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Parkin Suppresses Dopaminergic Neuron-Selective Neurotoxicity Induced by Pael-R in *Drosophila*

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Summary

Parkin, an E3 ubiquitin ligase that degrades proteins with aberrant conformations, is associated with autosomal recessive juvenile Parkinsonism (AR-JP). The molecular basis of selective neuronal death in AR-JP is unknown. Here we show in an organismal system that panneuronal expression of Parkin substrate Pael-R causes age-dependent selective degeneration of *Drosophila* dopaminergic (DA) neurons. Coexpression of Parkin degrades Pael-R and suppresses its toxicity, whereas interfering with endogenous *Drosophila* Parkin function promotes Pael-R accumulation and augments its toxicity. Furthermore, overexpression of Parkin can mitigate α-Synuclein-induced neuritic pathology and suppress its toxicity. Our study implicates Parkin as a central player in the molecular pathway of Parkinson's disease (PD) and suggests that manipulating Parkin expression may provide a novel avenue of PD therapy.

Introduction

PD is the most common movement disorder and the second most common neurodegenerative diseases. PD patients suffer from rigidity, slowness of movement, tremor, and postural instability. The movement disorder in PD is largely due to the deficiency of brain dopamine contents caused by the degeneration of DA neurons in the midbrain. Although the mechanism underlying the selective degeneration of DA neurons is still poorly understood, both exogenous environmental toxins and endogenous proteotoxins have been implicated in PD pathogenesis (Zhang et al., 2000a; Giasson and Lee, 2001; Lansbury and Brice, 2002).

The molecular cloning of genes linked to familial forms of the disease has provided tremendous insights into the pathogenesis of PD. Missense mutations in the α-*Synuclein* (α-*Syn*) gene have been shown to be the cause of rare forms of autosomal dominant familial PD (Polymeropoulos et al., 1997; Kruger et al., 1998). α-Syn is an abundant brain protein enriched at presynaptic terminals (Clayton and George, 1998). Mice with α-*Syn* gene deleted show increased striatal dopamine release but do not develop neurological phenotypes observed in PD. This suggests that familial α-Syn mutations may cause PD through a dominant gain-of-function mechanism (Abeliovich et al., 2000). Significantly, wild-type α-Syn protein was found to be a major component of Lewy bodies, the proteinaceous aggregates found in PD and other diseases termed "Synucleinopathies" (Spillantini et al., 1997; Baba et al., 1998). This suggests that the accumulation and aggregation of α-Syn is intimately involved in disease pathogenesis. Further support came from transgenic animal studies in which overexpression of wild-type and mutant forms of α-Syn in mouse and *Drosophila* led to α-Syn aggregate formation and neuronal dysfunction (Feany and Bender, 2000; Masliah et al., 2000; Giasson et al., 2002; Lee et al., 2002).

Mutations in the *parkin* gene have been linked to AR-JP (Kitada et al., 1998). AR-JP patients develop the typical parkinsonian symptoms also as a result of loss of midbrain DA neurons. This usually occurs in the absence of Lewy body formation (Mizuno et al., 1998). Biochemical studies have shown that Parkin has E3 ubiquitin-protein ligase activity and that AR-JP-linked *parkin* mutations abolished this activity (Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000b). Ubiquitin-protein ligases are components of the ubiquitin-proteasome pathway that degrades proteins with abnormal conformations (Hershko and Ciechanover, 1998; Sherman and Goldberg, 2001). In this pathway, ubiquitin moiety is transferred to substrate proteins through ubiquitin-activating enzyme (E1), ubiquitin-carrier protein (E2), and ubiquitin ligase (E3). After a polyubiquitin chain is attached to the substrate protein, it is recognized by the 26S proteasome and targeted for degradation. Substrate specificity in this reaction is largely conferred by the interaction between E3 and the substrate.

A number of proteins have been identified as Parkin substrates in vitro. CDCrel-1, a synaptic vesicle-associated protein, has been shown to interact with and can be ubiquitinated by Parkin (Zhang et al., 2000b). Synphilin-1, which was originally identified as a α-Syn-interacting protein, has also been shown to be a substrate of Parkin-mediated ubiquitination and degradation (Chung et al., 2001). It has not been determined whether CDCrel-1 or Synphilin-1 accumulates to higher levels in AR-JP patient brain as a result of loss of Parkin activity. Parkin has also been shown to promote the ubiquitination and degradation of an *O*-glycosylated form of α-Syn (αSp22) (Shimura et al., 2001). The abundance of αSP22 is very low in normal brain, but it was found at modest levels in AR-JP brains lacking Parkin activity. It is not known whether *O*-glycosylation is a strict prerequisite for α-Syn interaction with Parkin and its subsequent ubiquitination and whether these modifications play any significant roles in PD pathogenesis. It is possible that through Synphilin-1, Parkin and unmodified α-Syn could functionally interact in the disease process.

Parkin has recently been shown to ubiquitinate Pael-R, a putative G protein-coupled transmembrane polypeptide (Imai et al., 2001). When overexpressed in cultured cells, Pael-R tends to become unfolded and insoluble and induces endoplasmic reticulum (ER) stress.

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Parkin ubiquitinates Pael-R and promotes the degradation of the insoluble form of the protein. Moreover, the insoluble form of Pael-R has been found to accumulate in AR-JP patient brains (Imai et al., 2001). However, the causal relationship between Pael-R accumulation and AR-JP is not clear.

To test the functional significance of Parkin's biochemical interactions with Pael-R and α-Syn, we have used transgenic flies expressing human Pael-R, Parkin, or α-Syn to analyze their in vivo relationships and their roles in the pathogenesis of PD. Panneuronal expression of Pael-R results in age-dependent selective degeneration of *Drosophila* DA neurons, suggesting that DA neurons are especially susceptible to Pael-R toxicity. Coexpression of human Parkin causes the degradation of Pael-R and suppresses the DA neuron degeneration phenotype, whereas interference of endogenous *Drosophila parkin* function promotes Pael-R accumulation and enhances Pael-R-induced neurotoxicity. These data provide strong in vivo evidence that Pael-R is a genuine substrate for Parkin and that the accumulation of Pael-R is a cause of DA neuron death in AR-JP. Interestingly, overexpression of Parkin can also suppress the toxicity of α-Syn, at least in part by mitigating α-Syn-induced neuritic pathology. Our study implicates Parkin as a central player in the pathogenesis of different forms of PD.

Results

Targeted Expression of Pael-R in *Drosophila*

In the sequenced *Drosophila* genome, there is no clear fly homolog of human Pael-R. To address the role of Pael-R in causing neurodegeneration, we used the bipartite *UAS-GAL4* system to target the expression of human Pael-R protein to different fly tissues and cell types (Brand and Perrimon, 1993). This system involves two transgenic lines, a *UAS-Pael-R* line, in which the human *Pael-R* cDNA is placed under the control of upstream activating sequence (UAS) for the yeast transcription factor GAL4, and a *GAL4* line, which expresses GAL4 in a tissue- and cell type-specific pattern. In the progenies resulting from a cross between these two lines, Pael-R is expressed in the same tissue and cell type where GAL4 is expressed.

Multiple independent *UAS-Pael-R* transgenic lines were generated through P element-mediated germline transformation. To assess the levels of Pael-R expression in these different transgenic lines, we crossed these *UAS-Pael-R* lines to the *scabrous-GAL4* line, a strong *GAL4* driver line that directs transgene expression in the precursor cells and their differentiated progenies in the central and peripheral nervous system. Resulting transgenic embryos were examined for transgene expression by immunostaining with antibodies against Pael-R. Of ten independent transgenic lines analyzed, four give detectable expression of the Pael-R protein as determined by the immunostaining method. Two lines, a strong expression line and a moderate expression line, were chosen for subsequent studies.

Overexpression of Pael-R Leads to a Loss of DA Neurons

To target the expression of Pael-R to DA neurons, we used the *Ddc-GAL4* driver line, in which the expression of GAL4 is under the control of the *DOPA decarboxylase* gene promoter (Li et al., 2000). Sections of aged control and transgenic fly brains were immunostained for tyrosine hydroxylase (TH), which specifically identifies DA neurons. We focused our analysis on the dorsomedial (DM) clusters of DA neurons, which have been well characterized and shown to preferentially degenerate in α-Syn transgenic flies (Feany and Bender, 2000; Auluck et al., 2002). The DM clusters are composed of approximately 18 DA neurons in wild-type adult fly brain. The two clusters are distributed in a bilaterally symmetric fashion with respect to the midline (Figure 1A). The number of DA neurons in the DM clusters does not change significantly, even in 60-day-old senescent wild-type flies. In 40-day-old transgenic flies expressing Pael-R, however, we observed that the number of DA neurons in the DM clusters was reduced compared to the controls (Figure 1B). This reduction of DA neurons was correlated with the expression level of the Pael-R transgene, as the strong expression lines consistently showed a smaller number of DA neurons (10.8 ± 0.5, mean ± SEM) than the moderate expression line (13.5 ± 0.6). Expression of a control green fluorescent protein (GFP) has no effect on DA neuron number (see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/37/6/911/DC1).

