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Scienze Morfologiche e Funzionali

Ciclo XXVIII

Drosophila melanogaster Genetic Model of Parkinson's Disease: phytotherapy approach for a new and more sustainable pre-clinical investigation

1. Drug effects of *Mucuna pruriens* in a PINK1^{B9} *Drosophila* model
2. Drug effects of *Withania somnifera* in a LRRK2 *Drosophila* model

Settore scientifico disciplinare di afferenza: BIO/09

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Section 2

Drug effects of *Withania somnifera* in a LRRK2 *Drosophila* model

List of Publications

This thesis is based on the work contained in the following papers:

1. Poddighe S[*], **De Rose F**[*], Marotta R[*], et al. (2014) *Mucuna pruriens* (Velvet bean) Rescues Motor, Olfactory, Mitochondrial and Synaptic Impairment in PINK1^{B9} *Drosophila melanogaster* Genetic Model of Parkinson's Disease.
PLoS ONE 9(10): e110802. doi:10.1371/journal.pone.0110802

2. **De Rose F**[*], Marotta R[*], Poddighe S[*], Talani G[*], et al. (2016) Functional and Morphological Correlates in the *Drosophila* LRRK2 *loss-of-function* Model of Parkinson's Disease: Drug Effects of *Withania somnifera* (Dunal) Administration.
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Abbreviations

PD	Parkinson's disease
Dm	*Drosophila melanogaster*
$PINK1^{B9}$	PTEN-induced putative kinase 1
LRRK2	Leucine-rich repeat kinase 2
Mpe	*Mucuna pruriens* extract
Wse	*Withania somnifera* extract
$LRRK2^{WD40}$	LRRK2 *loss-of-function* in the WD40 domain
WT	Wild type
L-Dopa	L-3,4-dihydroxyphenylalanine; levodopa
DA	Dopamine
MPTP	Methyl-phenyl-tetrahydropyridine
GFS	Giant Fiber System
DLM	Dorsal Longitudinal Muscles
EAG	Electroantennogram recording
ALs	Antennal lobes
TEM	Trasmission electron microscopy
BRP	Bruchpilot
TH	Tyrosine hydroxylase
DDC	Dopa decarboxylase
LID	L-Dopa-induced dyskinesias

Aim of the project

The aim of his project is to investigate the pathogenic mechanisms underlying Parkinson's disease (PD), the second most common neurodegenerative disorder after Alzheimer's disease, and to develop and validate an alternative therapeutic approach to common treatments using plant extracts in preventive and/or medicative roles against the progression of Parkinson's disease.

Specifically, this research intends to gain more knowledge on some aspects of the disease in a powerful PD genetic model, *Drosophila melanogaster* (*Dm*), a relatively simple organism that shares many basic biological, physiological and neurological features with vertebrates, including humans. Nearly 75% of human disease-causing genes have a functional homolog in the fly, the manipulation of which allows the creation of excellent experimental models.

Two types of transgenic mutants of *Drosophila melanogaster*, expressing human variants of Parkinson's disease, have been used in this project: *loss-of-function* $PINK1^{B9}$ (with a deletion of 570 bp in PINK1 Kinase domain) and *loss-of-function* $LRRK2^{WD40}$ (with a deletion of 464 bp in LRRK WD40 domain). The validity of the $PINK1^{B9}$ model derives from many lines of research [1,2], while $LRRK2^{WD40}$ is a variant of LRRK2 mutation still scantily studied (previously named $LRRK^{ex1}$; [3]).

Mutations in the human gene PINK1 are traditionally associated with familial forms of early-onset PD. The encoded protein acts as a mitochondrial kinase and is involved in many biological processes, often in response to oxidative stress. Mutations in the LRRK2 human gene are linked to the autosomal dominant inheritance type of PD. The protein expressed is involved in neurogenesis, endocytosis/vesicles trafficking and autophagy system as well as in other mechanisms. The specific phenotypic differences open the way to potential specific pharmacological treatments, even with a neuroprotective type mode.

The aim of the project is also to test the efficacy of plant species *Mucuna pruriens* (*Mpe*) and *Withania somnifera* (*Wse*) - used for centuries in Ayurveda medical therapy - in preventing or delaying the degeneration of dopaminergic neurons and motor/non-motor deficits in *Drosophila* mutants.

The parameters considered for the experimental design are lifespan, motor activity, olfactory response, synaptic protein expression, mitochondria and synaptic active zone morphology, both in treated and untreated flies.

Introduction

Parkinson's disease

Parkinson’s disease (PD) is the second most common neurodegenerative disorder after Alzheimer’s disease, affecting more than 1% of the population over age 60, with an increased incidence closely correlated with aging. PD is characterized by the selective degeneration of dopaminergic (DA) neurons in the *substantia nigra pars compacta* and, in many cases, by the accumulation of cytoplasmic aggregates of eosinophilic proteins (Lewy bodies), including α-synuclein, ubiquitin and parkin, in brain stem, spinal cord, and cortical regions [4–6]. A precise diagnosis that identifies a specific type of the disease is still extremely difficult, also due to the lack of diagnostic tests [7]. Parkinson’s disease belongs to a wide spectrum of diseases named “parkinsonisms" that share common symptoms and causes - such as striatal DA deficiency or striatal damage - of which PD represents ~80% of cases [8].

Parkinson’s disease presents with motor defects, such as resting tremor, akinesia, postural instability and muscle rigidity, and with non-motor defects, such as neuropsychiatric, autonomic, gastrointestinal and sensory symptoms, but the onset is gradual and the earliest symptoms might be unidentified by clinicians and not readily related to the syndrome. Events from depression, anxiety, fibromyalgia to shoulder pain, commonly occur about 4-6 years before the onset of typical severe symptoms, as well as olfactory dysfunctions, constipation and sleep behavior disorders which might precede the manifestation of motor deficits by 10 years [9–11]. The impairment of locomotor function appears when at least 50% of nigral DA neurons and 70% of putaminal DA have been lost and the degeneration is complete by about 4-5 years post-diagnosis [6,12].

Unfortunately, therapeutic treatments to limit the onset of PD are not yet available: up to now the common approach to treating patients is to intervene by relieving symptoms. To this end, the drug most commonly used for symptomatic therapy is levodopa (l-3,4-dihydroxyphenylalanine; l-dopa), the physiological amino acid precursor of dopamine [13]. For many years the positive effects of the

administration of levodopa in Parkinson patients have been known: they include alleviating some of the locomotor complications, but the side effects of high doses and long-term treatment, such as dyskinesia, postural hypotension and motor fluctuation have also been discovered [11,12 review]. Moreover, a review by Chaudhuri and Schapira [11], suggests that treatment with levodopa might also be used to counteract non-motor symptoms, but it is necessary to point out that in some cases the therapy may produce the opposite result, causing a worsening of the events. The action mechanism of levodopa in counteracting Parkinson's disease remains uncertain and a valid therapy without side effects in preventing motor and non-motor symptoms is not currently available.

Genes Associated with Parkinson's disease

At present, the pathogenesis of Parkinson's disease has still not been completely defined, but it is clear that the etiology of the disorder is multifactorial, i.e. a complex interaction between genetic and environmental factors [4]. About 90% of PD cases are sporadic but, in the remaining cases, the disease is inherited in autosomal recessive or dominant modality [8].

Several loci have been identified for monogenic forms of PD, but there are five clearly defined genes for which a single mutation is sufficient to cause the phenotype: LRRK2 (PARK8), SNCA (PARK1), PINK1(PARK6), Parkin (PARK2), DJ-1(PARK7).

Hundreds of distinct genetic variants of these five genes have been reported in Mendelian (familial) forms of disease. Mutations in SNCA and LRRK2 genes are associated with a dominant form of the disease, while mutations in PINK1, Parkin and DJ-1 cause recessive early onset parkinsonism (age of onset <40 years) [16].

Drosophila melanogaster as a PD model

Research on PD in human subjects is limited by technical and ethical issues. Cellular models commonly used for molecular, biochemical, and pharmacological approaches are not completely reliable. For this reason it has been necessary to develop a genetic modeling system to allow the *in vivo* study of functional processes related to the pathology to confirm data obtained on cell lines. Among the animal models used in the study of neurodegenerative disorders, a valuable system for basic studies of neuronal development, activity and dysfunction is the fruit fly *Drosophila melanogaster* (*Dm*). The fruit fly is considered a valuable model for studying several complex biological processes [17–19] owing to several features, such as ease of maintenance, rapid reproductive cycle and short lifespan and a complex behavior driven by a sophisticated nervous system. It is of particular interest to underline that the *Dm* nervous circuit is composed of ~100,000 neurons and, recalling that the most characteristic symptom of PD is the progressive loss of dopaminergic neurons, 200 neurons are led by the neurotransmitter DA. Moreover, the genome of *Dm* has been completely encoded and most of the human genes, including those involved in PD, are evolutionarily conserved in the fly [19]. *Dm* gene expression is easily manipulable with several techniques and tools, therefore their *loss-of-function* (LOF) or *gain-of-function* (GOF) can be analyzed and genetic and pharmacological modulation of the phenotypes can be evaluated [20]. Besides, most of the genes implicated in familial forms of the disease have at least one fly homolog [21]. This work focuses on PINK1 and LRRK2 *Drosophila melanogaster* homolog genes.

