EGCG ameliorates neuronal and behavioral defects by remodeling gut microbiota and TotM expression in Drosophila models of Parkinson’s disease

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Abstract
Parkinson’s disease (PD) is the second most common neurodegenerative disease. Epigallocatechin-3-gallate (EGCG), the major polyphenol in green tea, is known to exert a beneficial effect on PD patients. Although some mechanisms were suggested to underlie this intervention, it remains unknown if the EGCG-mediated protection was achieved by remodeling gut microbiota. In the present study, 0.1 mM or 0.5 mM EGCG was administered to the Drosophila melanogaster with PINK1 (PTEN induced putative kinase 1) mutations, a prototype PD model, and their behavioral performances, as well as neuronal/mitochondrial morphology (only for 0.5 mM EGCG treatment) were determined. According to the results, the mutant PINK1^B^9 flies exhibited dopaminergic, survival, and behavioral deficits, which were rescued by EGCG supplementation. Meanwhile, EGCG resulted in profound changes in gut microbial compositions in PINK1^B^9 flies, restoring the abundance of a set of bacteria. Notably, EGCG protection was blunted when gut microbiota was disrupted by antibiotics. We further isolated four bacterial strains from fly guts and the supplementation of individual Lactobacillus plantarum or Acetobacter pomorum strain exacerbated the neuronal and behavioral dysfunction of PD flies, which could not be rescued by EGCG. Transcriptomic analysis identified TotM as the central gene responding to EGCG or microbial manipulations. Genetic ablation of TotM blocked the recovery activity of EGCG, suggesting that EGCG-mediated protection warrants TotM. Apart from familial form, EGCG was also potent in improving sporadic PD symptoms induced by rotenone treatment, wherein gut microbiota shared regulatory roles. Together, our results suggest the relevance of the gut microbiota-TotM

Abbreviations: AP, Acetobacter pomorum; APa, Acetobacter pasteurianus; DA, dopaminergic; EGCG, Epigallocatechin-3-gallate; FPKM, fragments per kilobase of transcript sequence per Million base pairs sequenced; GM, Gut microbiota; LB, Lactobacillus brevis; LP, Lactobacillus plantarum; MGB, microbiome-gut-brain; PCoA, principal coordinates analysis; PD, Parkinson’s disease; PINK1, PTEN induced putative kinase 1; PPL1, protocerebral posterior lateral 1; ROS, reactive oxygen species; TotM, Turandot M.

Yi Xu and Mengmeng Xie contributed equally to the work.

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pathway in EGCG-mediated neuroprotection, providing insight into indirect mechanisms underlying nutritional intervention of Parkinson’s disease.

**KEYWORDS**

*Lactobacillus plantarum*, mitochondria, PINK1, rotenone

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**1 | INTRODUCTION**

Parkinson’s disease (PD) is the second most common neurodegenerative disease in humans. PD is generally characterized by dopaminergic neuronal loss in the substantia nigra, abnormal accumulation of alpha-synuclein protein inclusions, and motor dysfunction. Despite extensive interest, the etiology of PD is highly complex and largely unknown, but is believed to associate with both genetic and environmental factors. Among the proposed mechanisms, mitochondrial dysfunction, neuroinflammation, oxidative stress, and impaired autophagy are credited as major pathways of neurodegeneration. Most compellingly, while sporadic forms of PD account for up to 90%-95% of cases, familial PD can be frequently caused by a single mutation of PINK1 (PTEN induced putative kinase 1), a mitochondrial Ser/Thr kinase which acts to maintain healthy mitochondria via regulating mitochondrial dynamics and quality control.

*Drosophila melanogaster* is a model organism essentially applied in genetic research contributing to the understanding of evolutionarily conservative mechanisms. The ease of genetic manipulation in *Drosophila* has sparked the development of numerous models of PD. Among them, mutations of PINK1 or Parkin, an E3 Ubiquitin ligase, are the most prevalent cause of early onset familial PD, as well as, to a lesser extent, the sporadic forms. *PINK1* mutant flies display selective DA neuron degeneration and motor deficits, and likewise exhibit a wide range of non-motor symptoms, including memory deficits and disrupted sleep-wake cycles, allowing it to be a model of choice for intervention studies.

EGCG (epigallocatechin-3-gallate) is a polyphenol of the flavonoid family belonging to the class of falvan-3-ols. As the most abundant bioactive compound of green tea, EGCG receives increasing interest for its anti-oxidative and health-promoting effect. Compelling evidence supports a correlation between EGCG intake and a reduced risk of PD, and its neuroprotective effect has been demonstrated in both in vitro and in vivo PD models, including nonhuman primates. For instance, Ng et al discovered that EGCG acted as a potent suppressor of dopaminergic dysfunction in mutant LRRK2-null flies via the augmented activity of AMPK. In terms of mechanisms underlying the EGCG-mediated neuroprotection, reduced oxidative stress, neuroinflammation, protein aggregation, and mitochondrial dysfunction were previously proposed. EGCG was shown to redirect the aggregation of α-synuclein monomers and remodel synuclein fibrils into disordered oligomers. These findings are promising in explaining the merits of EGCG; however, it might be difficult for EGCG to make direct contact with α-synuclein in vivo. EGCG crosses the blood-brain barrier primarily in the form of epicatechin glucuronide and 3-O-methyl epicatechin glucuronide, with free epicatechin hardly detectable. Therefore, it remains to be understood how EGCG exerts its indirect effect on the dopaminergic neurons. A possible pathway is the in vivo inter-organ communications that could be potentially employed by EGCG, like gut-brain cross talk.

A notion prevails that Parkinson’s disease originates from outside of the central nervous system, which is supported by the finding that intestinal infection acts as a triggering event in disease progression, highlighting the relevance of microbiome-gut-brain (MGB) axis in the disease. MGB refers to a bidirectional communication between gut microbiota (GM)/gut and brain via hormonal, neuronal or immune pathways. According to the epidemiological survey, gastrointestinal symptoms such as colonic inflammation and constipation are found in over 80% of PD cases and generally precede the motor signs for several years. While gut microbiota regulates motor deficits and neuroinflammation in PD models, whether it bridges natural compounds and disease intervention needs to be further clarified.

Growing evidence suggests that EGCG remolds gut microbiota architecture. Remely et al. stated that EGCG interventions resulted in a reduced *Clostridium* spp., increased *Bacteroides*, and an altered propensity of *Bifidobacterium* and *Prevotella* in the murine gut. In another instance, intestinal bacteria accounted for the degrading products of EGCG *bona fide* existing in rat brains. In light of these previous publications, efforts should be made to decipher the roles of gut microbiota in the EGCG-mediated alleviation of PD.