The loss of DA neurons could be caused by late onset neurodegeneration or early developmental abnormalities. To distinguish these possibilities, we examined younger flies. In newly eclosed transgenic flies (1-day-old) expressing Pael-R, the number of DA neurons in the DM clusters (18.6 ± 0.6) was similar to that observed in control flies (18.8 ± 0.8, Figure 1C). This suggests that the DA neurons are initially formed normally in the transgenic flies, but over time some of these neurons degenerate as a consequence of the overexpression of Pael-R protein.

The Toxicity of Pael-R Is Relatively Selective toward DA Neurons

We next examined the effects of expressing Pael-R ubiquitously in all the differentiated neurons of the fly nervous system using the strong *UAS-Pael-R* line and the panneuronal driver *elav-GAL4* (Lin and Goodman, 1994). Western blot analysis on adult fly heads showed that the amount of transgene-produced Pael-R protein per milligram of brain tissue in *elav-GAL4 /UAS-Pael-R* flies is 45%–55% that of endogenous Pael-R protein in mouse brain (see Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/37/6/911/DC1). TH immunostaining revealed that, similar to that observed in *Ddc-DAL4* driven transgenic flies, the number of DA neurons in the DM clusters is also reduced in *elav-GAL4* driven transgenic flies (12.0 ± 1.7; Figure 2B).

We then analyzed other neuronal types to see if they are also affected after panneuronal expression of Pael-R. The monoclonal antibody 22C10, which recognizes the Futsch antigen (Zipursky et al., 1984; Hummel et al., 2000), is highly expressed in the visual system as well as the central brain complex in adult flies. The staining of lamina and medulla neuropils by the 22C10 antibody was similar between the control and Pael-R transgenic flies (Figures 2C and 2D). Overall morphology of the central brain was preserved in Pael-R transgenic

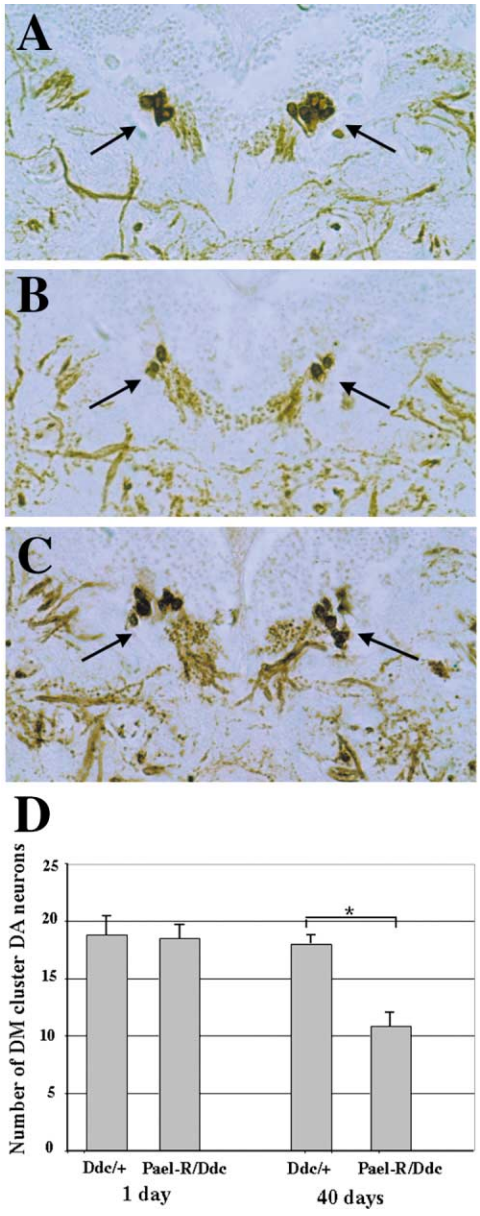

Figure 1. Age-Dependent Loss of DA Neurons in the DM Clusters of Pael-R Transgenic Flies

Frontal sections of aged fly brain were stained with anti-TH antibody to identify DA neurons. The section containing most of the DA neurons in the DM clusters (marked by arrows) was shown for each brain.

(A) A 40-day-old *Ddc-GAL4/+* control fly brain.

(B) A 40-day-old *Ddc-GAL4/+; UAS-Pael-R/+* fly brain.

(C) A 1-day-old *Ddc-GAL4/+; UAS-Pael-R/+* fly brain.

(D) Bar graph summarizing quantitative analysis of DA neuron number in the DM clusters in Pael-R transgenic flies and control flies. Asterisk indicates that the difference in DA neuron number between 40-day-old Pael-R transgenic flies and control flies is statistically significant ($p < 0.01$, Student's t test).

flies, suggesting that ubiquitous expression of Pael-R in the brain does not cause widespread degeneration. We also examined specific neuronal subtypes. Immunostaining with the antibody against choline acetyltransferase (Yasuyama et al., 1996), an enzyme in the biosynthetic pathway of acetylcholine, showed that the distribution and number of cholinergic neurons in major brain cholinergic centers were similar between control and Pael-R transgenics (data not shown). Immunostaining of serotonergic neurons with anti-5HT antibody also did not reveal any obvious reduction of these neurons in major brain serotonergic centers (data not shown). Although we can not exclude the possibility that a small percentage of serotonergic or cholinergic neurons degenerate in Pael-R transgenic flies, our analysis indicates that there are no clusters of these neurons that are vulnerable to Pael-R toxicity like the DM clusters of DA neurons.

We also examined whether the expression of Pael-R is toxic to cell types outside of the central nervous system (CNS). For this purpose, we crossed the *UAS-Pael-R* transgenic lines to *GAL4* driver lines that have known expression patterns in non-CNS tissues. Previous studies showed that expression of many human disease-causing proteins such as polyglutamine repeat-containing proteins, tau, and α-Syn in fly eye caused defects in eye morphogenesis and retinal degeneration (Warrick et al., 1998; Feany and Bender, 2000; Kazemi-Esfarjani and Benzer, 2000; Wittmann et al., 2001). In contrast, Pael-R expression in the eye has no phenotypic consequence (Figures 2E and 2F). We have targeted Pael-R expression in the eye at different stages of eye development using the *elav-, sevenless-, gmr-,* and *eyeless-GAL4* lines. In all four cases, Pael-R-expressing flies had normal eye morphology and retina structure. Similarly, no phenotype was observed when the *dpp-GAL4* and *24B-GAL4* lines were used to express Pael-R along the anterior-posterior boundary of imaginal discs and in muscle cells, respectively.

The only phenotype outside of the nervous system was observed when we used the *apterous-GAL4* line to direct Pael-R expression. *apterous-GAL4* is expressed in several larval imaginal discs including the dorsal mesothoracic and metathoracic discs. In newly eclosed flies from a cross between the *apterous-GAL4* and the strong Pael-R lines, body fluids leaked from two spots located in a bilaterally symmetric fashion on the dorsal thorax at the junction between the notum and scutellum. The fluids quickly solidified and formed two dark patches adhering to the cuticle (Figure 3B). This phenotype is presumably caused by the degeneration of the epithelial tissue or the underlying musculature at those sites.

Coexpression of Human Parkin Suppresses Pael-R Toxicity

Given the propensity of overexpressed Pael-R to become unfolded and insoluble and elicit ER stress in cell culture (Imai et al., 2001), it is possible that the degeneration phenotype observed in Pael-R transgenic flies is caused by the accumulation of abnormal and toxic forms of Pael-R protein. To test this possibility, we asked if coexpression of human Parkin protein could promote the degradation of these aberrant proteins and therefore suppress Pael-R toxicity. In transgenic flies that coexpressed Pael-R and human Parkin in DA neurons under the control of the *Ddc-GAL4* driver, we observed that the number of DA neurons in the DM clusters is restored to near wild-type levels (16.9 ± 0.6; Figure

