R}::DVMATi flies at 2 weeks (gray lines) compared with *ddc*::parkin ^{T240R} (red lines) and *ddc*::parkin ^{T240R}::DVMAT (green lines). At least six flies were scored for each genotype. ***B***, The righting reflex demonstrates earlier onset of postural instability in *ddc*::parkin ^{T240R}::DVMATi flies (gray bars at 1 week) compared with *ddc*::parkin ^{T240R} (red bar at 1 week). At 2 weeks, the progressively impaired righting ability of *ddc*::parkin ^{T240R} (red bar) was ameliorated in *ddc*::parkin ^{T240R}::DVMAT flies (green bar), whereas *ddc*::parkin ^{T240R}::DVMATi flies continued to show a more pronounced motor deficit compared with *ddc*::parkin ^{T240R} (red bar). The values represent the mean ± SEM; $n = 20$. $^*p < 0.05$, $^{**}p < 0.01$ relative to control *ddc*::parkin ^{T240R} by one-way ANOVA with Bonferroni's multiple comparison correction. All genotypes at individual stages were compared with *ddc*::parkin ^{T240R}. ***C***, Modulation of DVMAT affects the incomplete pupal lethality phenotype produced using two copies of *ddc*-GAL4 driver to express mutant parkin. Overexpression of parkin ^{T240R} (solid red bar) or parkin ^{Q311X} (solid blue bar) causes incomplete pupal lethality, whereas parkin ^{wt} (solid green bar) produces an ~100% eclosion rate. Knockdown of DVMAT significantly worsens the parkin ^{T240R} lethality phenotype (red checked bar; $^*p < 0.05$), whereas overexpressing DVMAT significantly suppresses both parkin ^{T240R}-induced (red striped bar; $^{**}p < 0.01$) and parkin ^{Q311X}-induced (blue striped bar; $^*p < 0.05$) pupal lethality. Modulation of DVMAT expression has no effect on wild-type parkin (green bars; one-way ANOVA with Bonferroni multiple comparison test; $n \geq 4$). ***D***, Confocal images of *ddc*::parkin ^{T240R} (***a***, ***b***) or *ddc*::parkin ^{wt} (***d***, ***e***) coexpressed with either DVMAT (***a***, ***d***) or DVMATi (***b***, ***e***) brains at 2 weeks. A marked reduction of TH immunoreactivity is observed in *ddc*::parkin ^{T240R}::DVMATi (***b***) compared with *ddc*::parkin ^{T240R}::DVMAT (***a***). At this stage, *ddc*::parkin ^{T240R}::DVMAT and *ddc*::parkin ^{T240R} brains are indistinguishable (data not shown). Quantitation of TH-positive neurons shows significant decreases in both DM and DL clusters in mutant parkin brains coexpressing DVMATi (***c***) (mean ± SEM; unpaired *t* test; $n = 4$; $^*p < 0.05$; $^{**}p < 0.01$). Modulation of DVMAT expression had no evident effect on wild-type parkin brains (***d–f***). Confocal images were reoriented from adjacent brains imaged simultaneously for paired comparison (***a***, ***b***, ***d***, ***e***). ***E***, Confocal images of *ddc*::parkin ^{T240R} (***a***) coexpressed with DVMAT (***b***) brains at 4 weeks. Significant increases in TH immunoreactivity are observed in *ddc*::parkin ^{T240R}::DVMAT (***b***) compared with *ddc*::parkin ^{T240R} (***a***). Quantitation of TH-positive neurons showed significant increases in both DM and DL clusters in mutant parkin brains coexpressing DVMAT (***c***) (mean ± SEM; unpaired *t* test; $n = 8$; $^{**}p < 0.01$; $^{***}p < 0.001$). Confocal images were reoriented from adjacent brains imaged simultaneously for paired comparison.

number of mouse genetic strategies have failed to recapitulate the neuronal cell death that is characteristic of PD (Goldberg et al., 2003; Perez and Palmiter, 2005). Second, many PD-linked parkin mutants, including parkin ^{T240R} and parkin ^{Q311X}, retain substantial E3 ligase activity, and some show a surprising increase in ligase activity (Zhang et al., 2000; Chung et al., 2001; Wang et al., 2005a). Third, although some proposed substrates such as Pael-R accumulate in postmortem AR-JP brains (Imai et al., 2001; Shimura et al., 2001), parkin knock-out mice do not show elevated steady-state concentrations of other substrates such as CDCrel-1 and synphilin-1 (Von Coelln et al., 2004). Fourth, many parkin mutations show altered solubility and form aggresome-like structures in transfected cells (Ardley et al., 2003; Cookson et al., 2003; Gu et al., 2003; Muqit et al., 2004; Henn et al., 2005; Wang et al., 2005a), thereby conferring potentially toxic properties.