In this study, using the *PINK1* mutant flies as the PD model, we characterized the protective effect of EGCG, roles of gut microbiota and individual strains, global transcript alterations, performances of TotM-knockdown flies, as well as the applicability of the studied pathway in the sporadic PD model. This study provides insight into the mechanisms underlying the EGCG-mediated intervention of Parkinson’s disease.
2 | MATERIALS AND METHODS

2.1 | Fly strains and genetic crosses

Fly stocks and crosses were maintained according to the standard procedures at 25°C. The PINK1-mutant fly stock PINK1B9 (genotype: PINK1B9/FM6; TH-Gal4>mtiGFP/TM2) has been described elsewhere.32,33 This stock, along with w118, Mhc>PINK1-RNAi (genotype: mtGFP(Cyo; Mhc>PINK1-RNAi/TM2) and balancer flies (elav/Y, Cyo/+; TM2/+), were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN, USA). The following flies: UAS-TotMRNAi, UAS-CG4908-RNAi, UAS-mRpL55-RNAi, UAS-MiroRNAi, UAS-P32-RNAi, and UAS-mt:ATPase6-RNAi were purchased from Tsinghua Fly Center (Beijing, China).

The transgenic flies harboring elav-Gal4 element were then obtained by crosses and designated as the elav:PINK1B9 (genotype: PINK1B9/FM6; elav-Gal4/TM2). To generate double-mutant transgenic flies for studying the roles of respective genes in PD rescue, elav:PINK1B9 female flies were crossed with UAS-TotM-RNAi, UAS-CG4908-RNAi, UAS-mRpL55-RNAi, UAS-Miro-RNAi, UAS-P32-RNAi or UAS-mt:ATPase6-RNAi male flies to obtain PINK1B9;TotM-RNAi, PINK1B9;CG4908-RNAi, PINK1B9;mRpL55-RNAi, PINK1B9;Miro-RNAi, PINK1B9;P32-RNAi or PINK1B9:ATPase6-RNAi lines, respectively. Flies carrying TH-Gal4>mtGFP/TM2 and mtGFP/Cyo;Mhc/Mhc served as control flies for the corresponding experiments.

2.2 | Drug treatment

For EGCG treatment, flies were fed with cornmeal-agar medium containing 0.1 mM or 0.5 mM EGCG (Solarbio, Beijing, China; dissolved in dimethyl sulfoxide (DMSO)) immediately after eclosion till behavioral assessment at Day 3 or Day 20. The dosages were chosen based on previous reports.1,34 In rotenone-treated flies, 250 µM rotenone (Sigma-Aldrich, Shanghai, China; dissolved in DMSO) was administered immediately after eclosion and lasted till behavioral assessment at Day 10. This dosage was selected based on the previous investigation.35 To induce intestinal dysbiosis, flies were treated with tetracycline hydrochloride (BIOSHARP, Hefei, China, dissolved in MQ-H2O) at a concentration of 50 µg/mL.36 The antibiotics treatment began immediately after eclosion and lasted till Day 20.

2.3 | Behavioral assays

The climbing assay was performed as described previously37 with some modifications. In detail, 10 male flies from each group were randomly selected and placed into vertical glass columns (23 cm long, 2.5 cm in diameter) that were marked with a line at 15 cm. Flies were gently tapped to the bottom of the column and the climbing time required for the first five flies to arrive at the marked line was recorded. Six trials were performed for each group and repeated at least three times. The average climbing time (±SEM) was calculated and presented. The jumping/flight assay was performed as described previously38 with some modifications. In detail, 15 male flies from each group were randomly selected and guided into separate vials. The jumping/flight events were counted for two consecutive minutes, during which vials were gently tapped to initiate those events. Six trials were performed for each group and repeated at least three times. The average number of jumping/flight events (±SEM) was calculated and presented.

2.4 | Lifespan analysis

Lifespan analysis was performed as described previously37 with some modifications. In detail, groups of 10 newly eclosed male flies were placed into separate vials with food and maintained at 25°C. Flies were transferred into vials containing fresh food and six vials were used for calculation for each treatment group. Food was changed every 2–3 days and the number of dead flies from each group was recorded and the data were used to calculate the survival percentages. Data were presented as Kaplan–Meier survival distributions, and the significance was determined by log-rank tests.

2.5 | Immunohistochemistry

The 20-days-old male brains were freshly dissected out and prepared in PBS buffer according to the published protocols.39 The collected brains were fixed in 4% paraformaldehyde for 20 minutes, incubated with PTX (PBS buffer with 0.3% Triton X-100) for 20 minutes, and blocked in goat serum for at least 1 hour at room temperature. The whole-mount brains were then immunostained with 1:100 diluted anti-tyrosine hydroxylase (TH) mouse antibody (#22941, ImmunoStar, Hudson, WI, USA) at 4°C overnight, followed by incubation with Cy3-conjugated secondary antibody (#BA1031, Boster, Pleasanton, CA, USA; 1: 100) at 4°C overnight. DA neurons in the protocerebral posterior lateral 1 (PPL1) cluster were then imaged by LSM710 confocal microscope (Zeiss, Shanghai, China) and all the images shown were generated by Z-stack deconvolution. TH-positive neurons were counted in a blinded manner and cells with enlarged mitochondria (shown by mitoGFP) were counted.
2.6  |  Muscle histological analysis

Mitochondrial morphology analysis was performed as described previously,33 with some modifications. In detail, indirect fly muscle from 20-days-old male flies was dissected out in PBS and examined by an LSM710 confocal microscope (Zeiss, Shanghai, China) equipped with 63 × oil objectives. The number of abnormal mitochondria was counted in a blinded manner and the size of mitochondrion was quantified using the Image J software and expressed as mean ± SEM.

2.7  |  Bacterial Strains and intestinal colonization

*Lactobacillus plantarum* KJ01, *Acetobacter pomorum* KJ02, *Lactobacillus brevis* KJ03, and *Acetobacter pasteurianus* KJ04 were isolated from our laboratory fly stocks. Briefly, after dissecting the fly gut under sterile conditions, gut contents were suspended in sterilized saline solution. The diluted suspension was then plated onto the selective MRS (Thermo Scientific, Beijing, China) or YPM (25 g/L n-mannitol, 5 g/L yeast extract, 3g/L peptone) agar medium, respectively. The formed colonies were selected for 16S rRNA sequencing, leading to the identification of *Lactobacillus plantarum* KJ01 (accession number MN710419), *Acetobacter pomorum* KJ02 (accession number MN710420), *Lactobacillus brevis* KJ03 (accession number MN710421), and *Acetobacter pasteurianus* KJ04 (accession number MN710422), respectively.

To feed adult flies with single strains, the bacterial culture was harvested at the log phase (OD600 = 1, approximately 5 × 10^7 viable cells/mL) and used for this experiment. For oral administration, flies were fed with regular fly food mixed with single species of bacteria immediately after eclosion, whereas the weight/volume ratio was 5 g food/1 mL of bacterial suspension. The bacteria-containing fly foods were prepared fresh and replaced every 2 days. The administered flies were kept at 25°C till assays.