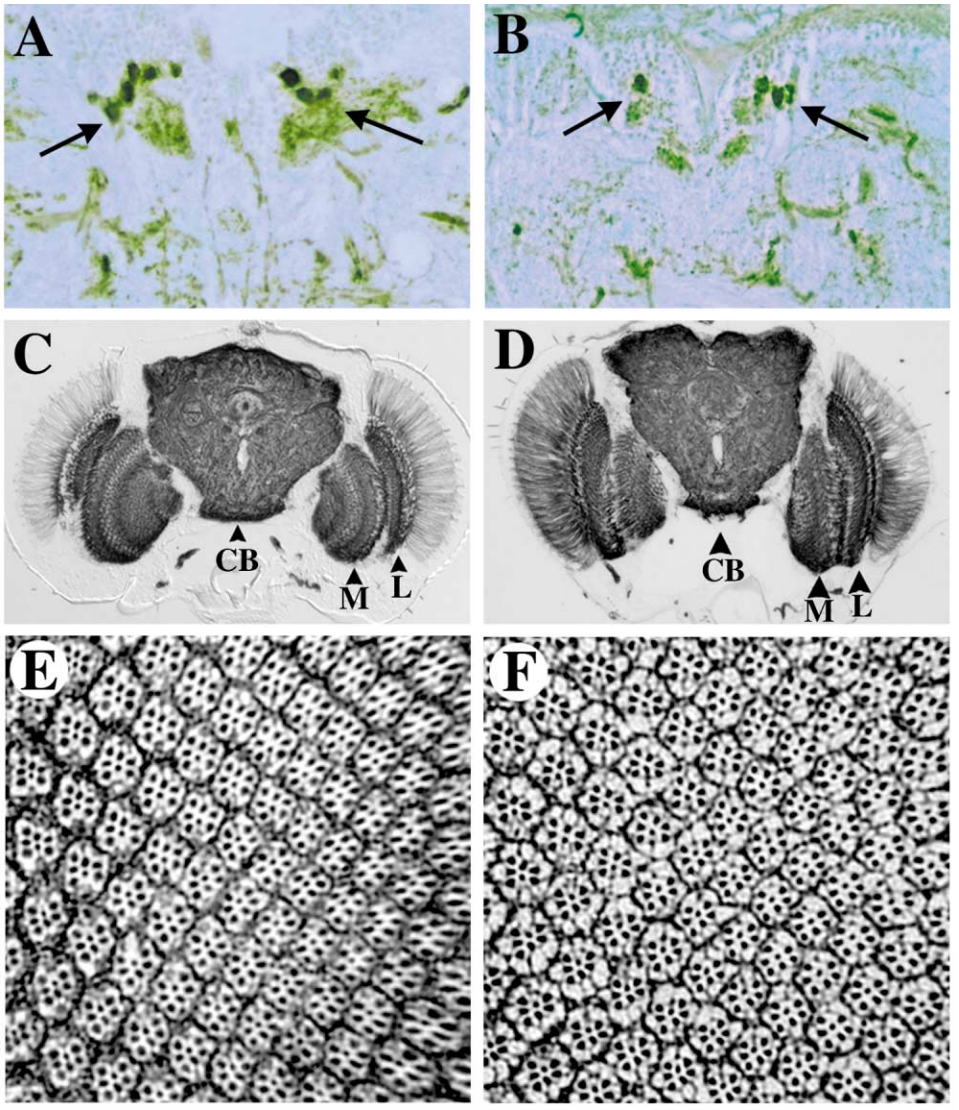

Figure 2. Cell Type Selectivity of Pael-R Neurotoxicity

(A–D) Frontal sections of 40-day-old *elav-GAL4/+* control flies (A and C) and 40-day-old *elav-GAL4/+; UAS-Pael-R/+* flies (B and D) were immunostained with anti-TH antibody (A and B) to identify DA neurons in the DM clusters (marked with arrows) or with 22C10 MAb (C and D) to reveal general morphology of optic lamina (L), medulla (M), and the central brain complex (CB).

(E and F) Toluidine blue staining of *elav-GAL4/+* (E) or *elav-GAL4/+; UAS-Pael-R/+* (F) adult retina to show that neither retinal architecture nor photoreceptor neuron number is affected by Pael-R expression.

3E). Expression of the human Parkin protein alone using the *Ddc-GAL4* driver did not affect the number of DA neurons in the DM clusters (Figure 3F).

When human Parkin and Pael-R were coexpressed using the *apterous-GAL4* driver, the thoracic toxicity of Pael-R was also suppressed and no dark patches formed on the dorsal thorax (Figure 3C). Coexpression of GFP did not suppress Pael-R-induced thoracic toxicity (see Supplemental Figure S3 at http://www.neuron.org/cgi/content/full/37/6/911/DC1). Expression of human Parkin alone using the *apterous-GAL4* driver had no effect on thoracic morphology (Figure 3D). These results indicate that coexpression of human Parkin suppressed Pael-R toxicity in two different cell types.

Human Parkin Promotes the Degradation of Pael-R in a Neuronal Cell Type-Specific Manner

We wished to gain further insights into the interaction between human Parkin and Pael-R at the molecular and cellular level. For this purpose, we performed immunohistochemical analysis of human Parkin and Pael-R proteins expressed in neural stem cell-derived DA neurons. Isolated *Drosophila* embryonic neural stem cells (called neuroblasts) undergo stereotyped divisions when cultured in vitro. After being in culture for 12 hr, the neuroblast progenies exit the cell cycle and form clusters of differentiated neuronal or glial cell types. The neuronal progenies, which include an appropriate proportion of TH-positive DA neurons (Figures 4G and 4H), are stable

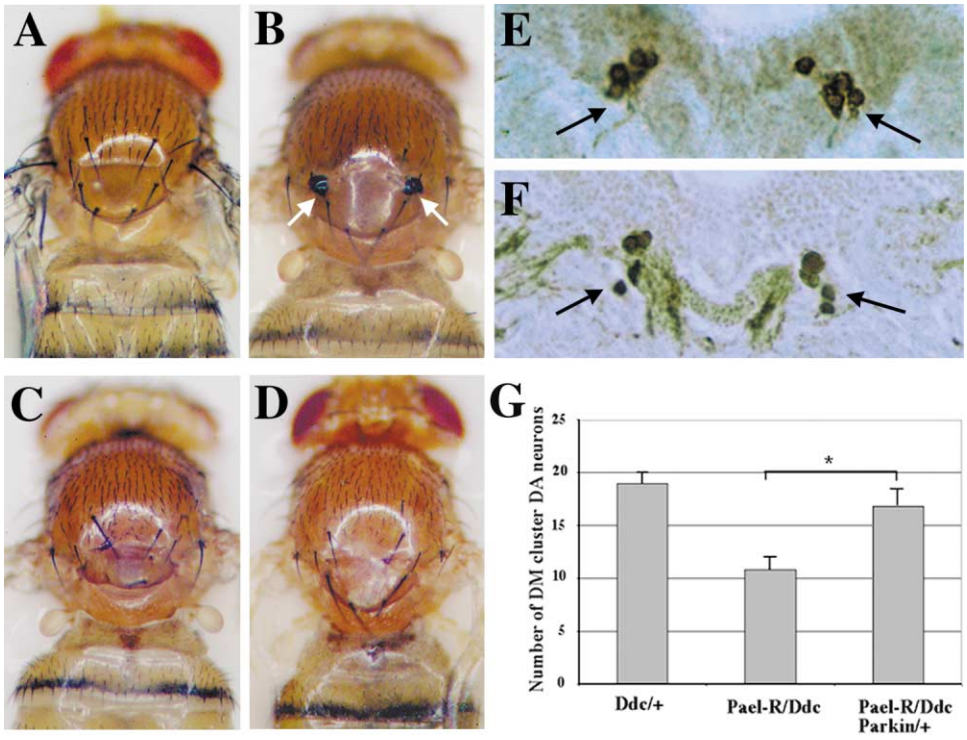

Figure 3. Suppression of Pael-R Toxicity by the Coexpression of Parkin

(A and B) Expression of Pael-R using the *apterous-GAL4* driver leads to degeneration of the notum, as indicated by the leakage of body fluids (marked by arrows in B), which is absent in control flies (A).

(C–F) Coexpression of a transgene expressing human Parkin completely suppressed this thoracic degeneration phenotype (C). Note that the scutellum bristles are pointing upwards and out of focus in this image. Coexpression of human Parkin also completely suppressed the loss of DA neurons in the DM clusters (marked by arrows in E). Expression of human Parkin alone has no effect on either thoracic morphology (D) or DA neuron number in the DM clusters (F). The genotypes are *apterous-GAL4/+* (A), *apterous-GAL4/+; UAS-Pael-R/+* (B), *apterous-GAL4/UAS-hParkin; UAS-Pael-R/+* (C), *apterous-GAL4/UAS-hParkin* (D), *Ddc-GAL4/+; UAS-hParkin/UAS-Pael-R* (E), and *Ddc-GAL4/+; UAS-hParkin/+* (F).

(G) Bar graph summarizing quantitative analysis of DA neuron number in the DM clusters in 40-day-old Pael-R transgenic flies with and without Parkin coexpression. Asterisk indicates that the difference in DA neuron numbers between the two conditions is statistically significant ($p < 0.01$, Student's t test).

in culture for weeks. In cultured DA neurons, Pael-R is mainly localized to the cell surface, as indicated by its colocalization with Numb, a membrane protein (Figures 4A–4C). There is also a lower level of expression in neuronal processes. Human Parkin protein exhibits similar localization pattern in cultured DA neurons, but its localization to neuronal processes is more pronounced and appears punctate. Double labeling with Synaptotagmin suggests that the punctate staining of Parkin in neuronal processes represents its association with synaptic vesicles (Figures 4D–4F). This is consistent with earlier findings in mammalian cell culture (Kubo et al., 2001).