PINK1 Domain Structure and Mutations

The PINK1 gene (phosphatase and tensin homolog (PTEN)-induced putative kinase 1; PARK6) that encodes the homonymous serine/threonine kinases protein consists of eight exons, 581 amino acid and shows two domain structures: a mitochondrial targeting motif and a kinase domain. In human the transcript is

ubiquitously expressed but, in the brain, the higher expression is localized in *substantia nigra,* hippocampus neurons and cerebellar Purkinji cells [22–24].

PINK1 protein is involved in synaptic vesicular transport system and in the response to mitochondrial oxidative stress [24,25], but its role is not yet completely clear. Moreover, several studies in flies and vertebrates highlight its closely link to Parkin protein activity (parkin RBR E3 ubiquitin protein ligase protein, encoded by the PARK2-Parkin gene), showing how the PINK1/Parkin pathway play a role in mitochondrial dynamics and morphology [16,23,26–28]. Different types of mutations (nonsense, missense, small insertions and deletions, and whole-gene or single/multiple exon variations mutations), distributed along the entire gene within and outside the kinase domain, are the most common cause of recessive familial Parkinsonism [23,24]. *Drosophila melanogaster* contains a single PINK1 homologue which shares with human PINK1 43% amino acid identity and 60% similarity [26,29].

LRRK2 Domain Structure and Mutations

The LRRK2 gene consists of a genomic region of 51 exons and is characterized by the presence several independent domains: Roc (Ras GTPase family), COR (C-terminal of Roc), Kinasi (serine/theronine and tyrosine kinase family) and protein-protein interaction domains LRR (leucine rich repeats) and C-terminal WD40 domain [30,31]. LRKK2 gene coding for an unusually large protein composed of 2527 amino acids is widely expressed in the brain and other organs; several studies have detected the presence of the protein in specific brain regions such as cortex, striatum, hippocampus, cerebellum and in the dopaminergic neurons of the *substantia nigra* [32–35]. The LRRK2 roles, listed in the Berwick and Harvey review [31], include neurogenesis and neurite outgrowth, cytoskeleton assembly, endocytosis/vesicles trafficking and autophagy coordination. LRRK2 mutations are the most common cause of both familial and sporadic forms of late onset PD worldwide. Li et al. [33, review] summarized the possible involvement of mutations of the LRRK2 gene in many pathogenic mechanisms of PD such as

inflammatory response, oxidative stress, mitochondrial dysfunction, synaptic dysfunction and lysosomal system disorder. *Drosophila melanogster* has a sole homologue of human LRRK2 which shares similar GTPase and kinase domains, with 46% and 44% homology respectively [37].

Mucuna pruriens

Mucuna pruriens, also known as velvet bean, is a perennial semi-woody climberis that belongs to the family of Fabaceae, endemic in India and other tropical countries. The extract of *Mucuna pruriens* (*Mpe*) is a popular Indian medicinal plant, which has long been used in traditional Indian Ayurvedic medicine for a variety of purposes, such as carminative, hypertensive and hypoglycemic agent. It is also used as an aphrodisiac, diuretic, vermifuge and used for cancer, cholera, asthma, pleuritis, cough, dog- and snakebite, etc. [38–40]. Mucuna seed composition shows several compounds: proteins, lipids, carbohydrates and numerous minerals as well as a series of active ingredients such a alkaloids, glutathione, nicotine and serotonin. Moreover, *Mpe* has been suggested to be useful against Parkinson's symptoms considering that the endocarps contain a fundamental amino acid compound, levodopa (L-3,4- dihydroxyphenylalanine, L-Dopa), a main precursor of the neurotransmitter dopamine, in 4-10% w/w amounts [39,41,42]. In this respect, some clinical studies have evaluated the therapeutic efficacy of *Mpe* as a natural alternative to commonly used (levodopa) anti-Parkinsonian drugs in humans [43–45]. *Mpe* provides alleviation of parkinsonism as well as levodopa treatment and has been reported not to cause drug-induced dyskinesia [43]. However, it may cause other side effects, such as gastrointestinal dysfunction due to absorption of oral treatments, as in the Lieu et al. [42] test on parkinsonian primates. It is assumed that the therapeutic activity of *Mucuna pruriens* may not be attributed to the L-dopa content of the plant alone, but may be due to the synergic action of one or more active components and secondary metabolites [40,44,46].

Withania somnifera

Withania somnifera, also known as Ashwagandha or Indian ginseng, is a perennial shrub that belongs to the family of Solanaceae, widely distributed in Africa, Southern Europe and Asia, but also geographically identified in the Italian islands - Sardinia and Sicily [47,48]. The plant is commonly used as a herbal drug in traditional Indian Ayurveda medicine for over 3000 years, owing to its several beneficial effects, including anti-oxidant, anti-inflammatory, anti-tumor and immunomodulatory activity [47,49,50]. The biochemical composition of *W. somnifera* root is complex, several biologically active chemicals are known: they include alkaloids (ashwagandhine, cuscohygrine, anahygrine, tropine etc.) and steroidal lactone (a class of constituents known as withanolides such as withaferin A, withanone, withanolide WS-1, Withanolide A-Y, withasomniferina-A, withasomidienone, withasomniferoli) [49,51]. These phytochemical constituents present in *Withania somnifera* are reported to counteract the excitotoxicity, oxidative damage and promote neuroprotective activity [52,53]. Therefore, the traditional uses and several studies suggest that *Wse* may possibly be useful also in treating, among others illnesses, neurodegenerative diseases [50,52,54]. Studies have revealed that *Wse* may be helpful in protecting against neuronal injury in Parkinson's disease in murine models [50,55,56].

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Section 1

Drug effects of *Mucuna pruriens* in a PINK1^{B9} *Drosophila* model

Mucuna pruriens (Velvet bean) Rescues Motor, Olfactory, Mitochondrial and Synaptic Impairment in PINK1^{B9} *Drosophila melanogaster Genetic Model of Parkinson's Disease*

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Abstract

The fruit fly *Drosophila melanogaster* (*Dm*) mutant for PTEN-induced putative kinase 1 (PINK1^{B9}) gene is a powerful tool to investigate physiopathology of Parkinson's disease (PD). Using PINK1^{B9} mutant *Dm* we sought to explore the effects of *Mucuna pruriens* methanolic extract (*Mpe*), a L-Dopa-containing herbal remedy of PD. The effects of *Mpe* on PINK1^{B9} mutants, supplied with standard diet to larvae and adults, were assayed on 3–6 (I), 10–15 (II) and 20–25 (III) days old flies. *Mpe* 0.1% significantly extended lifespan of PINK1^{B9} and fully rescued olfactory response to 1-hexanol and improved climbing behavior of PINK1^{B9} of all ages; in contrast, L-Dopa (0.01%, percentage at which it is present in *Mpe* 0.1%) ameliorated climbing of only PINK1^{B9} flies of age step II. Transmission electron microscopy analysis of antennal lobes and thoracic ganglia of PINK1^{B9} revealed that *Mpe* restored to wild type (WT) levels both T-bars and damaged mitochondria. Western blot analysis of whole brain showed that *Mpe*, but not L-Dopa on its own, restored bruchpilot (BRP) and tyrosine hydroxylase (TH) expression to age-matched WT control levels. These results highlight multiple sites of action of *Mpe*, suggesting that its effects cannot only depend upon its L-Dopa content and support the clinical observation of *Mpe* as an effective medication with intrinsic ability of delaying the onset of chronic L-Dopa-induced long-term motor complications. Overall, this study strengthens the relevance of using PINK1^{B9} *Dm* as a translational model to study the properties of *Mucuna pruriens* for PD treatment.