2.8  |  Microbiome analysis

Bacterial DNA of each genotype or treatment group was extracted from 30 to 40 adult flies at Day 20 using HiPure Stool DNA Kit B (Magen, Shanghai, China), according to the manufacturer’s instructions. Subsequently, 1 ng/µL of DNA was subjected to 16SV4 rRNA amplification. The primer pair used in V4 amplification is 515F (5’-GTGYCAGCMGCGCGGTAA-3’) and 806R (5’-GGACTACNVGGGTWCTTAA-3’). The PCR products were extracted and purified using the GeneJET gel extraction kit (Thermo Scientific, Beijing, China), according to the manufacturer’s instructions.

High-throughput sequencing was performed and analyzed as described previously.40,41 The library was constructed using the Ion Plus Fragment Library Kit (Thermo Scientific, Beijing, China). Following the Qubit quantification, the pooled amplicons were subjected to IonS5XL for sequencing. Data analysis was conducted with the following steps: data split, data filtration, and chimera removal. The species annotation was conducted following OTU production, with data normalized using a standard of sequence number corresponding to the sample with the least sequences.

Differences in microbial communities between groups were investigated through the phylogeny-based weighted UniFrac distance metrics. Beta diversity on weighted UniFrac was calculated by QIIME software (Version 1.7.0). R software (Version 2.15.3) was used to depict the PCA graph and analyze the inter-group differences of beta diversity. LEfSe analysis was performed using LEfSe software, with the default filter value of the LDA score set as 4.0. The datasets generated during microbiome analysis are available in the Mendeley repository, https://doi.org/10.17632/yn58mjk2fs.1.

2.9  |  RNA extraction and transcriptional profiling

The isolation of brain RNA from 8 to 12 flies per sample, all aged 20-d post-eclosion, was performed using the RNeasy Mini Kit (Qiagen, Shanghai, China) following the heads collection, according to the manufacturer’s instructions. A total amount of 1 µg RNA per sample (with a minimum concentration of 50 ng/µL) was used as the input material for RNA sample preparations. Sequencing libraries were generated using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer’s recommendations followed by cDNA synthesis. In order to select cDNA fragments of preferentially 250–300 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA).

RNA sequencing was performed based on the fundamentals described previously.42 The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina, NEB, USA). After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform and 125 bp/150 bp paired-end reads were generated.

For data analysis, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N, and low quality reads from raw data. All the downstream analyses were based on the clean data with high quality. Reference genome and gene model annotation files were downloaded from the genome website directly and clean reads were aligned to the reference genome using Hisat2 v2.0.5. FeatureCounts v1.5.0-p3 was used to count the reads.
number mapped to each gene. And then FPKM (Fragments Per Kilobase of transcript sequence per Million base pairs sequenced) of each gene was calculated based on the length of the gene and read counts mapped to this gene.

Differential expression analysis of two groups was performed using the DESeq2 R package (Version 1.16.1). The resulting P-values were adjusted using the Benjamini and Hochberg’s approach for controlling the false discovery rate and genes with an adjusted P value < .05 found by DESeq2 were assigned as differentially expressed. GO (Gene Ontology) and KEGG enrichment analysis were conducted using the clusterProfiler R package, whereas KEGG pathways were referenced to the database resource (http://www.genome.jp/kegg/). The datasets generated during transcriptomic analysis are available in the Mendeley repository, https://doi.org/10.17632/nc3cysy4fzv.1.

2.10 | Oxidative stress measurement

Oxidative stress measurement was performed as described previously43 with some modifications. The brains of flies were freshly dissected out in PBS buffer for analysis of the intracellular ROS (Reactive oxygen species) level by incubating with 10 μM DCF-DA fluorescent dye (Sigma-Aldrich, Shanghai, China) for 30 minutes at room temperature. The fluorescent signals were observed using an LSM710 confocal microscope (Zeiss, Shanghai, China) at 490 nm excitation and 525 nm emission wavelengths. The fluorescent densities were subsequently determined using ImageJ software for quantification.

2.11 | Quantitative PCR analysis

Following microbial DNA extraction, 100 ng DNA was subjected to qPCR quantification using the primer pairs listed in Table 1. Stain-specific qPCR was performed using the Roche LightCycler 96 (Shanghai, China). The Ct values for each strain or universal microbiota were obtained, and the relative abundance was calculated and normalized against the cases in control flies. The specificity of primer pairs is shown in Supplemental Figure S1.

For RT-PCR quantification, the derived RNA sample was reverse-transcribed using EasyScript One-Step gDNA

| Target bacteria/genes       | Forward/Reverse | Primer sequence                  | Amplicon length |
|-----------------------------|----------------|---------------------------------|-----------------|
| *Acetobacter pasteurianus*  | F, R           | ATTCGTAGATATTGGGAAGAACACC, ACATCGTTTACAGCTGGACTA | 127 bp          |
| *Acetobacter pomorum*       | F, R           | GGCTGCAAGCAGAGATGTGGTG, TCTAGAGTGGCACCACCA | 154 bp          |
| *Lactobacillus brevis*      | F, R           | GCAGTTGCCGGAGCTCCA, CCACACCTTTTCAGACATCA | 64 bp           |
| *Lactobacillus plantarum*   | F, R           | AGGCGCGGCTGTATGC, CGCAGTGTCTTGTT | 68 bp           |
| Universal bacteria          | F, R           | AAACCAAAGGAATTGACG, CTCACRRCACGAGCTGAC | 145 bp          |
| *Miro*                      | F, R           | CGGAAAGAGACACACAAACGA, CGGATGTCGGTGGGAGTGAG | 746 bp          |
| *CG4908*                    | F, R           | CTCGATCCCGGCTTAGTTC, CGGCTTTTGTCGTCACTGT | 125 bp          |
| mt:*ATPase6*                | F, R           | AAGGCTTTTTCCATTAAAGCGGGA, CGACGATGCGCATGTC | 93 bp           |
| mrPIL55*                    | F, R           | GCCGGCTATATCCACAGTT, CTACGTGGGCACATGAC | 74 bp           |
| *TotM*                      | F, R           | TCGACAAGCCTGGTGACCTTTTC, ACCAAAGACCACCACGAC | 124 bp          |
| *P32*                       | F, R           | CAGCCTAAGGACAAAGCACA, AACATCACCAGGGGCTGGTG | 294 bp          |
| *β-actin*                   | F, R           | CCTGAAGTAACCCATTTGAA, GAGGCTCTTACCGGATGCA | 681 bp          |
2.12 Statistical analysis

Graph data were presented as means ± SEM. Statistical analysis was performed using SPSS software. The unpaired, two-tailed t test was used to perform two-group comparisons and the p value was FDR-corrected when multiple comparisons (>3) were performed. The homogeneity of variances was assessed with the Levene’s test. In case of a non-parametric distribution of data, the Mann-Whitney U test was used. One-way ANOVA was used to analyze the groups with at least three different treatments. PCoA analysis was performed, centered, and scaled to unit variance (R function pcomp). PERMANOVA was performed to make multiple comparisons of inter-group community structure. The number of samples examined in each analysis was shown in the legends and all data were obtained from at least triplicate experiments. Probability values of $P \leq .05$ were regarded as statistically significant.