We next examined the interaction between Pael-R and human Parkin when they were coexpressed in postmitotic neurons under the control of the *elav-GAL4* driver. In 1- or 2-day-old neuronal culture, Pael-R and Parkin showed colocalization, and in most neurons (135/154) the level of Pael-R protein was similar to that observed in neurons expressing Pael-R alone (Figures 5A and 5B). In 6-day-old culture, however, the majority of these neurons (172/235) showed a significant reduction of Pael-R protein as judged by immunostaining (Figures 5C and 5D). Interestingly, there was a significant portion (63/235) of neurons that still expressed normal levels of Pael-R protein in the presence of Parkin (Figure 5D). Because the action of Parkin requires E1, E2, and possibly other cofactors, these neurons may miss certain cofactors required for Parkin-mediated degradation of Pael-R.

We then tested whether DA neurons have the capacity to degrade Pael-R in the presence of human Parkin. We coexpressed these two proteins in cultured DA neurons using the *Ddc-GAL4* driver. Similar to that observed in *elav-GAL4* neuronal culture described above, in younger cultures (1–2 days old) Pael-R and Parkin showed colocalization, and in most Ddc-GAL4-expressing neurons (27/31), the level of Pael-R was similar to that observed in neurons expressing Pael-R alone (Figures 5E and 5F). But in older neuronal culture (4–6 days), Pael-R protein level was markedly reduced in most Ddc-GAL4-expressing neurons (21/29; Figure 5H). In contrast, control neurons expressing Pael-R in the absence of Parkin showed

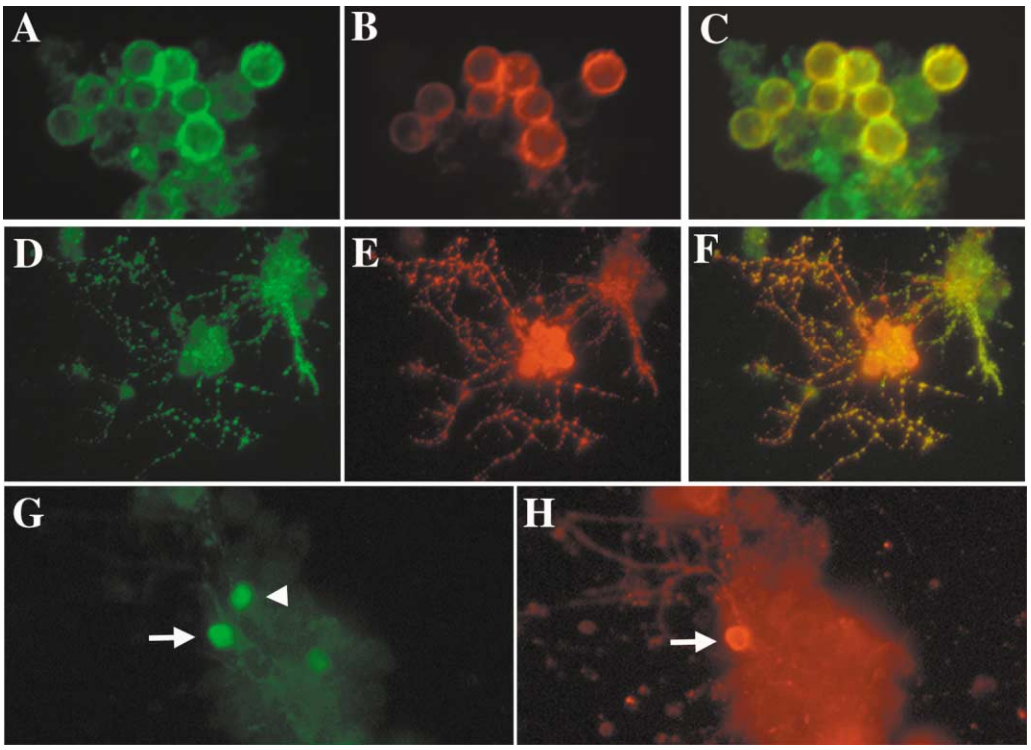

Figure 4. Subcellular Localization of Pael-R and Parkin in Cultured Neurons

(A–C) Membrane localization of Pael-R. A neuronal cluster generated from an *elav-GAL4/+; UAS-Pael-R/+* genotyped neuroblast was double-labeled for Pael-R (B) and Numb (A), a membrane protein. The merged image in (C) shows colocalization of Pael-R and Numb at the plasma membrane. Pael-R also shows lower level of expression in neuronal processes, which are out of focus in this image.

(D–F) Vesicular localization of Parkin. A neuronal cluster generated from an *elav-GAL4/+; UAS-hParkin/+* genotyped neuroblast was double labeled for Parkin (E) and Synaptotagmin (D), a synaptic vesicle protein. The merged image in (F) shows colocalization of Parkin and Synaptotagmin in vesicular structures of neuronal processes.

(G and H) Generation of DA neurons from cultured neuroblasts. A neuronal cluster generated from a *Ddc-GAL4/+; UAS-GFP/+* genotyped neuroblast was immunostained for TH (H). One of the two GFP-expressing neurons (marked by an arrow in H) is TH positive and thus dopaminergic; the other one is likely a serotonergic neuron, which also expresses Ddc-GAL4.

robust Pael-R expression in 6-day-old culture (Figure 5J). Thus, we conclude that human Parkin promotes the degradation of Pael-R in selective groups of *Drosophila* neurons including DA neurons.

To demonstrate the in vivo relevance of our results showing Parkin-mediated degradation of Pael-R in cell culture experiments, we used Western blot analysis to quantify Pael-R protein levels in transgenic flies with or without Parkin coexpression. Due to the small number of neurons expressing Ddc-GAL4 and the sensitivity of available Pael-R antibodies, we were unable to detect Pael-R protein in *Ddc-Gal4* driven transgenic flies, even in the absence of Parkin. Therefore, we used *elav-GAL4* driven transgenic flies, which express Pael-R protein in more cells. In head extracts obtained from 30-day-old *Pael-R* transgenic flies driven by *elav-GAL4*, we observed a significant reduction of Pael-R protein level in the presence of coexpressed Parkin (Figure 5K). In contrast, coexpression of Parkin has little effect on the level of a control GFP protein (see Supplemental Figure S4 at http://www.neuron.org/cgi/content/full/37/6/911/DC1). Together, our immunohistochemical analysis on cultured neurons and Western blot analysis on adult fly brain demonstrate that Parkin promotes the degradation of Pael-R under physiological conditions.

RNA Interference of the Fly Homolog of Parkin Promotes Pael-R Accumulation and Accelerates Pael-R-Induced Neurodegeneration

Our initial observation that in more than half of the Pael-R transgenic lines the protein is present at a low level undetectable by immunostaining suggests that there are mechanisms in *Drosophila* that limit its accumulation. Recent sequencing of the *Drosophila* genome has revealed a fly homolog of human Parkin, dParkin (Rubin et al., 2000). To test whether *dParkin* genetically interacts with the *Pael-R* transgene, we used the RNA interference (RNAi) technique (Fire et al., 1998) to inhibit *dParkin* expression and examined the effect on Pael-R-induced DA neuron degeneration. To inhibit the expression of *dParkin* in DA neurons of adult fly brain, we generated *UAS-IRdParkin* transgenic flies that express double-stranded *dParkin* RNA as hairpin RNA from an inverted repeat of *dParkin* cDNA (Kennerdell and Carthew, 2000). Ubiquitous expression of hairpin *dParkin* RNA using a *heat shock-GAL4* driver line resulted in a