Introduction

Several reports on antiparkinsonian activity of *Mucuna pruriens* (*Mp*) [13] [14] endorse the use of *Mp* seeds in PD. In addition to L-Dopa, *Mp* seeds contain genistein and polyunsaturated fatty acids which support its Parkinson's disease (PD) is, after the Alzheimer's disease, the second most prevalent neurodegenerative disease first affecting medulla oblongata, olfactory bulb and substantia nigra [1]. Loss of olfaction is a very consistent marker of PD occurring in 95% of patients early before the onset of motor symptoms [2]. Olfactory dysfunction is observed in PTEN-induced putative kinase 1 ($PINK1^{B9}$) Parkinsonism, both in humans [3] and in animal models of PD [4]. The *Drosophila melanogaster (Dm)* $PINK1^{B9}$ mutant model recapitulates several of the essential features of PD [5] and has been used to study neuronal dysfunction and molecular aspects of neurodegeneration [6]. In particular, $PINK1^{B9}$ model provides major information regarding pathogenic molecular basis of early onset PD and mitochondrial dysfunction [5]. Accordingly, it was recently reported that PINK1 mutation enhances mitochondrial stress-induced neurodegeneration in mice [7].

L-Dopa is the most effective symptomatic medication of PD and is still considered the gold standard in its treatment, although other drugs such as dopamine (DA) agonists, DA uptake and mono amino oxidase-B inhibitors are commonly used in the clinical management of PD patients [8] [9] [10]. Besides, other drugs such as adenosine A2A antagonists used as adjunct might be effective in the symptomatic treatment of PD [11]. In addition, the involvement of non-dopaminergic neurotransmitters such as noradrenaline, serotonin, glutamate, and acetylcholine in different brain areas like cortex, brainstem and basal ganglia has prompted many researchers to investigate the effects of non-dopaminergic drugs [12] indicating the involvement of multiple targets in treatment of PD. Several reports on antiparkinsonian activity of *Mucuna pruriens* (*Mp*) [13] [14] endorse the use of *Mp* seeds in PD. In addition to L-Dopa, *Mp* seeds contain genistein and polyunsaturated fatty acids which support its antiparkinsonian and neuroprotective actions [15].

Furthermore, phytic acid, another *Mp* constituent with antioxidant and iron sequestrant activity, has been reported to suppress methyl-phenyl-tetrahydropyridine (MPTP) induced hydroxyl radical generation [16]. Hence, in view of multiple phytoconstituents supporting antiparkinsonian activity of *Mp*, the present study was aimed at verifying if *Mpe*'s ability to ameliorate symptoms in this PD model might be attributable to L-Dopa only or to the *Mp* extract as a whole in which L-Dopa is present along with other ingredients.

On these bases we evaluated the antiparkinsonian profile of the standardized methanolic extract of the seeds of *Mp* (*Mpe*) on lifespan, climbing activity and olfactory function in PINK1^{B9} as compared to either wild type (WT) and untreated PINK1^{B9} *Dm.* In addition, in order to gain mechanistic insights on the neuroprotective and neuro-rescue properties of *Mpe*, we also evaluated the expression of bruchpilot protein and tyrosine hydroxylase, as well as the morphology of presynaptic active zones and mitochondria in flies'antennal lobes, i.e. the olfactory bulbs-equivalent structure, and thoracic ganglia, of both WT as well as untreated and *Mpe*-treated PINK1^{B9} mutants.

Materials and Methods

Fly Strains

For these experiments we used adult wild type (WT) Oregon-R (Oregon-R-C) and PTEN-induced putative kinase 1 PINK1^{B9} (w[*] Pink1[B9]) mutant *Drosophila melanogaster* (*Dm*) males (from Bloomington Stock Center; Fly Base: http://flybase.bio.indiana.edu). After emergence from pupae, male WT and PINK1^{B9} mutant flies were separated. WT and mutant flies were reared on a standard cornmeal-yeast-agar medium in controlled environmental conditions (24–25°C; 60% relative humidity; light/dark=12/12 hours). In detail, four groups of mutant flies were reared on a standard medium supplemented with *Mucuna pruriens* methanolic extract (*Mpe*) (Batch no. F*MPE*X/2012060001; Natural Remedies Ltd., Bangalore, India). PINK1^{B9} mutants were supplied with *Mpe* at

different concentrations (0 (i.e. untreated PINK1^{B9} mutants), 0.1, 1 and 10% w/w in their standard diet) both as larvae and adults (L^+/A^+). In addition, another group was reared on a standard medium supplemented with 0.01% (0.5 mM) L-Dopa (Sigma Aldrich, Milan, Italy), a percentage similar to that at which L-Dopa was supplemented with 0.1% *Mpe* [15]. The effects of *Mpe* were assayed at different age steps (I: 3–6; II: 10–15; III: 20–25 days old). A series of experiments on life span, using various concentrations of *Mpe* (see below in Survival curves) provided the basis for selecting the optimal concentration at which conduct the behavioral, morphological, and protein expression assessments. In particular, based on lifespan results, the olfaction behavior assessments, transmission electron microscopy (TEM) and western blot analyses were restricted to group II flies after 0.1% *Mpe* administration as L^+/A^+. Standard genetic procedures were used during the study.

Survival curves

With the aim of selecting the optimal *Mpe's* concentration, *Dm* were grown on standard diet supplemented with different concentrations of *Mpe* at 25°C. Cohorts of 40 flies (4 flies/tube) from each experimental group (i.e. WT, untreated and *Mpe*-treated PINK1^{B9}) were monitored every 2 days for their survival. Mortality was analyzed using Kaplan-Meier survival curves and the statistical comparisons were made with a Gehan-Breslow-Wilcoxon test. Experiments were done in duplicate with the exception of those on WT, untreated mutants, 0.1% *Mpe*- and 0.01% L-Dopa-treated PINK1^{B9} that were done in triplicate. Each experiment was conducted with the appropriate control group (i.e. WT, untreated PINK1^{B9} and treated PINK1^{B9}).

Climbing assay

The climbing assay (negative geotaxis assay) was used to assess locomotor ability [17]. Climbing data were obtained from groups I–III of untreated WT, untreated PINK1^{B9} and, as L^+/A^+, 0.1, 1 and 10% *Mpe*- and 0.01% L-Dopa-treated PINK1^{B9} mutants. Cohorts of 30 flies from each experimental group were subjected to the

assay. Flies were placed individually in a vertically-positioned plastic tube (length 10 cm; diameter 1.5 cm) and tapped to the bottom. Climbing time was recorded upon crossing a line drawn at 6 cm from the bottom. The number of flies that could climb unto, or above, this line within 10 seconds was recorded and expressed as percentage of total flies. Data were expressed as average + SEM from at least three separate experiments. The statistical evaluation was made by two-way ANOVA ($p<0.05$) followed by HSD post-hoc test.

Electroantennograms (EAGs) recordings

In vivo electroantennogram recordings (EAG) were performed following a previously described protocol [4]. Briefly, live adult WT *Dm* and untreated, *Mpe*- and L-Dopa-treated $PINK1^{B9}$ from group II (n=12/each strain) were singly positioned under the view of an Olympus BX51WI light microscope (Olympus, Tokyo, Japan). Electrodes were silver wires inserted in glass capillaries filled with a saline solution (NaCl 150 mM).The recording glass electrode was positioned on the tip of the left antenna while the reference was pierced through the compound eye. The EAG signal was amplified with an AC/DC probe and then acquired with an IDAC-4 interface board (Syntech, Hilversum, NL). The antennae were constantly blown by a flow of charcoal purified and humidified air (speed 0.5 m/s) via a glass tube. Odor stimuli were administered by injecting a puff of purified air (0.5 s at 10 mL/s airflow) through the pipette using the stimulus delivery controller (Syntech, Hilversum, NL).

Odor stimuli were prepared in 3 step-dose concentration (0.01, 0.1, and 1% v/v) diluted in hexane. Odor stimulus, 1-hexanol, was chosen according to Fishilevich and Vosshall [20], for its well-known stimulant activity in *Dm*. Mean values of EAG amplitude were calculated and then analyzed by comparing the results obtained in untreated $PINK1^{B9}$, *Mpe*- and L-Dopa-treated flies with matched WT. The significance of differences was tested by one-way ANOVA (followed by HSD post hoc test) with a threshold level of statistical significance set at $p<0.05$. EAG results are expressed as average values ± S.E.M and represented by histograms.

