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Glutathione S-transferase omega suppresses the defective phenotypes caused by *PINK1* loss-of-function in *Drosophila*

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ABSTRACT

Loss-of-function mutation of the *PTEN-induced kinase 1* (*PINK1*) gene is a common cause of early-onset Parkinson's disease (PD). Glutathione S-transferase omega (GSTO) is a phase II detoxification enzyme that conjugates targets to glutathione, and has recently been implicated in *parkin*-associated PD. In this study, we found *Drosophila* GstO2 to be a novel genetic suppressor of the *PINK1* loss-of-function mutant. We show that GstO2A expression is reduced in *PINK1* mutants. Moreover, the upregulation of GstO2A restores muscle degeneration and dopaminergic neuron loss in *PINK1* mutants. Given the previous data of a reduced expression of GstO2A and decreased glutathionylation of ATP synthase β subunit in *parkin* or *PINK1* mutants, these results suggest that the function of GstO2 is regulated by the PINK1/*parkin* pathway and that GstO2 also has a protective role in *PINK1*-associated PD.

1. Introduction

Glutathione S-transferase omega (GSTO), the most recently identified member of the glutathione S-transferase (GST) family, is a phase II detoxification enzyme that is responsible for conjugating an electrophilic substrate with glutathione (GSH). The GSTO enzymes have a N-terminal thioredoxin-like domain, as well as a cysteine residue in their active site at the N-terminus that binds to GSH [1]. The GSTOs show thiol transferase, dehydroascorbate reductase, and monomethyl arsenate reductase activities [2]. Recently, the roles of the GSTO enzymes have been investigated using *in vitro* assays. The human GSTOs have been associated with the modulation of the ryanodine receptor and activation of interleukin-1β [3,4]. Polymorphisms in the human GSTO1 gene have been associated with the risks of breast cancer and ovarian cancer [5–7]. In addition, genetic variations in human GSTOs were reported to be associated with the age at the onset of Alzheimer's disease and Parkinson's disease (PD) [8].

PD is the second most prevalent neurodegenerative disease, characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta and striatum, but the mechanism of its pathogenesis remains unknown [9,10]. Although PD is mostly a sporadic disorder, several genes known to be responsible for PD have been found in many patients afflicted with this disease. So far, 7 genes (*α-synuclein*, *UCH-L1*, *LRRK2*, *DJ-1*, *parkin*, *PINK1*, and *ATP13A2*) have been identified as pathological candidate genes for PD [11–18]. Among these genes, mutations in *parkin* (which encodes an E3-ubiquitin ligase) and *PINK1* (which encodes a serine/threonine kinase) cause early-onset autosomal recessive Parkinsonism. Previous researches have revealed that *parkin* and *PINK1* act in a common pathway that maintains mitochondrial function and integrity [19–21]. The overexpression of parkin significantly rescued all of the defective phenotypes in *PINK1* mutants. Consistent with these findings in *Drosophila*, animals with *parkin* or *PINK1* mutations also show mitochondrial defects [22,23]. Recent studies suggested that the *parkin* and *PINK1* genes regulate the mitochondrial remodeling mechanism [24–26]. Thus, these results suggest that mitochondrial dysfunction is a major cause of PD pathogenesis.

In a previous research, we reported that the upregulation of GstO2A significantly suppresses the defective phenotypes in *park*^{1} mutants by restoring ATP synthase (complex V) activity [27]. Furthermore, consistent with *parkin* mutants, we have shown that *GstO2A* and *parkin* mRNAs are decreased in *PINK1* mutants, and the glutathionylation of the ATP synthase β subunit, which is a catalytic core component of ATP synthase in mitochondria, is dramatically decreased in *PINK1* mutants [27]. In addition, the loss of *parkin* or *PINK1* mutants displays a defective assembly in the ATP synthase complex, which leads to impaired mitochondrial function [27,28]. Therefore, it seems possible that upregulation of GstO2A contributes to the protection of neurodegeneration in *PINK1* loss-of-function mutants.

In the present study, we investigated the physiological role of GstO2A in *PINK1* mutants, which is one of the models of PD.