3 RESULTS

3.1 EGCG rescues the locomotive and neuronal defects of PINK1-null mutant flies

EGCG is an abundant bioactive substance in green tea. While its neuroprotective activity was demonstrated in other Drosophila models with the dosage of 0.1 mM or 0.5 mM, we first investigated if the nutritional merits could be afforded to the PINK1 flies, a valid model of Parkinson’s disease. As shown in Figure 1A, B, the PINK1 loss-of-function mutation led to the significant climbing and flight defects, which were then rescued by EGCG supplement ($P = .004$ for climbing latency; $P < .001$ for flight ability). The injuries were only prevented when 0.5 mM EGCG was administered, while a lower dose of 0.1 mM did not yield a similar outcome ($P = .682$ for climbing latency; $P = .358$ for flight ability). Regarding growth periods, 3-days-old flies did not display the similar locomotive deficits/recovery as 20-days-old flies ($P = .811$ for climbing latency; $P = .863$ for flight ability), indicating that the protective effect of EGCG against the studied PD model varies depending on the developmental phase. Since 0.5 mM EGCG was potent in improving locomotive performances in 20-days-old flies, this condition was adopted in the subsequent studies.

In addition, PINK1-null mutation showed a decreased lifespan compared to control flies ($P < .001$), a phenomenon also rescued by EGCG supplement (Figure 1C, $P < .001$). EGCG addition did not extend the lifespan of normal flies ($P = .358$).

PD is generally characterized by dopaminergic (DA) neuronal loss in the substantia nigra. To profile DA neurons in response to EGCG treatment, the neurons were immunostained with anti-TH antibody and the number of TH-positive neurons in the PPL1 region was counted. According to the results (Figure 1D-F), EGCG prevented the DA neuronal loss in PINK1 flies, as evidenced by both DA neuron number ($P = .01$ against PINK1) and the percentage of TH-positive cells with enlarged mitochondria in the PPL1 region ($P = .03$ against PINK1).

Flight muscle abnormality represents another important aspect of PD symptoms. Due that genotype of PINK1 fly is “PINK1/FM6; TH-Gal4>mitoGFP/TM2” and is designed to express mitoGFP only in DA neurons, to better visualize the muscle alterations induced by EGCG, we introduced mitoGFP into muscles by obtaining PINK1-RNAi line under the direction of a muscle-specific promoter (Mhc). The muscle-specific siPINK1 flies showed obvious locomotive defects, as manifested by climbing and flight behaviors (Supplemental Figure S2A,B). With the treatment of EGCG, the durations required to climb over 15 cm were shortened and the flight activity was increased, suggesting a comparable restoration with EGCG activity in PINK1 cases. By fluorescent graphing, a number of mitochondria with aberrantly enlarged morphology was detected in the disease organisms (Figure 1G). Quantification of enlarged mitochondria (Figure 1H, $P = .003$) and analysis of mitochondrial size distribution (Figure 1I) showed that EGCG markedly rescued the mitochondrial abnormalities. Taken together, the data suggest that EGCG alleviates morphological and behavioral defects in various PD models.

3.2 Gut microbiota mediates the EGCG-mediated protective effect

In order to figure out the gut microbial compositions influenced by PINK1 mutation and EGCG intervention, 16S rRNA sequencing was performed using the IonS5TMXL sequencing platform. Average 80 128 reads per sample were generated, resulting in 74 564 taxon reads. According to the weighted UniFrac β-diversity (Figure 2A), species diversity differed between control and PINK1 flies, as well as PINK1+EGCG groups. Contrarily, control and PINK1+EGCG flies shared a similar β-diversity profile, suggesting that EGCG addition potentially normalized the microbial diversity. PCoA (Principal Coordinates...
Analysis) analysis also indicated a distinct microbial composition of $PINK1^{B9}$ from the control flies, a propensity partially restored by EGCG supplementation (Figure 2B).

Concerning bacterial abundance at the phylum level, EGCG intake led to a sharp drop of Proteobacteria in the $PINK1$-null flies (Figure 2C). In addition, the relative...
egcg-mediated rescue of locomotive and neuronal defects of PINK1-null mutant flies. A, Climbing latency of control (TH-Gal4>mtGFP/TM2) and PINK1^{B9} flies with or without EGCG treatment at a concentration of 0.1 mM or 0.5 mM. At 3d or 20d post-eclosion, 60 male flies were tested for each genotype or treatment group. B, Jumping/flight ability of each group. About 90 male flies were tested for their jumping/flight events within two consecutive minutes. C, Survival percentages of control and 60 male flies were tested for each genotype or treatment group. B, Jumping/flight ability of each group. About 90 male flies were tested for their

abundance of Firmicutes and Bacteroidetes was increased by EGCG, allowing the bacterial structure switched to a comparable profile with control flies. Subsequently, the relative abundance of bacteria was manifested at the genus level (Figure 2D). EGCG treatment led to higher abundance of some taxa of interest compared to PINK1^{B9} flies. Of note, as the major commensal bacteria inhabiting the fly guts, the abundance of both Acetobacter and Lactobacillus was decreased via the intervention of EGCG (Figure 2D). On the basis of findings here, the disrupted microbial architecture was partially repaired by EGCG intervention. Besides, the dominant species enriched in the corresponding group was further shown in the LEfSe cladogram (Supplemental Figure S3). We further explored the roles of gut microbiota in EGCG-mediated protection. The gut microbial architecture could be disrupted by tetracycline treatment. In this study (Figure 2E,F), intestinal dysbiosis or EGCG treatment did not significantly change the locomotive performances of control flies, suggesting the robustness of healthy organisms to maintain body homeostasis. Of note, in the case of PD model of PINK1^{B9}, while EGCG rescued the locomotive defects of PINK1^{B9} flies (P = .012 for climbing latency, P < .001 for jumping activity), the damage was reproduced by the disruption of gut microbiota (Figure 2E,F, P = .007 for climbing latency, P < .001 for jumping activity). These data indicate that EGCG-mediated protection against PD requires intestinal homeostasis.

3.3 Introduction of individual Lactobacillus plantarum strain reproduces locomotive defects

One consideration Drosophila merits is the limited number of commensal intestinal bacteria, which favors the dissection of individual strain contributions. Given it, four strains constituting major symbiotic gut bacteria in flies were isolated through selective growth and identified with 16S rRNA sequencing (Figure 3A). The sequence alignment with reference database revealed that the isolated strains should be assigned to Lactobacillus plantarum, Acetobacter pomorum, Lactobacillus brevis and Acetobacter pasteurianus (Table 2), and be named as KJ01, KJ02, KJ03, and KJ04, respectively. Their relative abundance was then compared in distinct flies using strain-specific qPCR methods. The primer sequence used is shown in Table 1 and their specificity in amplifying genes from target organisms is measured in Supplemental Figure S1. As revealed by Figure 3B, EGCG did not alter the relative abundance of Lactobacillus brevis (LB) (P = .065) and Acetobacter pasteurianus (APa) (P = .7) strains, but inhibited the growth of Lactobacillus plantarum (LP) (P < .001) and Acetobacter pomorum (AP) (P < .001) in the guts of PINK1-null flies. This result may implicate LP KJ01 or AP KJ02 in further inspections.