Olfactory behavior

Free-walking bioassay was performed following the experimental procedures used by Dekker et al. [18]. In particular, group II WT, untreated PINK1^{B9} and, as L^+/A^+, 0.1% *Mpe*- and 0.01% L-Dopa-treated PINK1^{B9} mutants were given the opportunity to choose between vials containing water with or without odor. Two 4 mL glass vials were placed symmetrically in a large petri dish (arena) and then fitted with truncated pipette tips. The vials were filled with 300 µL of water with 0.25% Triton X (Sigma-Aldrich, Milan, Italy) with or without the odorant (0.1% (v/v) 1-hexanol; Sigma-Aldrich, Milan, Italy). As mentioned above, in order to allow detection of possible *Mpe's* effects independently from the circuit, octopaminergic -for appetitive- and dopaminergic -for aversive stimuli [19], 1-hexanol was chosen, according to Fishilevich and Vosshall [20], because the mechanism(s) of olfactory transduction signal involve several glomeruli and complex neural pathways. Flies were starved for 8 hours prior to starting the experiments. These, done in triplicate, were performed in controlled environmental conditions (n=12 bioassays/each experimental group of flies; n=20 flies/arena). The assays lasted 18 hours, a streamlined range of time to overtake the possible influence of motor impairment in mutants. The dehydration of flies was prevented by placing a cotton ball with 3 mL of water in the arenas. Data obtained were expressed as average of percentages of flies reaching the 1-hexanol or water trap and statistically evaluated by one-way ANOVA ($p<0.05$) followed by HSD post-hoc test.

Electron microscopy analysis

Group II WT, untreated PINK1^{B9} and, as L^+/A^+, 0.1% *Mpe*-treated PINK1^{B9} mutants were anesthetized using carbon dioxide and carefully decapitated. The brains and the thoracic ganglia, once rapidly removed, were fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, washed several times in the same buffer, post-fixed in 1% osmium tetroxide in distilled water for 2 hours, and stained overnight at 4°C in an aqueous 0.5% uranyl acetate

solution. After several washes in distilled water, the samples were dehydrated in a graded ethanol series, and embedded in SPURR resin. To identify the antennal lobes (ALs), semi-thin coronal sections of the whole brains were cut with a Leica EM UC6 ultramicrotome, stained with toluidine blue and observed with a Leica DM2700 P light microscope. Sections of about 70 nm corresponding to portions of the ALs and thoracic ganglia were cut with a diamond knife on a Leica EM UC6 ultramicrotome. Transmission electron microscopy (TEM) images were collected with a FEI Tecnai G2 F20 (FEI Company, The Netherlands) and a Jeol JEM 1011 (Jeol, Japan) electron microscopes, working respectively at an acceleration voltage of 80 and 100 kV, and recorded with a 1 and 2 Mp charge-coupled device (CCD) camera (Gatan BM Ultrascan and GatanOrius SC100, respectively). T-bars density (expressed as number of T-bars/m^2) in both ALs and thoracic ganglia presynaptic boutons was assessed on a total of ten animals (three WT, three untreated $PINK1^{B9}$ and four 0.1% *Mpe*-treated $PINK1^{B9}$ mutants). 459 and 683 T-bars were randomly sampled respectively in the ALs and the thoracic ganglia on a total 496 non-overlapping micrographs at a final magnification of 6000, corresponding to a total sampled area of more than 6000 μm^2. T-bars were unambiguously identified at presynaptic active zones by the presence of T-shaped electron-dense projections typically tethered by a large number of presynaptic vesicles.

The number of damaged mitochondria within ALs (expressed as percentage of the total number of mitochondria/sampled area) was evaluated in WT, untreated $PINK1^{B9}$ and 0.1% *Mpe*-treated $PINK1^{B9}$ mutants. More than 3000 mitochondria were randomly sampled on 191 non-overlapping micrographs at a final magnification of 4000, corresponding to a total sampled area of more than 5000 μm^2. Damaged mitochondria were recognized for the presence of swollen external membrane, clearly fragmented cristae and inhomogeneous electron transparent mitochondrial matrix. The mean differences were tested using a two tailed *t*-test and a $p<0.01$ level was considered statistically significant.

Protein extraction and western blot analysis

Group II WT, untreated PINK1^{B9} and, as L^+/A^+, 0.1% *Mpe*- and 0.01% L-Dopa-treated PINK1^{B9} mutants flies were collected and immediately stored at −80°C. Head lysate preparations of adult males were performed by homogenization in RIPA buffer (9.1 mmol/L dibasic sodium phosphate, 1.7 mmol/L monobasic sodium phosphate, 150 mmol/L sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate [pH adjusted to 7.4]) containing fresh protease inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA). Two centrifugations were performed at 4°C at 10,000 g for 15 minutes, before protein quantification by DC Protein assay (Biorad, Hercules, CA, USA). 20 µg of proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis using the mini-PROTEIN 3-electrophoresis module assembly (Biorad, Hercules, CA, USA) and then transferred to immobilon-polyvinylidenedifluoride membranes (Amersham Biosciences). The membranes were incubated with primary antibodies overnight at 4°C. Immune complexes were detected with horseradish peroxidase–conjugated secondary antibodies and chemiluminescence reagents (ECL, Amersham Biosciences) and visualized by Image Quant LAS 4000. Densitometric analysis was performed by Image Studio Lite software for quantitative assessment.

Primary antibodies used in this study were against nc82 (1:100 dilution, DSHB); Tyrosine Hydroxylase (1:1000 dilution, MAB 318 Merk Millipore); actin (1:100, sc1616 Santa Cruz Biotechnology); Horseradish-peroxidase–conjugated secondary antibodies were purchased from Life Technologies. Statistical significance of the results was evaluated by one-way ANOVA ($p<0.05$) followed by a HSD post-hoc test.

Results

Effects of *Mucuna pruriens* and L-Dopa on life span of PINK1^{B9} mutants

As shown in Fig. 1A, in agreement with our previous report [4], PINK1^{B9} mutants displayed a significantly shorter lifespan with respect to WT flies. To assess the ability of *Mpe* to affect lifespan of PINK1^{B9} mutants, they were supplied *Mpe* at different concentrations (0 (untreated), 0.1, 1 and 10% w/w in their standard diet) both as adults only (L^-/A^+) (Fig. 1B and Fig. S1A), and as larvae and adults (L^+/A^+) (Fig. 1C and Fig. S1B). The effects of L-Dopa (supplied as L^+/A^+ at the concentration, 0.01%, at which is present in the *Mpe* 0.1%) on life span of PINK1^{B9} are also reported in Fig. 1D. The comparison between untreated and *Mpe*-treated PINK1^{B9}, as shown by Kaplan-Meier survival curves, revealed a statistically significant effect of *Mpe* on lifespan of PINK1^{B9} mutants only when L^+/A^+ flies were fed 0.1% *Mpe* (Fig. 1C, $p<0.05$ by Gehan-Breslow-Wilcoxon test). No effect was observed following the L-Dopa administration in L^+/A^+. As shown in Fig. S1A, no significant effects were detected in in L^-/A^+ flies, no matter the concentration tested, nor in L^+/A^+ flies fed 1% or 10% *Mpe* enriched standard diet (Fig. S1B).

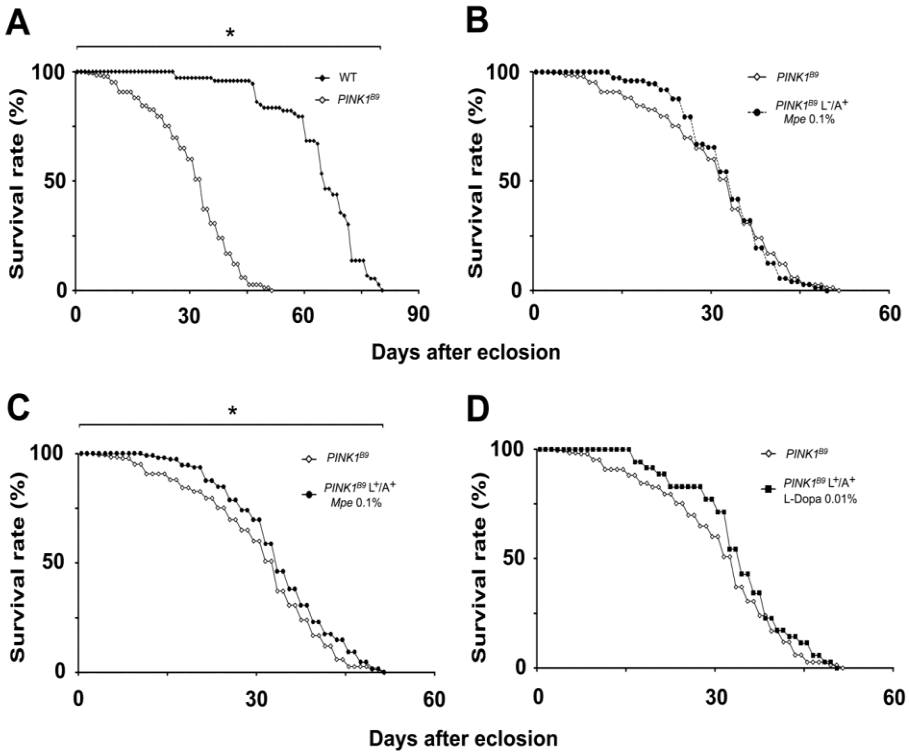

Figure 1. Effects of *Mpe* and L-Dopa on lifespan.