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2. Materials and methods

2.1. Fly stocks

Flies were grown on standard food condition at 25 °C. The UAS-*GstO2A* transgenic line used in this study has been previously described [27]. The *PINK1* mutant line, *PINK*^{B9}, was a kind gift from J. Chung (Seoul National University) [20]. The *TH*-Gal4 driver line was a gift from S. Birman (CNRS-Université de la Méditerranée) [29]. The *mef*-Gal4 fly line was obtained from the Bloomington Stock Center. All fly experiments were carried out at 25 °C.

2.2. Semi-quantitative RT-PCR

Total RNA from the flies was extracted with a Trizol reagent (Invitrogen), and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega). For the semi-quantitative RT-PCR, the following *dPINK1* and *GstO2A* primers were used: dPINK1-For (TTC TGC CAC CAC CGC CCC CAC ACT TC), dPINK1-Rev (CCG CAG CAC ATT GGC AGC GGT GG) [27], GstO2A-For (CAT ATG GCC CTG CCG CAA AAG CAC T) and GstO2A-Rev (CTC GAG CTA TGG TGT ACC CTT GAA GGC AAT GTC) [30].

2.3. Western blot analysis

Protein extracts for western blot analysis were prepared by homogenizing ten 3-day-old male flies. The total protein extracts were separated by 10% SDS–PAGE and transferred to PVDF membranes (Millipore). The membranes were incubated for 1 h in a blocking solution (Tris-buffered saline with 4% bovine serum albumin or non-fat dry milk) and immunoblotted with rabbit anti-GstO2A antibody (1:1000) [27] or mouse anti-β-tubulin antibody (1:3000; Sigma–Aldrich). Detection was carried out by using an ECL-Plus kit (Amersham).

2.4. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay

Apoptosis in the flight muscles of 3-day-old male flies was detected using the *In Situ* Cell Death Detection Kit (Roche). The fly thoraces were fixed in 4% formaldehyde in PBS for 20 min at 25 °C. For permeation, samples were incubated in 0.5% Triton X-100 in PBS for 5 min. The thorax muscles were dissected and subjected to TUNEL analysis according to the procedure in standard manuals.

2.5. Immunohistochemistry

Adult fly brains for whole-mount immunostaining were dissected from 20-day-old flies and fixed with 4% formaldehyde in a fixative buffer. The brains were then stained overnight at 4 °C with rabbit anti-tyrosine hydroxylase (TH) antibody (1:100; Pel-Freeze) and mouse anti-TH antibody (1:100; Immunostar). Then, the samples were incubated with Cy3-conjugated secondary antibodies (1:200; Jackson Immunoresearch). The number of DA neurons was counted using a DE/LSM510 NLO Carl Zeiss confocal microscope.

3. Results and discussion

3.1. GstO2A expression is decreased in PINK1 loss-of-function mutant flies

The loss of 2 genes, *parkin* and *PINK1*, results in early-onset autosomal recessive Parkinsonism [12,14]. *PINK1* or *parkin* mutant flies show morphological defects caused by mitochondrial dysfunction [19,20,31]. We have previously investigated the mRNA expression level of *Drosophila* GSTOs in *parkin* mutant flies. Of the 4 *GSTO* genes (*sepia*, *GstO1*, *GstO2*, and *GstO3*) in *Drosophila melanogaster* [30,32], we found the expression of *GstO2A* mRNA was decreased in the *park*^{1} mutant [27]. To determine whether *GstO2A* gene expression is also decreased in *PINK1*^{B9} mutant flies, we examined its mRNA expression by semi-quantitative RT-PCR analyses, using total RNA extracted from adult thoraces. Consistent with the *park*^{1} mutants, *GstO2A* mRNA expression was decreased in the *PINK1*^{B9} mutants (Fig. 1A), while *Drosophila PINK1* (*dPINK1*) mRNA expression was completely eliminated (Fig. 1A). The results of immunoblot analysis also confirmed that the level of GstO2A protein expression was decreased in the *PINK1*^{B9} mutant (Fig. 1B). These results suggested that *GstO2A* gene expression is regulated by the PINK/parkin pathway.