The distinct groups of flies were then fed on the individual strain, with their locomotion performance and neuronal morphology subsequently examined. The data showed that the sole administration of LP KJ01 resulted in a prolonged climbing latency in the PINK1^{B9} (P = .013) and PINK1^{B9}+EGCG (P = .005) flies (Figure 3C). It suggests that excessive introduction of LP KJ01 exacerbated PD symptoms in the context of the PINK1^{B9}-induced gut dysbiosis and also abolished the EGCG-mediated rescue. The similar cases arose from the quantification of flight activity of mutant flies (Figure 3D, P = .008 for LP+EGCG+PINK1^{B9} vs. EGCG+PINK1^{B9}). Contrarily, the addition of AP KJ02 did not lead to a consistent adverse outcome as specified by behavioral assessment (Figure 3C,D), whereas no evident alterations were observed in the case of LB KJ03 and APa KJ04 strains.

In light of DA neuron number in the PPL1 region (Figure 3E,F), the addition of LP KJ01 strain aggravated the neuronal loss in PINK1^{B9} flies (P = .013), and prevented the rescue effect of EGCG (P = .008). Mitochondrial dysfunction is a crucial subcellular event accompanied by the dopaminergic abnormality of PD. As evidenced in Figure 3G, EGCG
significantly decreased the ratio of neurons with abnormal phenotype \((P = .004, \text{ EGCG versus NF})\), a valid intervention further prevented by the introduction of \(LP\ KJ01\) strain into the fly intestine \((P = .009, LP+EGCG versus EGCG)\). Combined with the observation that EGCG decreased aberrantly enriched \(LP\ KJ01\) of \(PINK1^{B9}\) flies (Figure 3B),
the normalization of $LP$ abundance within gut microbiome should be viewed as an essential step for EGCG to interfere with disease progression.

3.4 | EGCG remodels transcriptomic profiles of PINK1 mutant flies

To further delineate the molecular basis underlying the studied neuroprotection, transcriptomic profiling was conducted. The fly heads were collected and RNA samples were prepared and then subjected to RNA sequencing analysis. This approach generated an average 48 540 948 raw reads per sample, accounting for 46 850 700 clean reads filtered by adapter removal. The representative sequence coverage of $BRP$ ($Bruchpilot$) gene, which expression was inhibited in $PINK1^{B9}$ flies, was visualized in Figure 4A. In the overall perspective, gene expression levels within each group did not show remarkable variations (Supplemental Figure S4A). This approach identified 1615 genes differentially expressed (FDR < .05) between $PINK1^{B9}$ and control flies, as well as 291 genes (FDR < .05) between $PINK1^{B9}$ and EGCG-rescue flies. Of note, 73 genes were commonly regulated by $PINK1$ mutation and EGCG rescue, including $TotM$ ($Turandot M$), a stress responder-encoding gene	extsuperscript{45} (Figure 4B). In detail, as visualized by the volcano graphs, $PINK1$ mutation upregulated 1032 genes and downregulated 583 genes compared to control flies (Figure 4C). In addition, 188 genes were upregulated by EGCG intervention against $PINK1$ mutant flies, with another 103 genes downregulated (Figure 4D).

GO (Gene Ontology) analysis unveiled potential gene clusters affected by EGCG supplement. As shown in Figure 4E, EGCG altered an array of gene expression in $PINK1^{B9}$ flies, primarily incorporated into GO terms of enzymatic activity, response to stimulus, carbohydrate metabolism, etc. The top 20 most enriched clusters are presented in the pie graph of Supplemental Figure S4B. In terms of KEGG pathways, the EGCG-enriched gene function involved drug metabolism, phototransduction, arginine, and proline metabolism (Supplemental Figure S4C).

Based on differential expression and $PINK1$-related gene function, we selected a list of genes of interest for additional analysis. These genes and their transcript levels were shown in a heatmap, according to the peaking data in sequencing (Figure 4F). Moreover, STRING analysis gave the entire picture of the interactive network of genes of interest (Figure 4G).

qPCR was performed to validate the expression profiles of genes of interest. According to the results (Figure 4H), six genes, namely $TotM$, $Miro$, mt:ATPase6, P32, CG4908, and $mRpL55$ (listed in Table 3), were proved to be dysregulated by $PINK1$ mutation and to a variable extent, restored by EGCG supplement. Of note, the transcript levels of TotM were decreased upon $PINK1$ mutation ($P < .001$), which were then partially resumed by EGCG administration ($P < .001$). With the additional antibiotic treatment, the inhibition of TotM was reproduced ($P = .029$ for T+EGCG+$PINK1^{B9}$ vs. EGCG+$PINK1^{B9}$). Moreover, the antibiotic treatment also led to the expression deregulation of mt:ATPase6 or P32 (two mitochondrion-related proteins). (Figure 4H, $P < .001$ for mt:ATPase6 or P32, T+EGCG+$PINK1^{B9}$ vs. EGCG+$PINK1^{B9}$). The divergent deregulation might implicate TotM, P32 or mt:ATPase6 in the microbiota-mediated pathology and intervention. The expression changes of TotM were also illustrated by sequence coverage at the corresponding gene locus (Figure 4I). In summary, these findings suggest that EGCG remodels gene expression in $PINK1^{B9}$ flies.