(A): Lifespan, expressed as % survival rates, of wild type (WT) and PINK1^{B9} flies. (B) and (C): Lifespan of PINK1^{B9} treated with *Mucuna pruriens* extract (*Mpe*) 0.1%,only when adults (L^-/A^+) (panel B) or from their larval stage to the end of their life-cycle (L^+/A^+) (panel C), respectively, as compared to lifespan of untreated PINK1^{B9} flies. (D): Lifespan of PINK1^{B9} flies treated with L-Dopa (L^+/A^+) 0.01%. *indicates $p<0.05$ at Kaplan-Meier survival curves (Gehan-Breslow–Wilcoxon - GraphPad Prism 5.01) between WT and untreated PINK1^{B9} (A) and between untreated PINK1^{B9} and PINK1^{B9} fed *Mpe* 0.1% (C).

Mucuna pruriens rescues impaired climbing behavior of PINK1^{B9} mutants

To investigate the locomotor ability the negative geotaxis assay, as described previously [17], was used. An impairment of climbing behavior was observed in untreated PINK1^{B9} at different age steps (I: 3–6; II: 10–15; III: 20–25 days old) with a worsening trend with aging, while WT flies fulfilled the evaluation criterion without differences among age groups.

As shown in Fig. 2A, the mutants took longer times to accomplish the task than the WT ($p<0.001$). The *Mpe* 0.1% treatment significantly ameliorated the climbing activity in mutants and also reduced the worsening trend with aging although the score obtained by treated mutants still remained higher than that measured in WT. Interestingly, the climbing time of L-Dopa-treated mutants from groups I and III did not significantly differ with respect to age-matched untreated PINK1^{B9}, the performance of only group II flies being significantly ameliorated.

As shown in Fig. 2B, L^+/A^+ 1% *Mpe*-treated mutants reached similar rescue of climbing activity as observed in 0.1% *Mpe*-treated ones only when tested at early ages (groups I and II). On the other hand, 10% *Mpe* administration failed to significantly ameliorate motor behavior in groups I and III with respect to untreated PINK1^{B9} mutants, while a significant effect was detected in treated flies from group II. We also considered the percentages of flies that were able to complete the test and the results are depicted in histograms shown in Fig. 2C and D. In this respect, most of WTs of all age steps (97–98%) were able to complete the test, while only 76% of PINK1^{B9} from group I, 46% from group II and 36% from group III accomplished it, showing a clear age-dependent worsening. Administration of 0.1% *Mpe*, as L^+/A^+, greatly rescued PINK1^{B9} mutants (groups I–III) from motor impairment and restored to WT values the percentages (86–94%) of flies able to accomplish the task according to the evaluation criterion (10 sec). Furthermore, at variance with the above results, the effects of L-Dopa worsened over time. In particular, 0.01% L-Dopa administration determined a decrease of the

number of flies able to complete the task showing a negative trend with aging. In fact, percentages of flies were 91%, 82% and 62%, in groups I, II and III, respectively.

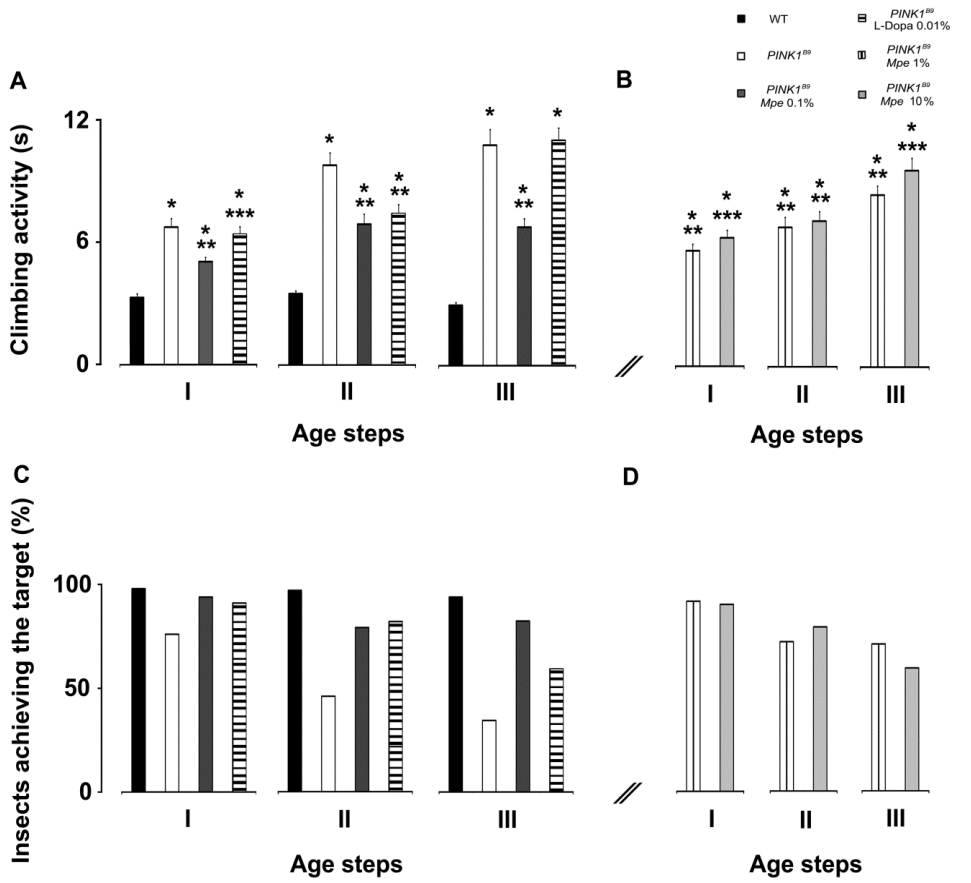

Figure 2. Effects of *Mpe* and L-Dopa on climbing activity.

(A): Climbing activity of adult males wild-type (WT), untreated PINK1^{B9}, PINK1^{B9} treated with *Mucuna pruriens* extract (*Mpe*) 0.1% and PINK1^{B9} treated with L-Dopa 0.01% (L-Dopa 0.01%). (B): Climbing activity of PINK1^{B9} adult males treated with *Mpe* 1 and 10% as compared with WT and untreated PINK1^{B9}. (A) and (B): Treatments were administered to flies from their larval stage to the end of their life-cycle (L^+/A^+) and their effects were assayed at three different age steps (I: 3–6; II: 10–15; III: 20–25 days) of flies' life-span. Values are average +

SEM. *indicates $p<0.05$ at two-way ANOVA followed by HSD post-hoc test as compared to WT; **indicates $p<0.05$ at two-way ANOVA followed by HSD post-hoc test as compared to PINK1^{B9}; ***indicates $p<0.05$ at two-way ANOVA followed by HSD post-hoc test as compared to PINK1^{B9} *Mpe* 0.1%. (C) and (D): Percentages of adult males WT, PINK1^{B9}, *Mpe* 0.1%, L-Dopa 0.01% (C) and *Mpe* 1 and 10% (D) that could climb unto, or above, the line drawn at 6 cm from the bottom of the tube within 10 seconds.

Mucuna pruriens and L-Dopa effects on the EAG amplitude

As expected, the olfactory stimulations of flies' antennae elicited responses with the typical EAG wave form, i.e. a rapid depolarization followed by a slower recovery phase, ending with the hyperpolarized wave before complete reversal to the baseline.

The results, summarized in Fig. 3A and 3B, show the olfactory response to 1-hexanol (0.01, 0.1 and 1%) elicited in WT, untreated PINK1^{B9}, 0.1% *Mpe*- and 0.01% L-Dopa-treated mutants from age group II. In details, the average EAG signal amplitudes evoked by stimuli were significantly lower in PINK1^{B9} specimens in respect to WT thus substantially confirming data previously reported [4]. The stimulation with 1-hexanol at 1% did not elicit a significant increase in the EAG amplitude as compared with the stimulation at 0.1% in all strains of flies with the exception of mutants flies treated with L-Dopa 0.01%. This result indicates that at the highest odor concentrations (0.1 and 1%) a saturation of response was reached by all groups but by the L-Dopa treated mutants. Besides, we observed that the responses to stimuli in WTs elicited a greater hyperpolarized phase in the EAGs (Fig. 3B and S2).