3.2. GstO2A suppresses the morphological defects in PINK1^{B9} mutants

The loss of *PINK1* and *parkin* functions in *Drosophila* results in similar phenotypes, with muscle degeneration and DA neuron loss [19,20,31,33]. Furthermore, in recent *Drosophila* genetic studies, the overexpression of parkin highly suppressed the phenotypes of *PINK1* mutants. However, the phenotypes of *parkin* mutants could not be rescued by the overexpression of PINK1 [19,20]. These results suggest that PINK1 acts upstream of parkin, in a common pathway that maintains mitochondrial function. We have recently shown that the upregulation of GstO2A rescues the defective phenotypes of *park*^{1} mutants in *Drosophila*, by regulating mitochondrial function. Thus, we hypothesized that GstO2A may have a protective function in *PINK1* null mutants. To clarify the effect of GstO2A on *PINK1*^{B9} mutants, we conducted a genetic interaction study with *PINK1*^{B9} mutants. The *PINK1*^{B9} mutants show collapsed thorax phenotypes (Fig. 2B and D). Upregulation of GstO2A under the control of a muscle-specific *mef-Gal4* driver significantly restored the collapsed thorax phenotypes (Fig. 2C and D). Thus, GstO2A expression suppressed the morphological defects in both *parkin* and *PINK1* mutants.

3.3. GstO2A restores apoptotic cell death in the flight muscles of PINK1^{B9} mutants

Histological analysis of the flight muscles in *PINK1* mutants revealed a severe defect of muscle integrity [19,20]. The degeneration of thorax muscles in *PINK1*^{B9} mutants occurs through apoptotic cell death. We examined whether GstO2A upregulation could restore the apoptosis in the thorax muscles of *PINK1*^{B9} mutants, by subjecting the muscles to a TUNEL assay. As shown in

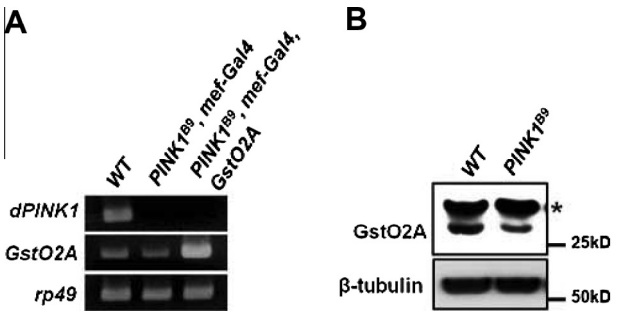

Fig. 1. Expression of *GstO2A* in *Drosophila PINK1*^{B9} mutant flies. (A) *GstO2A* mRNA expression in *PINK1*^{B9} mutants. The amount of *GstO2A* mRNA was visualized by qRT-PCR. *rp49* was used as a loading control. (B) Western blot analysis of GstO2A in wild-type and *PINK1*^{B9} mutant flies (*nonspecific band). *β*-Tubulin was used as a loading control.