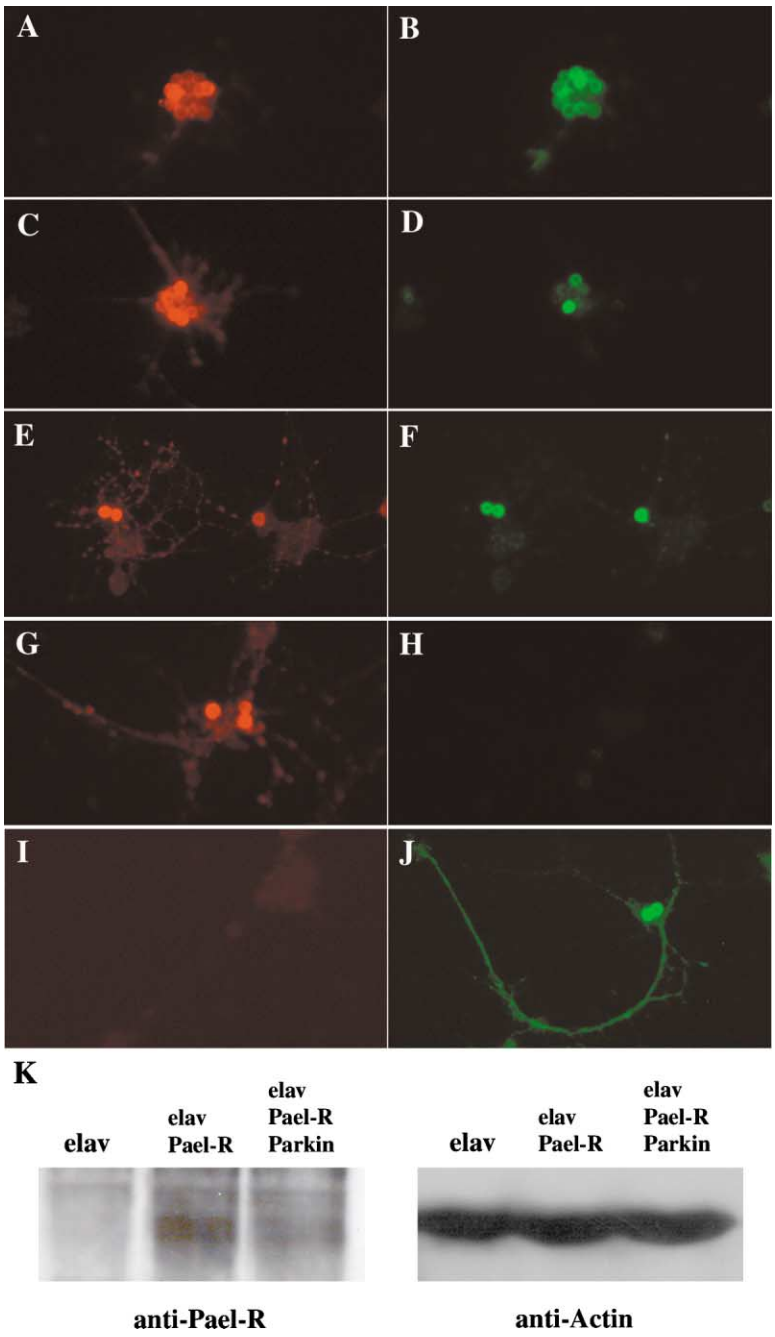

Figure 5. Parkin Promotes the Degradation of Pael-R

(A–D) Double labeling of neuronal clusters derived from *elav-GAL4/+; UAS-Pael-R/UAS-hParkin* genotyped neuroblasts using anti-Parkin (A and C) and anti-Pael-R (B and D) antibodies. The neuronal culture shown in (A) and (B) is 2 days old and the one in (C) and (D) is 6 days old.

(E–H) Double labeling of DA neurons derived from *Ddc-GAL4/+; UAS-Pael-R/UAS-hParkin* genotyped neuroblasts using anti-Parkin (E and G) and anti-Pael-R (F and H) antibodies. The neuronal culture shown in (E) and (F) is 2 days old and the one in (G) and (H) is 6 days old. Note that in 6-day-old culture many of the Parkin-coexpressing neurons have diminished Pael-R protein expression. Diluted anti-Pael-R antibody (1:10,000) was used to detect the difference in Pael-R protein level.

(I and J) Double labeling of DA neurons derived from *Ddc-GAL4/+; UAS-Pael-R/+* genotyped neuroblasts using anti-Parkin (I) and anti-Pael-R (J) antibodies to show that in the absence of Parkin, Pael-R is robustly expressed in 6-day-old DA neurons.

(K) Western blot analysis showing that Pael-R protein level is greatly reduced in adult flies coexpressing Parkin. Head extracts made from 30-day-old *elav-GAL4* control flies (elav), *elav-GAL4/+; UAS-Pael-R/+* (elav Pael-R), and *elav-GAL4/+; UAS-Pael-R/UAS-hParkin* (elav Pael-R Parkin) transgenic flies were probed with anti-Pael-R and then reprobed with anti-Actin.

significant reduction of endogenous *dParkin* mRNA as determined by quantitative RT-PCR analysis (Figure 6E).

When *IRdParkin* and *Pael-R* transgenes were coexpressed in transgenic flies using the *Ddc-GAL4* driver, we observed a dosage-dependent acceleration of Pael-R-induced DA neuron degeneration. Transgenic flies coexpressing Pael-R and two copies of the *IRdParkin* transgene showed the degeneration phenotype when they were analyzed at 14 days of age (Figure 6B). At this age, transgenic flies expressing Pael-R alone or coexpressing Pael-R and one copy of the *IRdParkin* transgene showed a relatively normal number of DA neurons in the DM clusters (Figures 6A and 6F). Furthermore, transgenic flies coexpressing Pael-R and two copies of *IRdParkin* showed more severe DA degeneration phenotypes when analyzed at 30 days of age, with the average number of DA neurons in the DM clusters reduced by more than 50% to 7.8 ± 0.5 (Figures 6D and 6F). Thus, inhibition of endogenous *dParkin* function accelerated the kinetics and enhanced the severity of Pael-R-induced degeneration of DA neurons. In transgenic flies that expressed *IRdParkin* alone, the number of DA neurons in the DM clusters was not significantly affected in 30-day-old flies (17.8 ± 1.0; Figure 6F). This indicates that activation of the RNAi pathway by itself is not deleterious to DA neurons. The lack of DA neuronal loss by dParkin RNAi alone may be due to the absence of toxic substrates such as Pael-R in *Drosophila* or that

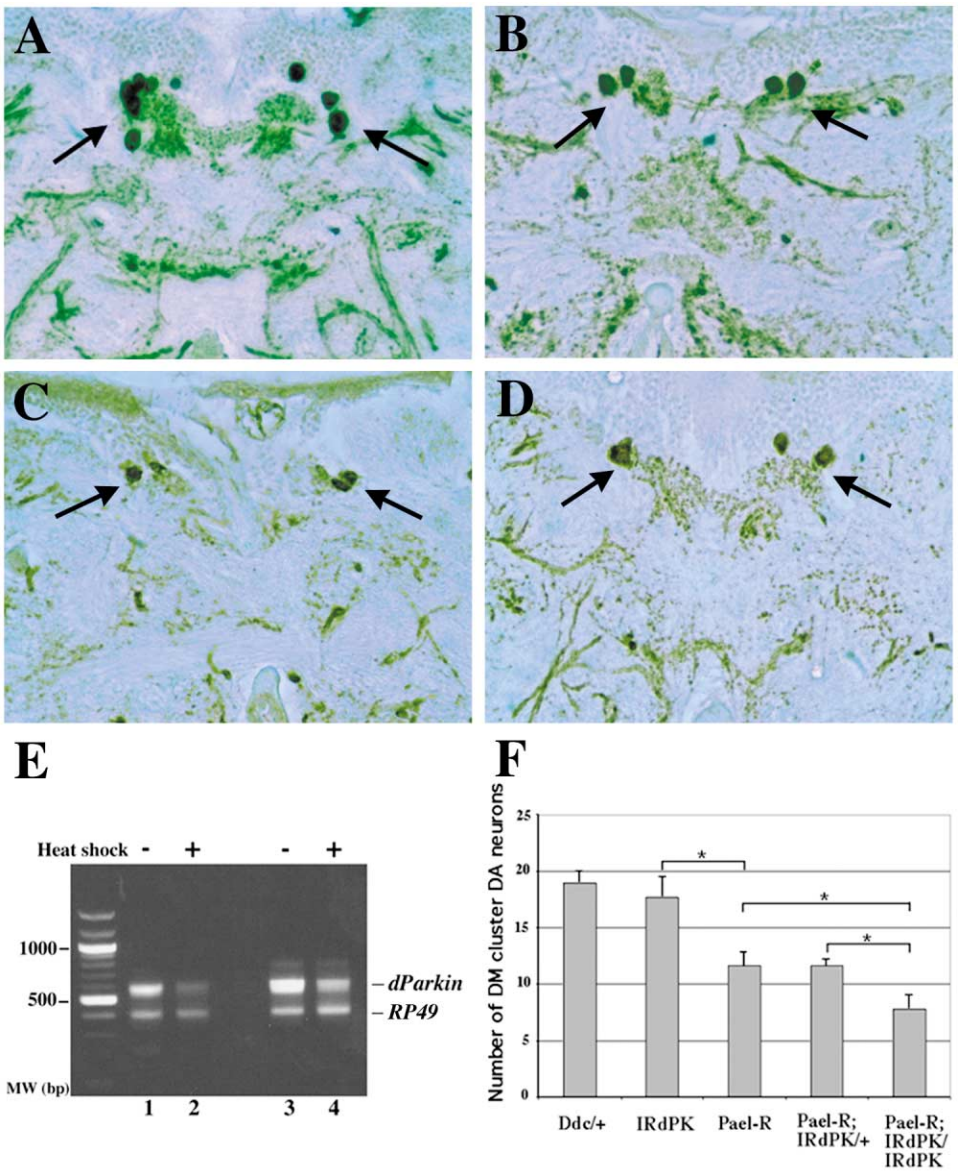

Figure 6. Interference of dParkin Function by RNAi Enhances Pael-R Toxicity

(A–D) Frontal sections showing TH immunostaining of DA neurons in the DM clusters of *Ddc-GAL4/+; UAS-Pael-R/+* (A and C) and *Ddc-GAL4, UAS-IRdParkin/UAS-IRdParkin, UAS-Pael-R* (B and D) genotyped flies that are 14 days (A and B) or 30 days (C and D) old.