Even if a positive trend in treated mutants exists in the signal amplitude in response to 1-hexanol, a statistical difference between untreated, *Mpe*- and L-Dopa-treated PINK1^{B9} was not detected. The lowest odor concentration tested elicited a significantly higher response in WT as compared to the all strains of mutants

($p<0.05$). This difference shrank when the 0.1% concentration of odor was administered. A reduced response, although not statistically significant ($p>0.05$), was still detected in untreated mutants with respect to WTs ($p<0.05$), while treated flies showed on average an increased response with respect to untreated flies. The response measured in treated flies was therefore halfway between the highest of WTs and the lowest of untreated $PINK1^{B9}$. Samples of EAGs responses are shown in Fig. 3B and Fig. S2.

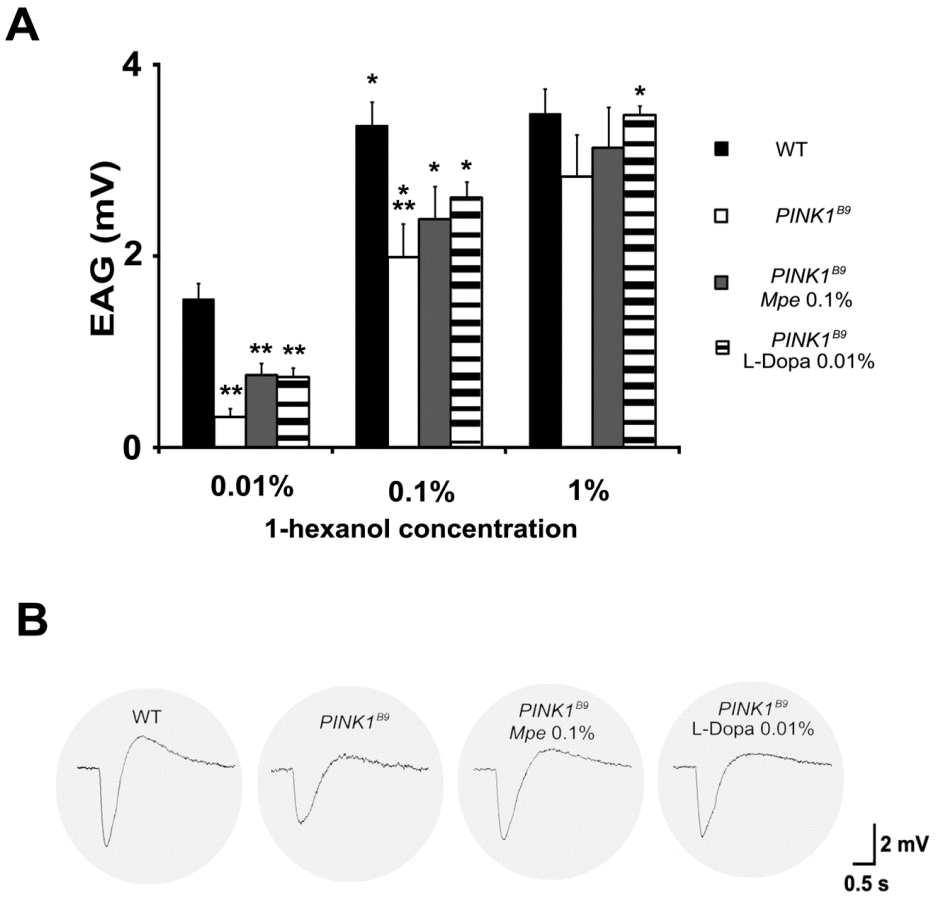

Figure 3. Electroantennogram responses to 1-hexanol.

Histograms in (A) show the dose-response relationship and their differences in signal for olfactory stimulations in WT, untreated PINK1^{B9} and in *Mpe* (0.1%)- and L-Dopa (0.01%)-treated PINK1^{B9}, recorded in flies from group II. As odor stimuli, the 1-hexanol was administered in a 3-step dose from 0.01 to 1% in hexane. Values are average + SEM. *indicates $p<0.05$ at one-way ANOVA followed by HSD post hoc test as compared to the previous concentration of the stimulus. **indicates $p<0.05$ at one-way ANOVA followed by HSD post-hoc test as compared to WT. (B) Samples of EAGs recordings in response to 1-hexanol 0.1%.

Mucuna pruriens rescues impaired olfactory behavior

The olfactory behavior assay was restricted to flies of group II, by testing the responses to 1-hexanol (0.1% v/v) of WT, untreated PINK1^{B9}, 0.1% *Mpe*- and 0.01% L-Dopa-treated, as L^+/A^+, PINK1^{B9} mutants. As expected, the analysis of the result, shown in Fig. 4, confirmed the olfactory behavioral impairment in PINK1^{B9} flies [4]. In fact, only 29.6±4.4% of mutant flies were odor-trapped, while the percentage of baited WT (52.9±6.6%) was significantly higher (p<0.004). PINK1^{B9} flies treated with 0.1% *Mpe* and 0.01% L-Dopa were able to reach the stimuli as WT controls (p>0.05). In fact, percentages of trapped flies were 45.2±5.8% and 44.2±3.6% for 0.1% *Mpe*- and 0.01% L-Dopa-treated mutants, respectively. Similar results were obtained concerning the numbers of trapped flies in the blank bait (H_2O) (p<0.05 between untreated PINK1^{B9}with respect to WT, 0.1% *Mpe*- and 0.01% L-Dopa-treated mutants).

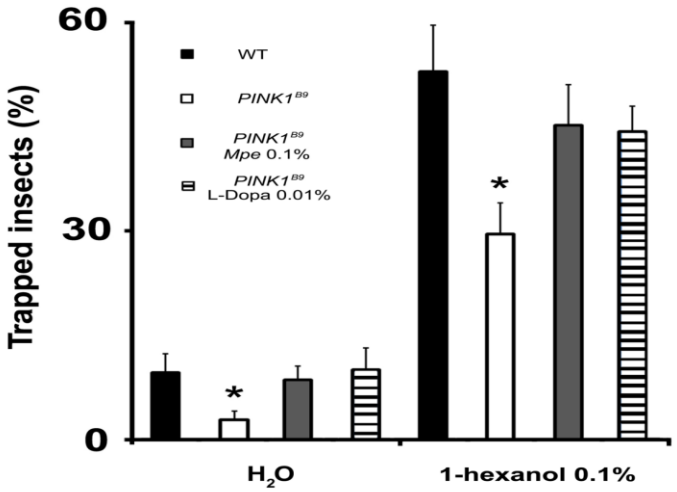

Figure 4. Effects of *Mpe* and L-Dopa on olfactory behavior.
Responses to 1-hexanol 0.1% and water (H_2O) of WT, untreated PINK1^{B9} and in *Mpe* (0.1%)- and L-Dopa (0.01%)-treated PINK1^{B9} flies. Values are average + SEM. *indicates p<0.05 at two-way ANOVA followed by HSD post hoc test as compared to WT, PINK1^{B9} *Mpe* 0.1%, PINK1^{B9} L-Dopa 0.01%.

Mucuna pruriens rescues loss of T-bars at active zones of presynaptic terminals and damaged mitochondria in the antennal lobes and thoracic ganglia

Transmission electron microscopy (TEM) analysis was restricted to flies of group II of untreated WT, untreated PINK1^{B9} and 0.1% *Mpe*-treated, as L^+/A^+, PINK1^{B9} mutants and results are shown in Fig. 5. A significant decrease of T-bars density was observed in the presynaptic bouton active zones of both ALs and thoracic ganglia of PINK1^{B9} mutants with respect to WT controls (panels A, B, E and F). More importantly, a significant increase of T-bars density was detected in the ALs and thoracic ganglia of PINK1^{B9} treated with 0.1% *Mpe*, as L^+/A^+, with respect to untreated PINK1^{B9} (panels A, B, E and F). Moreover the number of damaged, swollen and with clearly fragmented cristae, mitochondria was significantly lower in presynaptic boutons of ALs of PINK1^{B9} mutants treated with 0.1% *Mpe*, compared with untreated mutants (panels C, D and G).