3.5 | The EGCG-mediated protection is TotM dependent

In order to identify key genes responsible for EGCG-mediated protection against PD, we obtained brain-specific RNAi lines of $TotM$, $Miro$, mt:ATPase6, P32, CG4908, and $mRpL55$, under the direction of an $elav$ promoter on the basis of $PINK1^{B9}$, denoted as $PINK1^{B9};TotM$-RNAi, $PINK1^{B9};Miro$-RNAi, $PINK1^{B9};ATPase6$-RNAi, $PINK1^{B9};P32$-RNAi, $PINK1^{B9};CG4908$-RNAi, and
FIGURE 3  Introduction of individual *Lactobacillus plantarum* strain reproduces locomotive defects. A, Colony-forming morphology of the isolated commensal microbes from fly gut. *Apa*, *Acetobacter pasteurianus*; *AP*, *Acetobacter pomorum*; *LB*, *Lactobacillus brevis*; *LP*, *Lactobacillus plantarum*. B, Strain-specific real-time PCR analysis to quantify the relative abundance of each intestinal strain in flies (n = 3). C-D, Climbing latency (C) and jumping/flight activity (D) of the distinct group of 20-days-old male flies. *PINK1B9* flies were fed with the respective microbial strain (approximately 5g food/5× 10^7 viable cells) or treated with antibiotics (50 μg/mL of tetracycline) immediately after eclosion till 20 days. About 60 or 90 flies per group were subjected to climbing or jumping assessment, respectively. E, Representative immunostaining graphs of DA neurons in the PPL1 cluster. Whole-mount brains of 20-days-old male flies were marked by anti-TH antibody (red). mitoGFP was expressed in DA neurons using the *TH-Gal4* driver to visualize mitochondrial morphology (green). The scale bar represents 20 μm. F-G, Quantification of DA neurons (F) and percentage of cells with enlarged mitochondria (G) in the PPL1 cluster in each group of flies (n = 6). Unpaired, two-tailed *t* test was used to perform two-group comparisons. N values (n > 10) were indicated in the respective figures or presented as scattered points (n < 10). Ctrl, control group (*TH-Gal4>mitoGFP/TM2*); NF, normal food; T, tetracycline treatment; data are represented as Mean ± SEM; ***P < .001, **P < .01, *P < .05, NS P > .05
PINK1<sup>B9</sup>, mRpL55-RNAi, respectively. The recombinant flies were then subjected to the behavioral test in response to EGCG treatment. As determined by climbing latency and jump activity (Figure 5A-F), loss-of-function (LOF) mutation of TotM prevented the EGCG-mediated disease rescue (\(P = .01\), EGCG+PINK1<sup>B9</sup>;TotM-RNAi versus EGCG+PINK1<sup>B9</sup> for climbing assays; \(P = .002\), EGCG+PINK1<sup>B9</sup>;TotM-RNAi versus EGCG+PINK1<sup>B9</sup> for jumping assays), which demonstrates that TotM is required in the studied intervention (Figure 5A,D). What is more, when the expression of TotM was ablated, LPKJ01 could not reintroduce adversities to PINK1<sup>B9</sup> flies fed with EGCG (Figure 5A,D; \(P = .154\), EGCG+LP versus EGCG for climbing assays; \(P = .804\), EGCG+LP versus EGCG for jumping assays), contrary to its potency previously shown in PINK1<sup>B9</sup> flies (Figure 3C,D). This result may suggest that LPKJ01 impacted the EGCG-mediated locomotive repair via TotM. While TotM (Turandot M) in <i>Drosophila</i> is involved in defense response to Gram-positive bacterium,<sup>45</sup> it was shown here to respond to gut dysbiosis and mediate the disease symptoms of PD.

In terms of mt:ATPase<sup>6</sup>, its loss-of-function also prevented the therapeutic effect of EGCG as revealed by climbing assay (Figure 5B, \(P = .026\), EGCG+PINK1<sup>B9</sup>;mt:ATPase6-RNAi versus EGCG+PINK1<sup>B9</sup>), the similar outcome, however, did not appear in flight assessment (Figure 5E, \(P = .695\), EGCG+PINK1<sup>B9</sup>;mt:ATPase6-RNAi versus EGCG+PINK1<sup>B9</sup>). The case of P32-RNAi lines was intriguing, as EGCG decreased the mRNA level of P32 (Figure 4H, \(P = .007\)), additional suppression of its levels by genetic knockdown aggravated the locomotion performance compared to EGCG+PINK1<sup>B9</sup> flies (Figure 5C, \(P = .024\) for climbing assays; Figure 5F, \(P = .05\) for flight assessments). Since both ATPase6 and P32 are mitochondrial proteins responsible for neuronal oxidative phosphorylation, their loss-of-function may result in a compromised mitochondrial integrity, which partially impeded EGCG rescue. Considering the roles of Miro, CG4908, and mRpL55, their RNAi lines did not display consistent behavioral performances (Supplemental Figure S5A-F).

In terms of cellular basis leading to locomotive dysfunction, the DA neuron number in the PPL1 region was decreased from 9.833 ± .307 (EGCG+PINK1<sup>B9</sup>) to 8.167 ± .401 (EGCG+PINK1<sup>B9</sup>;TotM-RNAi) (Figure 5G, \(P = .008\)). Thus, the neuronal recovery in EGCG+PINK1<sup>B9</sup> group was disrupted by brain-specific TotM ablation, suggesting that TotM plays essential roles in EGCG-mediated neuronal repair. In addition, LP KJ01 administration did not yield significant neuronal loss in PINK1<sup>B9</sup>;TotM-RNAi flies supplemented with EGCG (Figure 5G, \(P = .076\) for EGCG+LP vs. EGCG). Along with findings that TotM expression responded to gut microbial status (Figure 4H), the GM-TotM pathway, a characterized form of gut-brain axis in the studied context, is required in the EGCG intervention of Parkinson’s disease.

Emerging evidence proposed PINK1 as a key regulator of mitochondrial dynamics and quality control,<sup>13,46</sup> which subsequently increased sensitivity to oxidative stress.<sup>12</sup> The data here also imply a close relationship of EGCG intervention with mitochondrial function (Figure 5B,C,F). Thus, to describe the physiological consequence due to the PD-related mitochondrial defects, ROS (Reactive Oxygen Species) levels were then determined. As evidenced in Supplemental Figure S6A,B, ROS was aberrantly stimulated by PINK1 mutation and gut dysbiosis, and rescued by EGCG intervention, implying the involvement of mitochondrial or EGCG metabolites anti-oxidative activities in flies’ response to these stimuli.

In summary, these results indicate that the GM-TotM axis is implicated in EGCG-mediated neuroprotection.

### 3.6 EGCG rescues the locomotion and morphology phenotypes of rotenone-exposed flies via the modulation of gut microbiota

The etiology of Parkinson’s disease is associated with the interaction of genetic and environmental factors.<sup>1</sup> Since sporadic form of PD accounts for 90%-95% of cases, any interventions will need to consider multiple toxic events.<sup>9,47</sup> A <i>Drosophila</i> PD model with environmental etiology was thus established by 250 μM rotenone exposure immediately after hatching.
post-eclosion till 10 days, as proposed previously. The exposed flies showed considerable damages in locomotion and neuronal morphology (Figure 6A-D, \( P = .002 \) versus WT for climbing assays, \( P = .011 \) versus WT for jumping assessment, \( P < .001 \) for DA neuron number), which was, to a variable extent, rescued when they were fed with EGCG (\( P = .031 \) for climbing assays, \( P = .006 \) for jumping assessment, \( P = .013 \) for DA neuron number). Similar with cases in \textit{PINK1} \textsuperscript{B9} model, gut microbial alterations induced by LP KJ01 strain blunted the EGCG-mediated rescue effect on the fly locomotion (Figure 6A, \( P = .003 \) column4 versus column3 for climbing assays; Figure 6B, \( P = .008 \) for jumping