Our data suggest that although a dominant mechanism could contribute to the pathological phenotypes caused by mutant parkin in *Drosophila*, it is unlikely that the phenotypes we observe are the result of a dominant-negative effect. Others have found that the *Drosophila* parkin homolog (*dparkin*) loss-of-function (null) flies show degeneration of indirect flight muscle (Greene et al., 2003). If human parkin mutants were capable of exerting a dominant negative effect on the endogenous fly ortholog, their misexpression in muscle would likely phenocopy the effects of the *dparkin* null mutation. However, we failed to detect any histological abnormalities in indirect flight muscle from *24B*::parkin ^{T240R} flies. Although it is possible that the use of a different driver line or multiple copies of the parkin transgene could produce effects in muscle, this experiment argues against a transdominant effect of mutant human parkin. Moreover, overexpression of wild-type fly parkin cannot rescue the pupal lethality phenotype of *ddc*X2::parkin ^{T240R} (data not shown), and wild-type human parkin cannot rescue the muscle phenotype of *dparkin* mutant animals. Although additional effort is required to completely exclude the possibility that misexpression of human parkin affects the function of endogenous parkin in the fly, our observations suggest that mutations of endogenous fly parkin and the expression of mutant human forms of parkin that we report here may confer cytotoxicity by different mechanisms.

Role of dopamine in mutant parkin-associated neurodegeneration

Most motor symptoms of PD are the result of progressive degeneration of DA neurons originating in the substantia nigra. The molecular pathways that lead to the death of this population of DA neurons are not known, and understanding the basis of selective mesencephalic DA neuron vulnerability may aid the rational design of therapeutics. Because all identified PD-linked genes are expressed ubiquitously in the CNS, it is unclear why such mutations give rise to selective pathology in the nigrostriatal system. It has been suggested that the pathogenesis of monogenic and perhaps idiopathic PD might involve proteins or neurochemicals that are particularly abundant in DA neurons. In vertebrates, nigral DA neurons are characterized by a distinct set of proteins that play a role in DA synthesis and metabolism, such as TH, Ddc, monoamine oxidase (MAO), and the plasma membrane dopamine transporter, as well as other proteins such as VMAT that may be differentially expressed (Vernier et al., 2004).

And of course, unlike other cells, DA neurons also store and release dopamine. The neurotoxic effects of dopamine may be mediated through general oxidative effects and perhaps more specific interactions with proteins implicated in familial PD. Yankner and colleagues (Xu et al., 2002) observed that α-synuclein-induced neurotoxicity is observed in primary cultures of DA neurons but not in non-DA cortical neurons. Moreover, mutant forms of α-synuclein mutation form neurotoxic adducts with DA quinone (Conway et al., 2001). In addition, the oxidative effects of dopamine may increase protein nitrosylation, and parkin activity is altered by this modification both *in vitro* and *in vivo* (Chung et al., 2004; Yao et al., 2004).

Fruit flies use similar sets of genes for dopamine synthesis and transport, but they metabolize dopamine differently from mammals. Insects do not express MAO (Roelofs and Van Haastert, 2001) and are thought to use conjugation as the primary route for amine degradation. This difference may be related to the unique use of dopamine for the hardening and pigmentation of cuticles in insects and other arthropods (Wright, 1987). Indeed, the need to maintain adequate levels of cytosolic dopamine for these pathways may account for the somewhat surprising effects of DVMAT in total head concentrations of dopamine. We find that overexpression of DVMAT decreases total dopamine and that inhibition of DVMAT using RNAi increases total tissue dopamine. Overexpression of mammalian VMAT2 in cultured cells decreases cytosolic dopamine (Sulzer et al., 2000), and decreased VMAT2 activity in mammals reduces total tissue dopamine (Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997; Mooslehner et al., 2001). We suggest that the cytoplasmic pool of dopamine may account for a larger proportion of total tissue dopamine in *Drosophila*, perhaps because of the requirement of dopamine for cuticle formation in other tissues. It is possible that this may render DA neurons in the fly particularly sensitive to neurotoxic mechanisms involving the conjugation of dopamine to cytoplasmic targets.

Based on previous findings in mammals, we speculate that dopamine and/or oxidized derivatives might contribute to mutant parkin-induced degeneration. If so, mutant parkin phenotypes should be relatively specific for DA neurons and sensitive to modulation of cytoplasmic dopamine levels. Indeed, we observe that DA but not cholinergic or histaminergic neurons degenerate in response to expression of mutant parkin. Interestingly, we observe a more limited degenerative phenotype in 5-HT neurons, consistent with the loss of other aminergic cell types in PD (Braak et al., 2003). We find that DVMAT knockdown enhances mutant parkin phenotypes, suggesting that an increase in cytoplasmic dopamine increases the vulnerability of neurons to mutant parkin. Conversely, increasing DVMAT partially rescues pupal lethality and DA neuron degeneration, presumably by reducing cytoplasmic dopamine. These data therefore suggest that mutations in parkin either increase susceptibility of neurons to dopamine or its metabolites or that dopamine is permissive for the toxic effects of parkin. Our model, which provides robust behavioral and neuropathological phenotypes, suggests that some mutations may give rise to disease by dominant mechanisms. Moreover, our model may prove a useful addition to other genetically based vertebrate and invertebrate models aimed at understanding PD and identifying potential therapeutic targets.

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