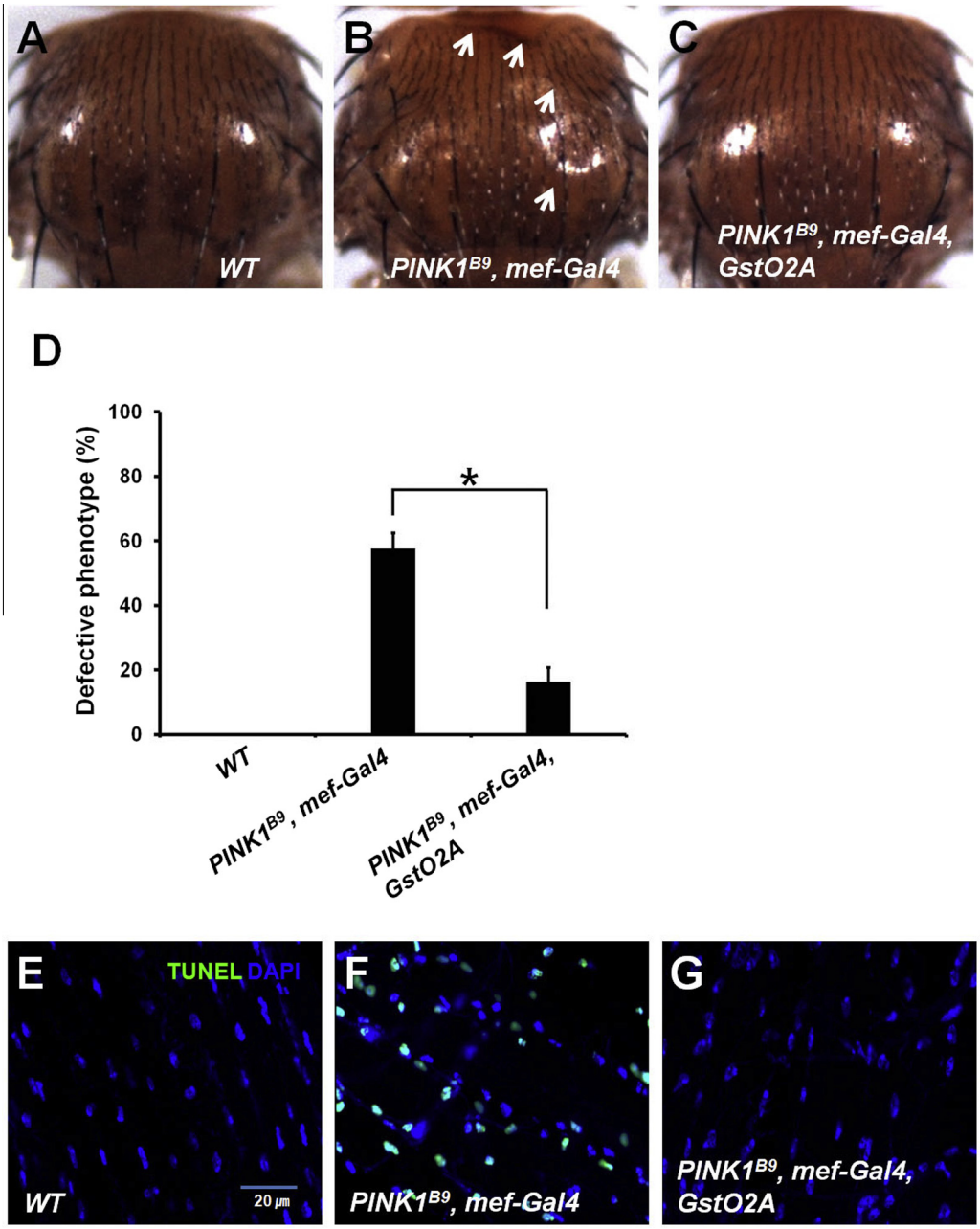

Fig. 2. Transgenic expression of GstO2A rescues the collapsed thorax defect in *PINK1*^{B9} mutant flies. (A–C) Upregulation of GstO2A by the *mef-Gal4* muscle-specific driver suppressed the collapsed thorax phenotypes of *PINK1*^{B9} mutant flies. (A) *Wild type*. (B) *PINK1*^{B9}, *mef-Gal4*. (C) *PINK1*^{B9}, *mef-Gal4*; *UAS-GstO2A*. (D) Statistical analysis of the percentage of collapsed thorax phenotypes in 3-day-old flies ($n > 140$ for each genotype). Error bars represent means ± S.D. of 3 independent experiments. The experimental significance was determined by one-way ANOVA (*$p < 0.001$). (E–G) The increased apoptotic signal in *PINK1*^{B9} mutant flies is suppressed by GstO2A expression. Merged images of apoptotic cells (TUNEL, green) and nuclei (DAPI, blue). (E) *Wild type*. (F) *PINK1*^{B9}, *mef-Gal4*. (G) *PINK1*^{B9}, *mef-Gal4*; *UAS-GstO2A*.

Fig. 2E–G, TUNEL-positive signals were ubiquitously detected in *PINK1*^{B9} mutant muscles. Interestingly, the increased TUNEL-positive signal was suppressed by GstO2A expression using the *mef-Gal4* driver (Fig. 2G). This indicates that GstO2A has a protective effect in the apoptotic muscle degeneration of *PINK1* mutants.