**FIGURE 4**
EGCG remodels the transcriptomic profiles of \textit{PINK1} mutant flies. A, Sequence coverage at \textit{BRP} (Bruchpilot) gene locus depicting transcriptional changes upon \textit{PINK1} mutation and EGCG treatment. The graphs were generated from the transcriptomic sequencing data. B, Venn diagram of the transcriptomic assay showing the number of genes differentially expressed in \textit{PINK1} \textsuperscript{B9} versus control flies (\textit{TH-Gal4>mtGFP/TM2}), as well as EGCG+\textit{PINK1} \textsuperscript{B9} versus \textit{PINK1} \textsuperscript{B9} flies. The overlapping region refers to the number of genes commonly regulated by these two pairs of comparisons (n = 3, FDR < .05). C-D, Volcano plots depicting the detailed analysis of differential gene expression between \textit{PINK1} \textsuperscript{B9} and control flies (C), as well as EGCG+\textit{PINK1} \textsuperscript{B9} versus \textit{PINK1} \textsuperscript{B9} flies (D). Blue data points are genes not differentially expressed; green data points are genes up-regulated, and red data points are genes down-regulated (n = 3, FDR < .05). E, GO (Gene Ontology) terms representing the top 30 differentially regulated pathways of \textit{PINK1} \textsuperscript{B9} flies in response to EGCG treatment. The FDR value was shown in a variety of colors, with the size of data points representing gene counts in the corresponding GO terms. F, Heatmaps showing a list of functional genes differentially regulated by \textit{PINK1} mutation and EGCG rescue. G, Interaction networks of functional genes differentially regulated in \textit{PINK1} \textsuperscript{B9} flies in response to EGCG treatment based on the STRING database. The circle colors represent the log\(_2\) values of gene expressional changes. The genes connected by a single line are functionally interactive. H, Real-time qPCR-based analysis to quantify the relative expression levels of genes in fly heads (n = 3). I, Sequence coverage at \textit{TotM} gene locus depicting transcriptional changes upon \textit{PINK1} mutation and EGCG treatment. The graphs were generated from the transcriptomic sequencing data. N values were presented as scattered points (n ≤ 10). Ctrl, control; T, tetracycline treatment; data are represented as Mean ± SEM; ***\( P < .001 \), **\( P < .01 \), *\( P < .05 \), NS \( P > .05 \).
assessments), but not on the DA neuron number (Figure 6C, D, P = .734 for column 4 versus column 3).

Moreover, in the case of rotenone-exposed PINK1^{B9} flies, a PD model with both genetic and environmental etiology, EGCG failed to rescue the climbing deficits (Figure 6E, P = .816 for EGCG+rotenone versus rotenone). However, EGCG still protected against the jumping dysfunction in the rotenone-exposed PINK1^{B9} flies (Figure 6F, P = .002 for EGCG+rotenone versus rotenone) and this protection is GM dependent (P = .004 for EGCG+rotenone+LP versus EGCG+rotenone). Together, EGCG alleviates disease symptoms in the rotenone-exposed PD model via regulating gut microbiota.

4 | DISCUSSION

One of the new findings in this study is to implicate the specific roles of gut microbiota in EGCG-mediated protection against Parkinson’s disease. Emerging evidence proposes EGCG as a multipotent therapeutic agent, which can potentially target a variety of molecules and signaling.\(^2\)\(^,\)\(^19\) Pertaining to the therapeutic intervention of PD, multiple mechanisms were raised to explain how EGCG worked, such as oxidative stress, neuroinflammation, protein aggregation and more recently, mitochondria homeostasis.\(^3\) While those mechanisms indicate the various aspects of EGCG functioning, its bioavailability in brains is very poor due to rapid metabolism and biochemical modification.\(^23\)\(^,\)\(^48\) Based on these observations, an inter-organ pathway is anticipated to exist to convert EGCG intake into the *bona fide* consequence in brains, that is, the indirect effect posed by EGCG. As evidenced in this study, a unique gut-brain communication is found to play the central part, linking EGCG rescue with the “gut origin” theory of PD development.

Research is emerging to uncover the “microbiome-gut-brain” axis by which microbiota can impact the body. Understanding how this inter-organ communication may contribute to diseases and homeostasis in the body is key to human health and well-being. In consistence with observations that PD patients are conventionally accompanied by intestinal symptoms, their microbial compositions are distinct from healthy participants, since microbial transplantation alters the disease pathophysiology.\(^24\)\(^,\)\(^28\) The relation between GM and PD was also demonstrated in this study, wherein gut dysbiosis abolished the potent rescue effect of EGCG (Figure 2E,F). Therefore, intestinal microbial architecture is not merely associated with disease progression, but also mediates the nutritional intervention originating from active compounds in vitro. In this case, EGCG impacted brains through adjusting intestinal microbiota; however, this indirect pathway should not be regarded as the unique one to bring desirable consequences, as no rationale was given to exclude the direct interaction between natural compound and target tissues. We reason that EGCG-mediated neuroprotection could be accomplished partially via the regulation of GM compositions.

EGCG has the highest anti-oxidative ability among common phenolic compounds, besides several tannin compounds\(^15\) and the anti-oxidative role was also viewed as an important mechanism for EGCG to protect against neuronal
FIGURE 5 The EGCG-mediated protection is TotM dependent. A-C, Climbing latency of 20-days-old male flies. *Elav;PINK1<sup>B9</sup>* flies were fed with 0.5 mM EGCG immediately post-eclosion till 20 d. The same protocols were also applied to *PINK1<sup>B9</sup>;TotM-RNAi* (A), *PINK1<sup>B9</sup>;mt:ATPase6-RNAi* (B) and *PINK1<sup>B9</sup>;P32-RNAi* (C) flies, a fraction of which were further fed with LP KJ01 strains (approximately 5g food/5× 10<sup>7</sup> viable cells). At 20 d, 60 flies per group were subjected to climbing assessment. D-F, Jumping/flight capacity of 20-days-old male flies. *Elav;PINK1<sup>B9</sup>* flies were fed with 0.5 mM EGCG immediately post-eclosion till 20 d. The same protocols were also applied to *PINK1<sup>B9</sup>;TotM-RNAi* (D), *PINK1<sup>B9</sup>;mt:ATPase6-RNAi* (E) and *PINK1<sup>B9</sup>;P32-RNAi* (F) flies, a fraction of which were further fed with LP KJ01 strains (approximately 5g food/5× 10<sup>7</sup> viable cells). At 20 d, 90 flies per group were subjected to climbing assessment. G, Quantification of DA neurons in the PPL1 cluster in each group of male flies with TotM knockdown following behavioral assessment (*n* = 6). Unpaired, two-tailed *t* test was used to perform two-group comparisons. N values (*n* > 10) were indicated in the respective figures or presented as scattered points (*n* ≤ 10). NF, normal food; LP, *Lactobacillus plantarum* KJ01; data are represented as Mean ± SEM; **P < .001, ***P < .01, *P < .05, NS P > .05.
This finding was supported by the cerebral ROS measurement in EGCG-fed flies, whereas EGCG significantly decreased the ROS level present in the disease flies (Supplemental Figure S6). While this oxidative stress responded to intestinal bacterial status (as shown by antibi-otic treatment) and concomitant mitochondrial alterations, direct radical scavenging by EGCG remains a potential integral or alternative route. This possibility needs a further...
check, due that the metabolite forms present in brains are primarily epicatechin glucuronide and 3-O-methyl epicatechin glucuronide, with free epicatechin hardly detectable, and epicatechins are dramatically more antioxidative in vitro than the conjugated glucuronide metabolites.