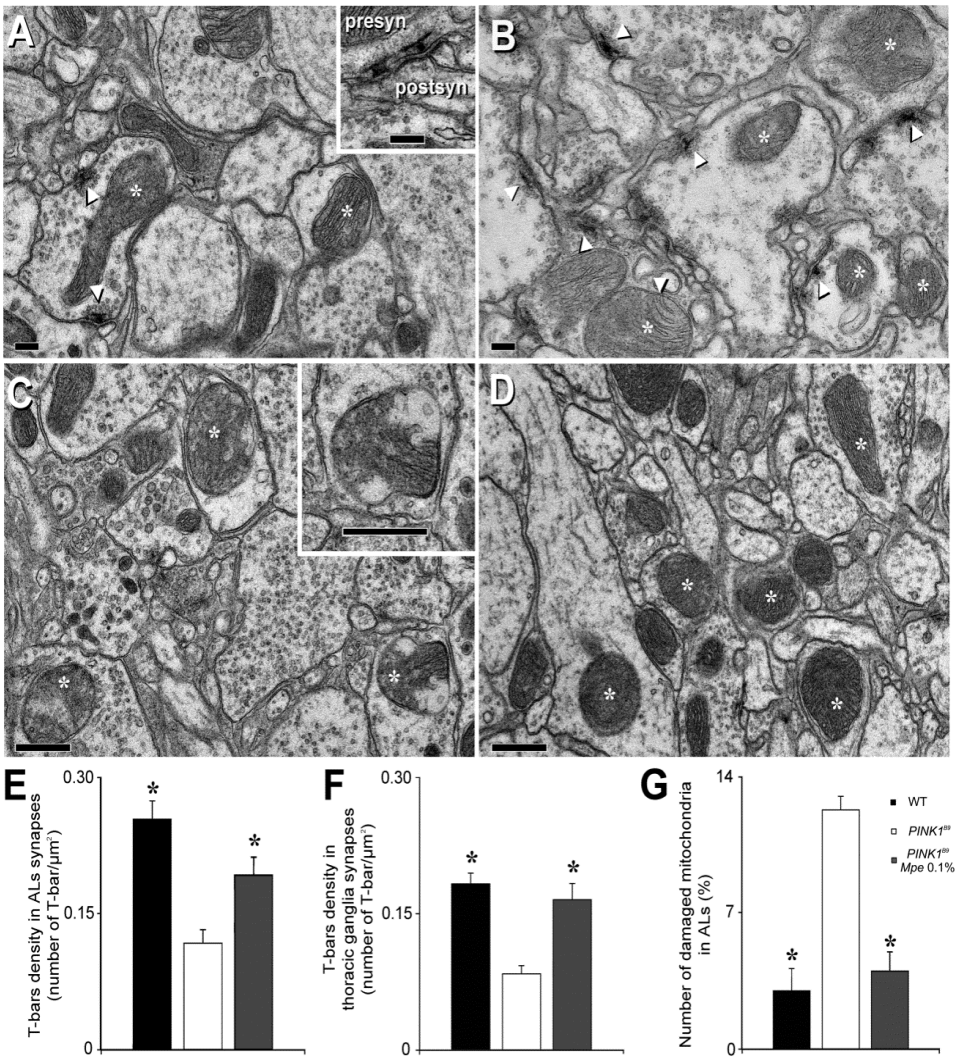

Figure 5. Effects of *Mpe* on T-bars and mitochondria in antennal lobes and thoracic ganglia.

Transmission electron microscopy (TEM) images of T-bars and mitochondria inside antennal lobes (ALs) of wild type (WT), untreated PINK1^{B9} and in *Mpe* (0.1%)-treated PINK1^{B9} flies. (A): T-bars in a presynaptic bouton of PINK1^{B9} ALs (arrowheads). Asterisks indicate mitochondria inside presynaptic boutons and neurites. Inset: high magnification of two T-bar in coronal section. (B): T-bars in presynaptic boutons of ALs of PINK1^{B9} *Mpe* 0.1% (arrowheads). Asterisks indicate mitochondria inside presynaptic boutons and neurites. (C): swelling on the

external mitochondrial membrane (at high magnification in the inset) and mitochondrial cristae widely degenerated (asterisks) in ALs of $PINK1^{B9}$. (D): Mitochondria of $PINK1^{B9}$ *Mpe* 0.1% (asterisks). (E): Presynaptic T-bar density in ALs of WT, $PINK1^{B9}$ and $PINK1^{B9}$ 0.1% *Mpe* flies. Values are average + SEM. *indicates $p<0.01$ at two tailed *t*-test with respect to $PINK1^{B9}$. (F): T-bar density in thoracic ganglia of WT, $PINK1^{B9}$ and $PINK1^{B9}$ 0.1% *Mpe* flies. Values are average + SEM. *indicates $p<0.01$ at two tailed *t*-test with respect to $PINK1^{B9}$. (G): Percentages of damaged mitochondria in ALs of WT, $PINK1^{B9}$ and $PINK1^{B9}$ 0.1% *Mpe* flies. Values are average + SEM. *indicates $p<0.01$ at two tailed *t*-test with respect to $PINK1^{B9}$. Abbreviations: postsyn: postsynaptic; presyn: presynaptic. Scale bars are 200 µm in A and B and 500 µm in C and D.

Mucuna pruriens and L-Dopa differentially affect whole brain bruchpilot (BRP) and tyrosine hydroxylase (TH) expression

Fig. 6 shows the results of western blot analysis of whole brain expression of BRP and TH of flies of group II WT, untreated $PINK1^{B9}$ 0.1% *Mpe*- and 0.01% L-Dopa-treated, as L^+/A^+, $PINK1^{B9}$ mutants. As shown in Fig. 6A, the expression of BRP and TH in untreated $PINK1^{B9}$ mutants was significantly lower ($p<0.05$) than in WT. Diet supply of 0.1% *Mpe* to $PINK1^{B9}$ mutants significantly recovered BRP and TH expression to WT controls levels ($p<0.05$) and these values did not differ statistically from those of WT. Notably, BRP and TH expression in $PINK1^{B9}$ mutants fed 0.01% L-Dopa resulted similar to BRP and TH expression in untreated $PINK1^{B9}$ mutants. ANOVA also revealed that both BRP and TH expression resulted statistically different as compared to their expression of both WT and $PINK1^{B9}$ mutants fed 0.1% *Mpe*.

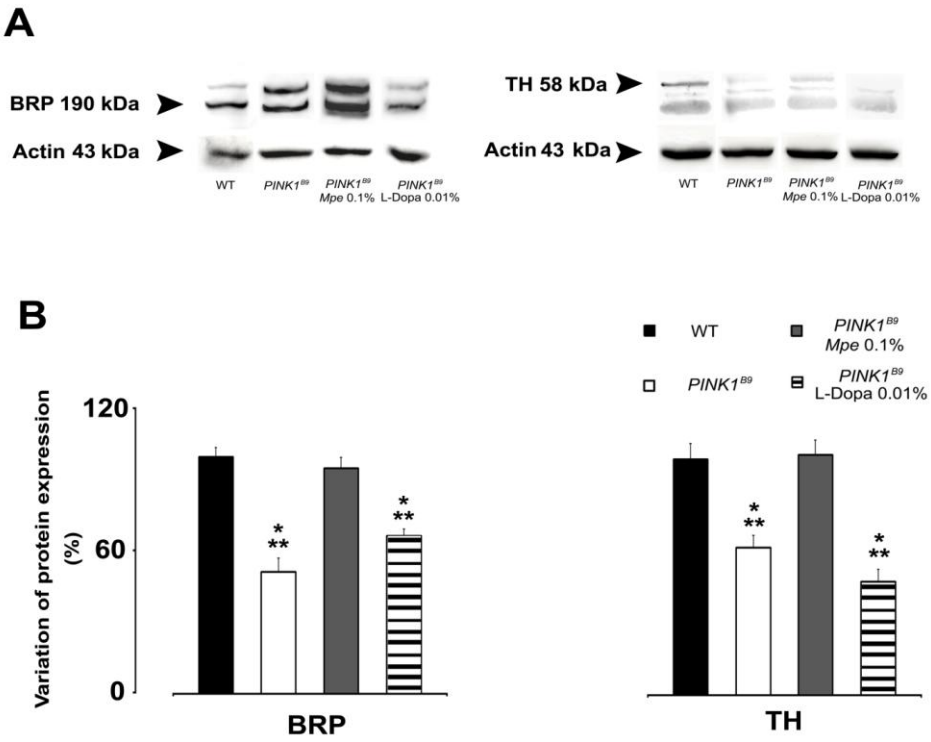

Figure 6. Effects of *Mpe* and L-Dopa on BRP and TH.

(A): Representative western blot analysis of head homogenates from adult wild type (WT), untreated PINK1^{B9} and in *Mpe* (0.1%)- and L-Dopa (0.01%)-treated PINK1^{B9} flies showing labeled bands of Bruchpilot protein (BRP), of Tyrosine hydroxylase (TH) and of the loading control actin (from top to bottom). (B): Percentage of protein expression variation of BRP and TH in WT, untreated PINK1^{B9} and in *Mpe* (0.1%)- and L-Dopa (0.01%)-treated PINK1^{B9} flies. Values are average + SEM. *indicates $p<0.05$ at one-way ANOVA with respect to WT; **indicates $p<0.05$ at one-way ANOVA (HSD post-hoc test) with respect to PINK1^{B9} *Mpe* 0.1%.