3.4. GstO2A rescues the dopaminergic neuron loss in *PINK1*^{B9} mutants

The loss of DA neurons is a major hallmark of PD. An age-dependent decrease in the number of DA neurons has been reported in *parkin* and *PINK1* mutants [20,21,31,33]. We recently showed that GstO2A was able to suppress the DA neuron loss in *park*^{1} mutants [27]. Therefore, to confirm the role of GstO2A in DA neuron protection, we examined and counted the number of DA neurons in the PPL1 cluster of *PINK1*^{B9} mutants. The 20-day-old adult fly brains were dissected and whole-mount immunohistochemistry was performed with anti-TH antibody. The number of DA neurons in the cluster of *PINK1*^{B9} mutants was not changed in 1-day-old flies. However, in 20-day-old adults, the *PINK1* mutants exhibited a decrease in the number of DA neurons in a specific cluster of the brain, compared with the wild-type controls. Protocerebral posterior lateral 1 (PPL1) clusters in the fly brain usually contain about 12∼14 DA neurons in wild-type flies (Fig. 3A and D). The *PINK1*^{B9} mutants displayed 20% decline in DA neurons of the PPL1 cluster (Fig. 3B and D). When the DA neuron-specific diver *TH-Gal4* was used to drive the GstO2A expression in the *PINK1*^{B9}

mutant background, the expression of GstO2A significantly suppressed the loss of DA neurons in the *PINK1*^{B9} mutants (Fig. 3C and D). These results suggest that GstO2A contributes to the rescue of DA neuron loss induced by *PINK1* loss-of-function.

3.5. A simple model for the GstO2-mediated restoration of mitochondrial dysfunction in PINK1/parkin loss-of-function

We used *Drosophila* as a model system to investigate the genetic modulators for PD and identified GSTO as a novel genetic suppressor of parkin dysfunction [27]. We showed that altering the level of mitochondrial ATP synthase β subunit glutathionylation by GstO2A in *parkin* mutants can regulate the efficiency of mitochondrial ATP synthase complex assembly [27]. Loss-of-function *PINK1* mutants display a defective assembly in the ATP synthase complex [28]. Furthermore, we found that the levels of the glutathionylated form of the ATP synthase β subunit were decreased in *PINK1* null mutant flies, *PINK1*^{B9} [27]. Therefore, we investigated the relationship between GstO2A and PINK1, and found that upregulation of GstO2A is able to restore *PINK1* mutant phenotypes, including the rescue of indirect fight muscle degeneration and DA neuron loss in *Drosophila* (Figs. 2 and 3). These results indicate that an increase in GstO2A activity is beneficial for protecting neurogeneration in the *PINK1*/*parkin* mutant.

From these results, we propose a simple model for the GstO2-mediated restoration of mitochondrial dysfunction in PINK/parkin loss-of-function (Fig. 4). The proposed model shows the relationship between GstO2A activity and mitochondrial function. Parkin acts downstream of PINK1 in a common pathway to regulate mitochondrial function [19–21]. The expressions of *GstO2A* mRNA and protein are dramatically decreased in *PINK1* or *parkin* mutants (Fig. 1) [27]. In addition, the upregulation of GstO2A alleviates the defective phenotypes in *PINK1* or *parkin* null flies, by regulating the mitochondrial ATP synthase activity. Therefore, it seems likely that the upregulation of GstO2A restores *PINK1* mutant phenotypes in a similar manner that it rescues *parkin* mutant phenotypes. However, because the *GstO2* mutants do not exhibit the *parkin* mutant-like phenotypes [27], we think there are other gene(s) that regulate the activity of ATP synthase complex. Although the exact mechanism is not clear, the restoration of mitochondrial ATP synthase activity by GstO2A expression is critically important for partial restoration of the mitochondrial function in PINK1/parkin-related PD.