(E) RT-PCR analysis showing a reduction of endogenous *dParkin* mRNA level after transgenic RNAi. Second instar *hs-GAL4/UAS-IRdParkin* larvae with (lanes 2 and 4) or without (lanes 1 and 3) heat shock treatment were used to prepare total RNAs for the RT-PCR analysis. PCR primers located outside of the inverted repeat region were used to amplify endogenous *dParkin*, and an *rp49* primer pair was used as an internal control. Samples in lanes 1 and 2 and lanes 3 and 4 are derived from two independent experiments.

(F) Bar graph showing quantitative analysis of DA neuron number in the DM clusters of 30-day-old Pael-R transgenic flies with one or two copies of *IRdParkin* transgene coexpression. Asterisks indicate that the differences in DA neuron numbers between the compared genotypes are statistically significant ($p < 0.01$, Student's t test).

endogenous dParkin substrates may never accumulate to levels sufficient to kill DA neurons. However, since RNAi in our *IRdParkin* transgenic flies results in a reduction but not complete loss of *dParkin* transcripts, it is possible that the residual dParkin may still provide neuroprotective function. Generation of genetic null mutations in the *dParkin* locus could address this issue.

The acceleration of DA neuron degeneration after *dParkin* RNAi could be due to defects in the turnover or subcellular distribution of Pael-R protein. To investigate the mechanisms involved, we analyzed Pael-R protein by immunostaining cultured DA neurons that coexpressed *IRdParkin* and Pael-R. As shown in Figures 7C and 7D, coexpression of *IRdParkin* and Pael-R in DA neurons results in the accumulation of Pael-R to higher levels compared to expression of Pael-R alone (Figures 7A and 7B). Some of the accumulated Pael-R protein was unevenly distributed (Figure 7D). Thus, interfering

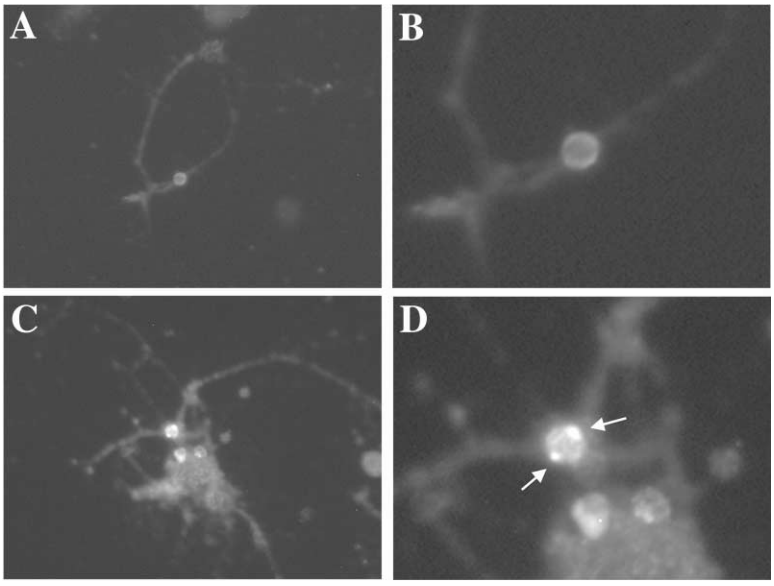

Figure 7. Interference of dParkin Function Promotes the Accumulation of Pael-R

DA neurons derived from *Ddc-GAL4, UAS-Pael-R/+* (A and B) or *Ddc-GAL4, UAS-IRdParkin/ UAS-IRdParkin, UAS-Pael-R* (C and D) genotyped neuroblasts were cultured for 6 days and then immunostained with anti-Pael-R antibody. (B) and (D) are higher-magnification views of the neurons shown in (A) and (C). Arrows in (D) mark the uneven accumulation of Pael-R protein at the periphery of cell membrane.

with endogenous dParkin function results in increased stability and accumulation of Pael-R protein. This may be responsible for the acceleration and enhancement of Pael-R-induced DA neuron degeneration observed in *dParkin* RNAi transgenic flies.

Overexpression of Parkin Suppresses α-Syn Toxicity

Biochemical interactions between Parkin and Pael-R or an *O*-glycosylated form of α-Syn suggest that Parkin may be a common player in the proteasome pathway that regulates the metabolism of these two proteins. The detection of Parkin in Lewy bodies of Parkinson's disease and dementia with Lewy bodies (Shimura et al., 2001; Schlossmacher et al., 2002) further suggests that either Parkin is actively engaged in degrading abnormal α-Syn proteins or that Parkin is sequestered by α-Syn into inactive complexes in the aggregates. In either case, overexpression of Parkin would be predicted to enhance the degradation of abnormal α-Syn or to compensate for the reduction of Parkin activity caused by α-Syn sequestration and therefore suppress α-Syn toxicity. To test this hypothesis, we coexpressed α-Syn together with human Parkin in DA neurons. While the expression of wild-type or pathological forms of α-Syn (A30P or A53T) consistently caused a 50% reduction of DA neurons in the DM clusters in 20-day-old flies (Figure 8A), this loss of DA neurons was suppressed when Parkin was coexpressed with α-Syn (Figure 8B). Interestingly, unlike Parkin suppression of Pael-R toxicity, which is accompanied by a dramatic reduction of overall Pael-R protein level, immunohistochemical analysis showed that α-Syn level is comparable in transgenic flies with or without Parkin coexpression (Figures 8C and 8D). This is further confirmed by Western blot analysis (Figure 8E). Detailed examination of α-Syn distribution in DA neurons in the DM clusters revealed that while α-Syn formed numerous grain-like structures in the processes, there was a marked reduction of such structures in Parkin-coexpressing flies (Figures 8F and 8G). Although it is not known whether all of these grain-like structures represent pathological forms of α-Syn, the suppression of α-Syn toxicity and concomitant reduction of these structures by Parkin coexpression suggest that at least some of them correspond to toxic forms of α-Syn. To further characterize the effect of Parkin overexpression on neuritic pathology induced by α-Syn, we stained α-Syn transgenic fly brain with ubiquitin antibody, which can recognize Lewy bodies and Lewy neurites in human PD patient brain. In *Ddc-GAL4* driven α-Syn transgenic fly brain, numerous ubiquitin-positive neurites could be detected. In Parkin-coexpressing flies, however, such abnormal neurites were almost completely eliminated (Figures 8H and 8I). Therefore, mitigation of α-Syn toxicity by Parkin overexppression is associated with reduced α-Syn-induced neuritic pathology and reduced aggregation of α-Syn.

Discussion

The underlying molecules and cellular pathways that mediate neuronal death in most human neurodegenerative diseases remain poorly defined. Modeling of these diseases in experimental organisms offers the power of genetic analysis to help understand the pathogenic processes. By expressing the human Parkin substrate protein Pael-R in *Drosophila*, we have developed a fly model of DA neuron degeneration in AR-JP. In addition to providing the first in vivo evidence that accumulation of Pael-R causes neurotoxicity and may contribute to DA neuronal death in AR-JP, our study has provided the following new insights into the mechanisms of neurodegeneration in PD. First, our panneuronal and targeted expression studies showed that DA neurons are particularly susceptible to Pael-R toxicity, thus providing new clues on cell type specificity of neurodegeneration in PD. Second, the effect of Parkin overexpression on α-Syn-induced neuritic pathology indicates that the main site of action for both proteins is in neuronal processes. Third, the suppression of neurotoxicity induced