How did gut microbiota impact brain behavior in the studied context? We found TotM played mediatory roles. The expression of TotM responded to GM signals, and its function was associated with DA neuron morphology and locomotion behavior, as discovered in this study (Figure 5A,D,G). Meanwhile, the enormous diversity of the resident microbiota community and the genetic complexity of the host system make it difficult to clearly establish the unique molecular links. Owing to the complex nature, the intermediate route conveying GM cues into cerebral molecular response awaits to be deciphered in future investigations. Of note, as TotM is a humoral factor that is implicated in stress response to injuries and consequent immune response, the roles of immune signals triggered by microbial status should not be overlooked. Moreover, a previous finding stated that EGCG metabolites sharply decreased when rat gut microbiota was disrupted, suggesting that GM was responsible for EGCG metabolism. Although microbial diversity is extremely low in flies compared with rats, the possibility could not be ruled out that GM acted through the metabolites pathway to achieve the studied inter-organ signaling.

High taxonomic diversity at the species level was observed among different natural and laboratory Drosophila populations. In the fruit flies used here, we isolated four dominant strains, identified as Lactobacillus brevis, Lactobacillus plantarum, Acetobacter pomorum, and Acetobacter pasteurianus, respectively (Table 2). By microbial profiling, we observed that EGCG decreased the relative quantities of LP KJ01 or AP KJ02 strains in PINK1 flies, as evidenced in Figure 3B. However, when EGCG was administered at control flies, their relative abundance was not significantly downregulated. These discrepancies were also observed in the overall microbial community structure (Figure 2C). This suggests that EGCG does not always remodel gut microbiome in a defined orientation, but relies on the physiological context in the body. This argument is supported by the previous finding that some autochthonous bacteria (such as Lactobacillus brevis and Gluconobacter morbillifer) are normally quiescent but provoke chronic inflammation under dysbiosis conditions.

Of interest, an increase in the abundance of LP KJ01 was always accompanied by adverse behavioral outcome in the studied context. EGCG prevented the loss of DA neurons by reducing LP KJ01 levels and the introduction of KJ01 reproduced the damage (Figure 3C-G). This finding is intriguing due that lactobacillus strains are normally viewed as health-promoting probiotics. As a reference study, we previously detected aberrantly high level of lactobacillus in lead-exposed rat intestine (data in press). A similar phenotype was discovered by microbial comparisons between PD patients and control subjects, which clarified a prominent increase of Lactobacillus and Lactobacillaceae strains in PD patients. Based on these observations, it is tempting to speculate that adverse effect accompanied by LP KJ01 increase is attributed to the microbial community characterized by LP dominance, rather than strain activity per se. The detailed interacting mechanism is warranted to discuss in the ensuing studies.

The transcripts remodeled by EGCG are relatively constrained compared to PINK1 mutation. In detail, 1615 genes were deregulated by PINK1 mutation, while 291 genes were impacted by the EGCG supplement (Figure 4B). The 73 commonly regulated genes should be categorized into some metabolism-related pathways such as sugar metabolism, drug metabolism, etc., most of which are associated with energy homeostasis. No apparent cell death-related pathways were suggested, as opposed to the previous publication, possibly due to the varying organisms and physiological context. Additionally, EGCG-mediated neuroprotection was previously shown as AMPK activity-dependent, thus there is a possibility that some unidentified molecules, apart from TotM and P32, may play key roles in regulating the studied intervention via their assigned protein function, since transcriptomic profiling can only be used to detect gene expression rather than enzymatic activity.

By means of transcriptomic screening and consequent validation, TotM was first found to regulate PD progression and EGCG-mediated intervention. In fruit flies, TotM was traditionally regarded as a stress effector regulated by the JAK-STAT pathway. Here it acts as mediators linking intestinal microbiota, shaped by either PINK1 mutation or EGCG intake, with neuronal and locomotive performance, which is consistent with the relevance of TotM with bacterial infectious response. In addition, when the expression of P32, a mitochondria-related protein, was blocked, EGCG also failed to improve the climbing activity of PD flies (Figure 5C). This may implicate mitochondrial dynamics in EGCG neuroprotection, an argument supported by the alterations of cell number with enlarged mitochondria (Figures 1F, and 3G) and ROS generation (Supplemental Figure S6). As both TotM and dopaminergic mitochondria responded to gut microbial changes and PINK1;TotM-RNAi lines possessed the decreased DA neurons in the PPL1 region, TotM is then hypothesized to encode neuronal mitochondria-related function or acted by impacting mitochondrial proteins. Based on it, a GM-TotM pathway could be proposed to mediate EGCG neuroprotection against PD, probably via regulating the mitochondrial dynamics in DA neurons.

Multiple Drosophila models were utilized here to mimic different aspects of PD symptoms. In these models, PINK1 flies provided the comprehensive locomotive abnormalities and DA neuronal dysfunction, a process believed to constitute the major part of PD manifestations. Besides, muscle-specific knockdown of PINK1 and rotenone exposure
were used to characterize muscle toxicity and environmental etiology, respectively. The investigations in these models proposed EGCG as a common effector against multiple aspects of PD. Meanwhile, gut dysbiosis prevented the EGCG-mediated protection in both PINK1\(^{59}\) and rotenone-exposed flies, suggesting that gut microbiota is a common route used by EGCG to improve disease symptoms. This argument was also supported by an earlier study\(^{53}\) that rotenone induced microbial alterations in a rat model of PD, consistent with changes described in PD patients and an abnormal increase of lactobacillus abundance. One of the limitations of this research is the limited evolutionary conservation of Drosophila with humans or mammals. The taxonomic diversity of gut microbiota in flies is extremely low compared to cases in humans\(^{54}\) and this situation renders it desirable to subject the current mechanism to the mammal context. However, as previously indicated\(^{7,11}\) the simplicity of fruit flies also provides a prominent advantage for the functional characterization of individual strain, such that the supplemented bacteria is likely to dominate over a relatively small community. Relying on this strategy, the unique role of Lactobacillus plantarum KJ01 strain was identified and highlighted here. Moreover, although it warrants additional tests in mammals, neurodegeneration studies in flies still have the medical significance of identifying promising therapeutic targets by pilot screens/validations, as well as the basic aspects of disease/intervention mechanisms, as revealed in this study.

In conclusion, the roles of gut microbiota and TotM in EGCG-mediated alleviation against Parkinson’s disease were investigated using Drosophila models. Gut microbial disruption, as well as LP KJ01 administration and TotM ablation, is sufficient to abolish EGCG-mediated repairment, suggesting that this protection varies depending on intestinal microbial status and TotM levels. The current study advances our understanding of how the gut-brain cross talk may have relevance with EGCG intervention and neurodegenerative progression, and the identified bacterial taxon and genetic molecule might serve as potential disease biomarkers or even drug targets available for further validations.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Y. Xu and H. Wang conceived of and designed research; M. Xie and Y. Xu performed the experiments; Y. Xu analyzed the data and wrote the paper; G. Xiao and H. Wang interpreted the results of the experiment; J. Xue and L. Xiang participated in the crosses of recombinant flies and immunostaining; Y. Li participated in primer specificity examination; J. Xiao participated in data analysis and blind counting; and all authors read and approved the final manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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