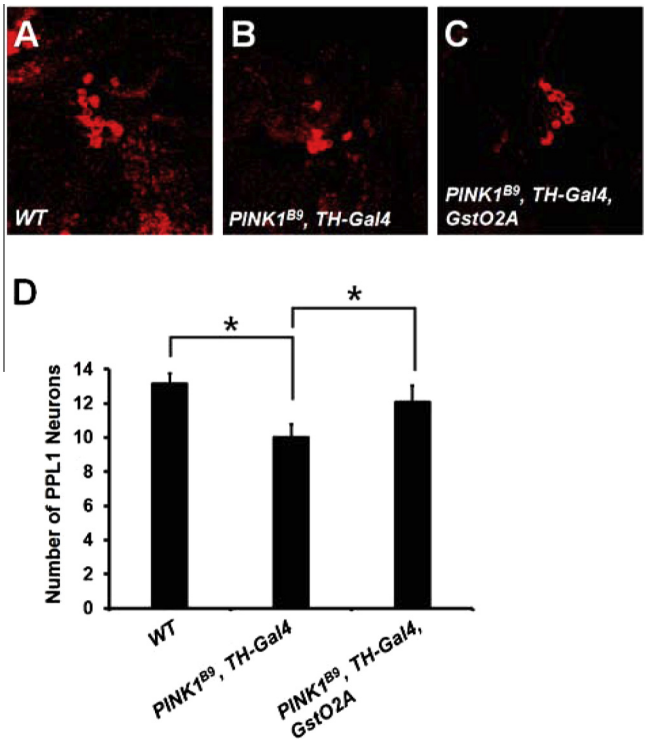

Fig. 3. GstO2A restores the dopaminergic (DA) neuronal loss in *PINK1*^{B9} mutant flies. (A–C) Whole-mount adult brains were immunostained with anti-tyrosine hydroxylase (TH) antibody. (A) *Wild-type*. (B) *PINK1*^{B9}; *TH-Gal4*. (C) *PINK1*^{B9}; *TH-Gal4*, *UAS-GstO2A*. (D) Quantification of the TH-positive neuron number in the PPL1 clusters in 20-day-old flies ($n > 10$ for each genotype). Upregulation of GstO2A improves the degeneration of DA neurons caused by *PINK1* loss of function. Error bars represent means ± S.D. of 3 independent experiments. The significance was determined by one-way ANOVA (*$p < 0.001$).

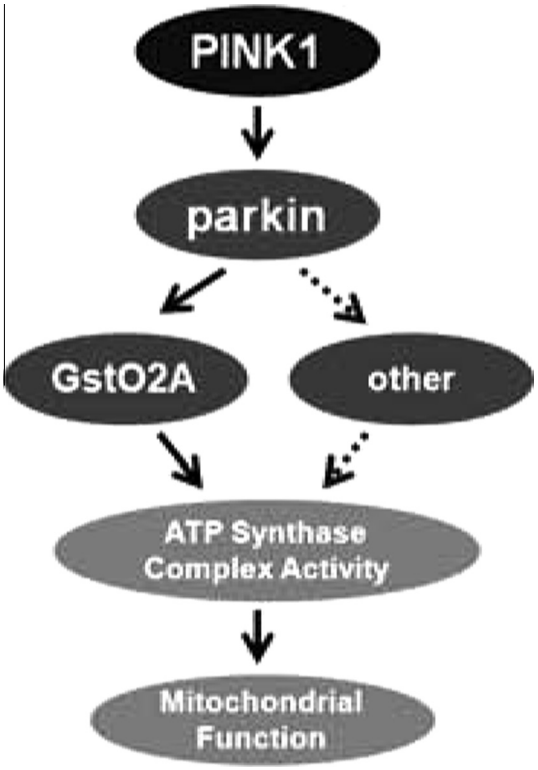

Fig. 4. Proposed model of how *GstO2A* relates to *PINK1*/*parkin* pathway in *Drosophila*. Because the defective phenotypes of the *PINK1* mutant are partially rescued by GstO2A expression, other possible gene(s) that act in the same manner as *GstO2A* may exist.

4. Conclusions

These findings support the possibility that GstO2 is linked to the pathogenesis of PINK1/parkin-associated PD. Furthermore, our results suggest that GstO2A may mediate the control of mitochondrial homeostasis through the PINK1/parkin pathway. Our findings on the GstO2-associated neuroprotection may lead to a deeper understanding of the protection mechanism due to GSTs in PD and help in the development of new therapeutic targets for this neurodegenerative disease.

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