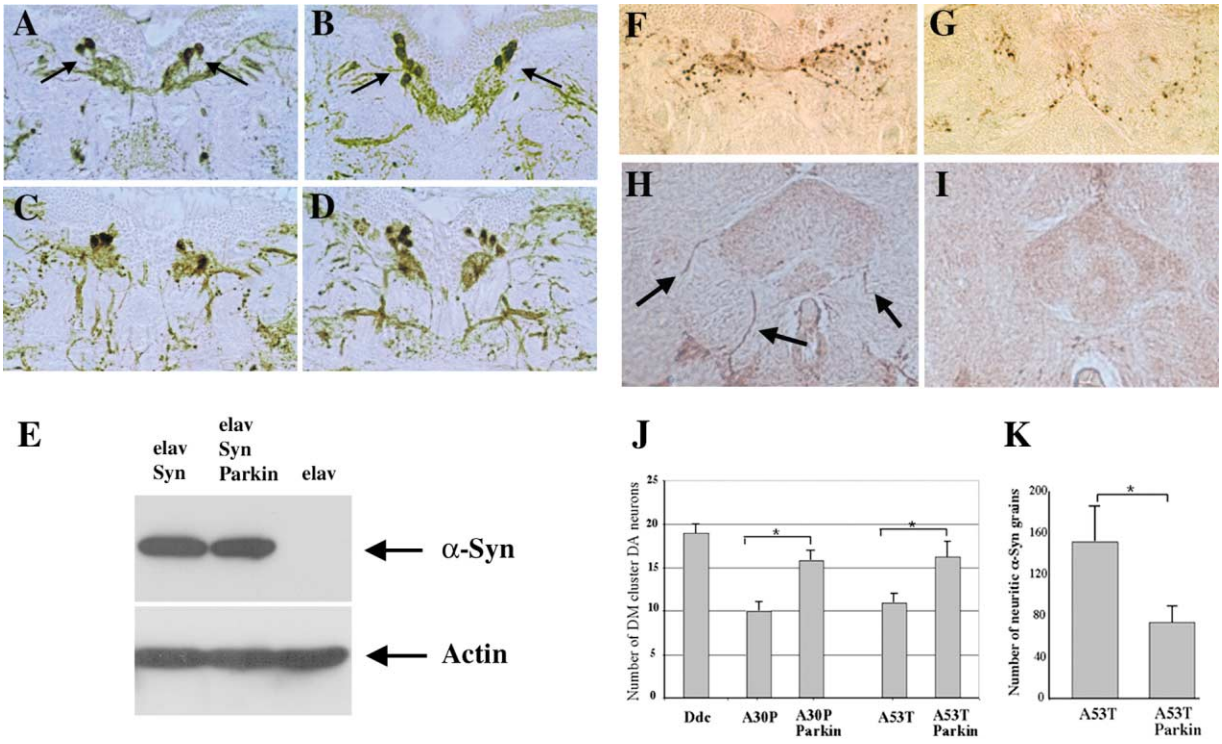

Figure 8. Suppression of α-Syn Toxicity by the Coexpression of Parkin

(A and B) TH immunostaining of frontal sections of 20-day-old fly brain expressing α-Syn alone (A) or coexpressing α-Syn and Parkin (B) under the Ddc-GAL4 driver control. Arrows mark the DM clusters of DA neurons.

(C and D) Immunostaining of total α-Syn protein in 20-day-old *Ddc-GAL4/+; UAS-α-Syn /+* (C) or *Ddc-GAL4/+; UAS-α-Syn /UAS-hParkin* (D) transgenic fly brain.

(E) Western blot analysis showing that Parkin coexpression does not cause a significant change in total α-Syn protein level. Head extracts made from 20-day-old *elav-GAL4* control flies (elav), or *elav-GAL4/+; UAS-α-Syn /+* (elav Syn), and *elav-GAL4/+; UAS-α-Syn /UAS-hParkin* (elav Syn Parkin) transgenic flies were probed with anti-α-Syn antibody (LB509) and then reprobed with anti-Actin as loading control.

(F and G) Immunostaining of grain-like structures in dopaminergic neurites in the area of the DM clusters in 10-day-old *Ddc-GAL4/+; UAS-α-Syn /+* (F) or *Ddc-GAL4/+; UAS-α-Syn /UAS-hParkin* (G) transgenic flies.

(H and I) Anti-ubiquitin staining of 10-day-old *Ddc-GAL4/+; UAS-α-Syn /+* (H) or *Ddc-GAL4/+; UAS-α-Syn /UAS-hParkin* (I) transgenic flies. Arrows in (H) mark ubiquitin-positive neurites.

(J and K) Bar graphs showing quantitative analysis of DA neuron number in the DM clusters in 20-day-old α-Syn transgenic flies with or without Parkin coexpression (J) or the number of α-Syn-positive grain-like neuritic structures (K) in 10-day-old α-Syn transgenic flies with or without Parkin coexpression. Asterisks indicate the differences in DA neuron number or the number of grain-like structure between the compared genotypes are statistically significant ($p < 0.01$, Student's t test).

by two different proteins by Parkin suggests that Parkin may play a central role in the pathobiology of different forms of PD.

A General Role for Parkin in Cellular Quality Control and Its Therapeutic Potential

The ability of overexpressed Parkin to suppress both Pael-R and α-Syn toxicity suggests that despite the difference in pathological manifestation between the recessive and dominant forms of PD, there is a common pathway of PD pathogenesis in which Parkin is a central player. The observation that Parkin is localized to Lewy bodies is consistent with this notion (Shimura et al., 2001; Schlossmacher et al., 2002). It is possible that Parkin is actively engaged in degrading abnormal α-Syn proteins in Lewy bodies. It is also possible that Parkin may be sequestered by α-Syn into inactive complexes, similar to the sequestration of transcription factor CBP by polyglutamine-repeat proteins into nuclear inclusions in Huntington's disease (Nucifora et al., 2001). This may interfere with Parkin's normal function in the ubiquitin/proteasome pathway and contribute to α-Syn toxicity. The latter scenario is consistent with our observation of accumulated ubiquitin immunostaining in α-Syn transgenic neurites and its disappearance after Parkin overexpression. A recent report showed that overexpression of mutant α-Syn protein increases the sensitivity to proteasome inhibitors by decreasing proteasome function in mammalian cell culture, an effect that can be suppressed by overexpression of Parkin (Petrucelli et al., 2002). This is also consistent with the latter possibility. However, our observation of reduced neuritic α-Syn aggregates in Parkin-coexpressing flies is also consistent with the former possibility that Parkin directly acts on abnormal forms of α-Syn, which may constitute only a minor portion of total α-Syn protein in the cell. It is likely that both mechanisms may be involved in the disease process.

Previous studies have shown that in response to unfolded protein response (UPR), Parkin becomes upregu-

lated at both the mRNA and protein level and that overexpression of Parkin can suppress UPR-induced cell death in cell culture models (Imai et al., 2000). Thus, Parkin may play a more general role in cellular quality control to protect neurons from unfolded protein-induced stress. It is therefore conceivable that overexpression of Parkin could also suppress neurotoxicity induced by unfolded proteins other than Pael-R and α-Syn. Given that overexpression of Parkin does not have obvious detrimental effects on the animal, manipulation of Parkin expression with small molecules may provide an effective strategy for PD therapy.

Contributions by the Ubiquitin-Proteasome Pathway and Molecular Chaperones in Removing Misfolded Proteins in Neurodegenerative Diseases

Our current and previous studies place AR-JP into the group of neurodegenerative disorders characterized by the accumulation and aggregation of aberrant forms of proteins. This includes Amyotrophic Lateral Sclerosis, Alzheimer's disease, Huntington's disease, the late onset dominantly inherited forms of PD, and spongiform encephalopathies (Sherman and Goldberg, 2001). In these various forms of neurodegenerative diseases, specific groups of neurons undergo degeneration as a result of the aggregation of distinct misfolded proteins. These proteins may cause cellular toxicity by affecting different aspects of neuronal physiology, from axonal transport (Gunawardena and Goldstein, 2001) and synaptogenesis (Walsh et al., 2002) to gene expression in the nucleus (Nucifora et al., 2001). In the case of Pael-R, abnormal forms of the protein may cause cellular toxicity through eliciting ER stress (Imai et al., 2001). Components of the ubiquitin-proteasome degradation pathway and molecular chaperones have been shown to associate with inclusions formed by these different proteins (Sherman and Goldberg, 2001). This may reflect the cell's strategy to eliminate these misfolded proteins by repairing/refolding them with the molecular chaperones or degrading them through the ubiquitin-proteasome system.

The importance of the ubiquitin-proteasome system in neurodegeneration is highlighted by the association of familial forms of PD with mutations in Parkin and ubiquitin carboxy-terminal hydrolase L1 (UCHL-1), another enzyme involved in ubiquitin metabolism (Leroy et al., 1998), and the acceleration of SCA1 phenotype by loss-of-function of an E3 ubiquitin ligase in mice (Cummings et al., 1999). Hsp70 molecular chaperones have been implicated in disease processes by whole animal studies in *Drosophila*, which showed that directed overexpression of Hsp70 attenuates whereas interference with endogenous chaperone activity exacerbates neurotoxicity associated with polyglutamine repeat-containing proteins and α-Syn (Warrick et al., 1999; Auluck et al., 2002). It remains to be determined whether Hsp70 molecular chaperones play significant roles in removing misfolded Pael-R protein. The fate of abnormal Pael-R protein depends on the relative kinetics of its interactions with the molecular chaperone pathway and the proteasome pathway (Imai et al., 2002). Unlike α-Syn, a small cytosolic protein, Pael-R is a large 7 transmembrane protein, the misfolded forms of which reside mainly in the ER. The Hsp70 class of molecular chaperones may have limited access to misfolded Pael-R or have limited ability to renature misfolded Pael-R proteins, and therefore the Parkin-mediated proteosome pathway may play a more dominant role in clearing them. Alternatively, other molecular chaperones may play more prominent roles in regulating the fate of misfolded Pael-R. One good candidate is the ER resident molecular chaperone BiP (GRP78), which associates with unfolded proteins in the ER and has been shown to be upregulated during ER stress and in AR-JP patient brain (Imai et al., 2000, 2001).

On the Selectivity of DA Neuronal Death in AR-JP

One of the most intriguing features of neurodegenerative diseases is the cell type specificity of neuronal death. Previous studies suggested that the restricted tissue distribution of Pael-R might contribute to the selective degeneration of DA neurons in AR-JP (Imai et al., 2001). It was shown that Pael-R protein is widely expressed in the mouse brain, predominantly in oligodendrocytes. Its neuronal expression is restricted to a subset of neurons such as DA neurons in the substantia nigra and hippocampal neurons in the CA3 region. While the restricted tissue distribution of Pael-R may partially explain the selectivity of neuronal degeneration in AR-JP, this study shows that there are some features specific to the DA neurons that contribute to their particular vulnerability to Pael-R toxicity. It is possible that this selective vulnerability is due to a smaller capacity of DA neurons to handle Pael-R-induced ER stress. It is also possible that certain DA neuron-specific cofactors may contribute to Pael-R toxicity. Two risk factors, oxidative stress and dopamine, have been suggested to contribute to the selective toxicity of α-Syn toward DA neurons. DA neurons in the substantia nigra are known to produce excess reactive oxygen species such as superoxide anion, which in reaction with nitric oxide (NO) can generate the more potent oxidant peroxynitrite and cause damages to proteins and other macromolecules (Giasson et al., 2000). It was also shown that dopamine can be ligated to α-Syn to form dopamine-α-Syn adducts (Conway et al., 2001). This adduct selectively inhibits the protofibril-to-fibril conversion and causes the accumulation of α-Syn protofibrils, which are the presumed toxic species. Future studies will test whether Pael-R is also modified by oxidative stress and dopamine and whether these modifications contribute to its toxicity.

Many studies have shown that there is unanticipated conservation of signaling pathways, regulatory mechanisms, and cellular and physiological processes between flies and humans. Our identification of Parkin as an important modulator of Pael-R and α-Syn toxicity suggests that we could use the fly models to further understand the role of Parkin and its substrates in the pathogenesis of PD. The ability to perform facile genetic loss-of-function and overexpression screens in this system will allow us to identify new genes that can either attenuate or exacerbate disease phenotypes. Such studies will allow us to delineate the molecular pathways involved in the pathogenesis of PD and possibly other related neurodegenerative diseases and identify potential therapeutic drug targets.

Experimental Procedures

Fly Stocks

All general fly stocks and *GAL4* lines were obtained from the Bloomington *Drosophila* stock center. Flies were grown under standard conditions at 25°C. The w^- strain was used to generate transgenic flies. The *UAS-hsp70* and the *UAS-α-Syn* lines were provided by Dr. Nancy Bonini (Warrick et al., 1998; Auluck et al., 2002). The *Ddc-GAL4* line was provided by Dr. Jay Hirsh (Li et al., 2000).

Constructs and Transgenics

To construct *UAS-hParkin* and *UAS-Pael-R* plasmids, the full-length cDNAs were released from *pcDNA3.1-hParkin* and *pcDNA3.1-Pael-R* plasmids with *EcoRI/XbaI* and *EcoRI/XhoI* double digests, respectively (Imai et al., 2001). The inserts were subsequently cloned into corresponding double digested *pUAST* vectors.

The *Drosophila parkin* homolog *dParkin* was amplified from a third instar larval cDNA library by polymerase chain reaction (PCR). The Advantage HF-2 PCR kit (Clontech) was used for PCR. The PCR product was cloned into *pCR2.1-TOPO* vector using the TOPO PCR Cloning Kit (Invitrogen). Two plasmids in which the *dParkin* PCR product was inserted in opposite orientations were chosen for constructing inverted repeats of *dParkin* (*IRdParkin*). Detailed cloning procedures for constructing *UAS-IRdParkin* can be found in the Supplemental Data at http://www.neuron.org/cgi/content/full/37/6/911/DC1.

To generate *UAS-Pael-R*, *-hParkin*, and *-IRdParkin* transgenic flies, 9 μg of *pUAST* transgenic plasmid was mixed with 3 μg of helper plasmid in 20 μl injection buffer (0.1 mM Na Phosphate [pH 7.8], 5 mM KCl). Standard procedures were followed for embryo injection and recovery of transgenic lines.

RT-PCR

Second instar larvae from a *UAS-IRdParkin* and *hs-GAL4* cross were heat shocked at 37°C for 2 hr and allowed to recover at room temperature for 2 hr. Total RNAs were prepared from heat shock treated and untreated control larvae using an RNeasy Kit (Qiagene). Details of the quantitative RT/PCR procedure can be found in the Supplemental Data online.

Neuronal Culture

Drosophila neuronal cultures were established using a protocol adopted from that of Huff et al. (1989). For immunofluorescence, neuronal cultures were fixed in 4% formaldehyde/PBS at 4°C for 20 min and rinsed for 4 × 5 min at room temperature. After blocking with 5% normal serum at room temperature for 30 min, they were incubated with primary antibodies at 4°C overnight. After washing for 6 × 10 min at room temperature, they were incubated with Cy2- or Cy3-conjugated secondary antibodies. The primary antibodies used were mouse monoclonal antibody against Pael-R (1:10,000; Imai et al., 2001), rabbit antibody against Pael-R (1:6000; Imai et al., 2001), rabbit anti-hParkin (1:400; Imai et al., 2001), rabbit anti-Synaptotagmin (1:1000; gift of H. Bellen), and guinea pig anti-Numb (1:1000; gift of Y.N. Jan). The secondary antibodies (Jackson Laboratories) used were Cy2-goat anti-rabbit (1:500), Cy3-goat anti-rabbit (1:1000), Cy2-goat anti-mouse (1:500), Cy3-goat anti-mouse (1:1000), and Cy2-goat anti-guinea pig (1:500).

Adult Brain Histology and Immunohistochemistry

Sections of paraplast-embedded adult fly heads were prepared and processed as described (Feany and Bender, 2000). The sections were incubated in primary antibody overnight at 4°C and subsequently processed using the Vectastain Universal Elite ABC Kit (Vector Laboratories). In the final step, the DAB Substrate Kit for Peroxidase (Vector Laboratories) was used. The primary antibodies used were anti-tyrosine hydroxylase polyclonal antibody (Pel Freeze, 1:100), 22C10 monoclonal antibody (Hybridoma Bank, 1:1000), rabbit anti-5HT (Chemicon, 1:500), 4B1 monoclonal antibody against *Drosophila* Choline Acetyltransferase (gift of Paul Salvaterra, 1:30,000), and mouse anti-α-Syn LB509 (Zymed). To stain total α-Syn protein, LB509 was used at 1:500 dilution. To preferentially stain aggregated form of α-Syn, LB509 was used at 1:5000 dilution. For the analysis of adult retina, eye sectioning and staining with toluidine blue was performed as described (Wolff, 2000). Between five and ten fly heads for each genotype per time point were examined and each experiment was repeated at least once.

Western Blot Analysis

To analyze Pael-R and α-Syn protein levels with or without Parkin coexpression, *UAS-Pael-R* or *UAS-α-Syn* transgenic male flies were first crossed to *elav-Gal4* females. F1 male progenies were crossed to w^- or *UAS-hParkin* transgenic females. Female flies resulting from above crosses were used to make head extracts for Western blot analysis. Half of these females express Pael-R or α-Syn proteins. Typically extracts made from five to ten fly heads were separated by SDS-PAGE and probed with mouse anti-Pael-R (1:1000) or LB509 anti-Synuclein (1:500). Antibody detection was done using the Amersham ECL system. To control for equal loading, the blots were stripped and reprobed with mouse anti-Actin (1:5000). In control experiments, we demonstrated that added expression of a control UAS-GFP transgene has no effect on the level of expression of Pael-R or α-Syn (see Supplemental Figure S5 at http://www.neuron.org/cgi/content/full/37/6/911/DC1).

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