Discussion

This study was aimed at characterizing the effects of the standardized extract of *Mucuna pruriens* seeds, known for possible neuroprotective effects in neurotoxin-induced models of PD [21] [22] and reduced risk of dyskinesias [14], in a genetic fly model of PD, the PINK1^{B9} mutant *Dm* [23]. Notably, mutations at PINK1 gene model a number of features of early onset PD such as cell energy maintenance [24] and compromised olfactory and mitochondrial function [4] enabling in-depth investigations into physiopathology of PINK1^{B9}-related molecular, morphological and functional bases of PD.

The present results show that addition of 0.1% *Mpe* to the feeding medium of PINK1^{B9} mutants significantly a) improved climbing ability and olfaction, b) rescued compromised T-bars density and damaged mitochondria in the ALs and thoracic ganglia, c) restored to WT control values the expression of BRP and TH proteins. Moreover, these results suggest that *Mpe* is an effective medication with intrinsic ability of delaying the onset of chronic L-Dopa-induced long-term motor complications (Fig. 2A and B). These findings are in general agreement with previous studies reporting antiparkinsonian activity of *Mp* [14] [25] associated with reduced risk of dyskinesias, both in the clinical [26] and in the experimental [14] setting, and suggest that its antiparkinsonian effects may be due to components other than L-Dopa or that its components might have L-Dopa-enhancing effects [25] [26] [27] on one hand, as well as L-Dopa-induced dyskinesias (LID)-preventive effects, on the other. Intriguingly, PINK1^{B9} mutations have been linked to both autosomal recessive and sporadic forms of PD and, given the role of PINK1-*parkin* pathway in regulating mitochondrial function, our findings highlight its role as a potential target for the described actions of *Mpe* [30] on mitochondria. This interpretation finds further support in the observation of mitochondrial stress-dependent neurodegeneration [7] and dysfunction in PINK1 knock-out mice [31].

A large body of literature documents that mutations of PINK1 gene are associated with mitochondrial dysfunction. In particular, complex I deficiency [24] [32] has

been characterized as a mechanism of energy balance failure [33] resulting also in dramatic loss of dopaminergic neurons [34]. Although in the present study we did not attempt any direct measurement of mitochondrial energy impairment, this dysfunction was indirectly determined by assessing the number of damaged, swollen and with clearly fragmented cristae, mitochondria and we found that 0.1% *Mpe* administration could dramatically recover their morphology to that of WT controls (Fig. 5). This indicates that *Mpe* may play beneficial actions by interfering with the mechanisms responsible of energy production [24] or linked to maintenance of membrane gradients as well as to protection against the raise of reactive oxygen species within mitochondria [16]. In this regard, it is intriguing to observe that *Mp* has antioxidant properties [35] and it was suggested that its "rescue" properties may be due to increased complex-I activity and presence of nicotinamide adenine dinucleotide and coenzyme Q-10 [25]. This interpretation is also supported by the observation that also enhancement of nucleotide production, by feeding PINK1 mutant *Dm* with folic acid, results in rescued loss of mitochondrial mass and function [31].

Thus, on the basis of these reports and of our results it seems possible to speculate that *Mpe* administration interferes with the pathway regarding the mitochondrial rescue from oxidative stress but not on the complex apoptosis mechanism. In fact, the clock of the end of the life is not modified as also suggested by the results regarding the effect of *Mpe* on life span according to which the amelioration is slight, albeit significant. In agreement with Poddighe et al. [4], $PINK1^{B9}$ mutants showed steeper slope life span curves and overall shortened lifespan with respect to WT. *Mpe* significantly attenuated these conditions only when administered to L^+/A^+ at 0.1%, but not when administered to adults only (L^-/A^+) no matter the concentration tested (Fig. S1A), nor when administered with 0.01% L-Dopa. These results can be explained by taking into account that in *Drosophila* the cluster of neurons is manly conserved from larval to adult stage [28]. Conversely in L^+/A^+ mutants treated at the highest concentration administered (1–10%) even if not significant, a worsening trend was observed (Fig. S1B). The effects of *Mpe* on

flies' lifespan resemble those of the *Mpe* component, nicotine, described in a *Drosophila* autosomal recessive-juvenile model of parkinsonism [29].

In addition to the observed rescue of damaged mitochondria in PINK1^{B9} mutants treated with 0.1% *Mpe*, we observed that this treatment significantly recovered the expression of BRP and the reduction of T-bars density in both PINK1^{B9} ALs and thoracic ganglia, strengthening the tenet that BRP is crucial for the correct formation of T-bars at active zones [36]. Mutation-induced mitochondrial degeneration may also have led to the observed diminished expression of BRP, known to be critical also for neurotransmitter release [37] [38]. Accordingly, PINK1^{B9} mutants show degeneration of flight muscle and of dopaminergic neurons accompanied by locomotive defects [23] [39] [40]. Humphrey et al. [40] also showed that climbing deficit is related to dysfunction of dopaminergic cells and we found that PINK1^{B9} mutants also showed compromised motor capabilities as assessed by climbing behavior (Fig. 2). Hence, *Mpe*-increased expression of BRP may have increased the ability to release neurotransmitters that would result in improved locomotion, as suggested by Yellman et al. [41]. Therefore, our data suggest that the effects of *Mpe* treatment on BRP expression, climbing and T-bars in PINK1^{B9} mutants may represent the convergence toward an unified mechanism grounded on mitochondria functional rescue.

Olfactory dysfunction is a clinical early non-motor symptom of PD [3] and, accordingly, we observed loss of olfaction in PINK1^{B9} mutant *Dm* [4]. The physiopathology of olfactory dysfunction is not known. However, many studies have suggested involvement of dopaminergic system [42] [43]. In our investigation we observed improved olfactory responsiveness underlined by both behavioral and electrophysiological experiments. It is interesting to observe that the shape of EAG responses recorded in the WT revealed a dose-related hyperpolarizing part (Fig. S2). This observation seems in accordance with the stimulating power of 1-hexanol that is reported to involve both the appetitive and the aversive stimuli [19]. The EAG represent the summed activity of all antennal sensory neurons involved in stimulation. This activity can result in the EAG recordings in a depolarization and/or hyperpolarization signal, that is elicited according to the stimulating effect

of the odor tested as well as of its concentration. In details, in WT strain, the rapid depolarization is followed by a slow recovery phase at the lower concentration (0.01%) while at the highest concentration (1%), a greater hyperpolarizing phase was recorded. This phase could represent the activation of a pool of receptors that hyperpolarize when stimulated at this high concentration. With regards to this, a similar response was not present in untreated PINK1^{B9} (Fig. S2). Future electrophysiological analysis of the olfactory response should take into account both shape and amplitude whose variations might be a promising tool to study peripheral olfactory responses. Furthermore, our behavioural results show that PINK1^{B9} mutants have a decreased responsiveness to 1-hexanol and water (Fig. 4) that reveals an impairment of also other chemoreceptors such as hygroreceptors [44]. In other words the mutants seem to present a general sensory impairment. In agreement with study by Katzenschlager and Lees [45], suggesting a possible association between olfaction, increased TH and dopamine in the olfactory bulbs, we observed that PINK1^{B9} mutation-dependent impairment of olfaction behavior and whole brain TH expression were improved by *Mpe* treatment (Figs. 4 and 6, respectively). Surprisingly, L-Dopa administration on its own failed to recover TH expression to WT controls levels. However, since our analysis was done in whole brain homogenates, if analysis was restricted to the ALs, the homologous structures of human olfactory bulbs, we cannot exclude the possibility that L-Dopa would have brought different results. The physiopathology of LID is still largely unknown and LID has consistently been related to excessive DA release [46]. Furthermore, in parkinsonian non-human primates [47], L-Dopa produces LID without enhancing striatal DA release. Interestingly, Katzenschlager et al. [26] observed a reduced severity of dyskinesias after *Mp* as compared to levodopa/carbidopa combination and an increased DDC expression associated with LID has been reported in rats [48]. This intriguing prospective remains to be fully demonstrated in the *Dm* mutant model. In conclusion, our study confirms in this translational model the validity of *Mucuna pruriens* as a valuable approach for PD treatment, discloses mechanistic insights at the basis of its effects and confirms the use of PINK1^{B9} *Dm* as a model of PD that fulfills the required face, construct and predictive validity criteria to follow up on these investigations.

Supporting Information

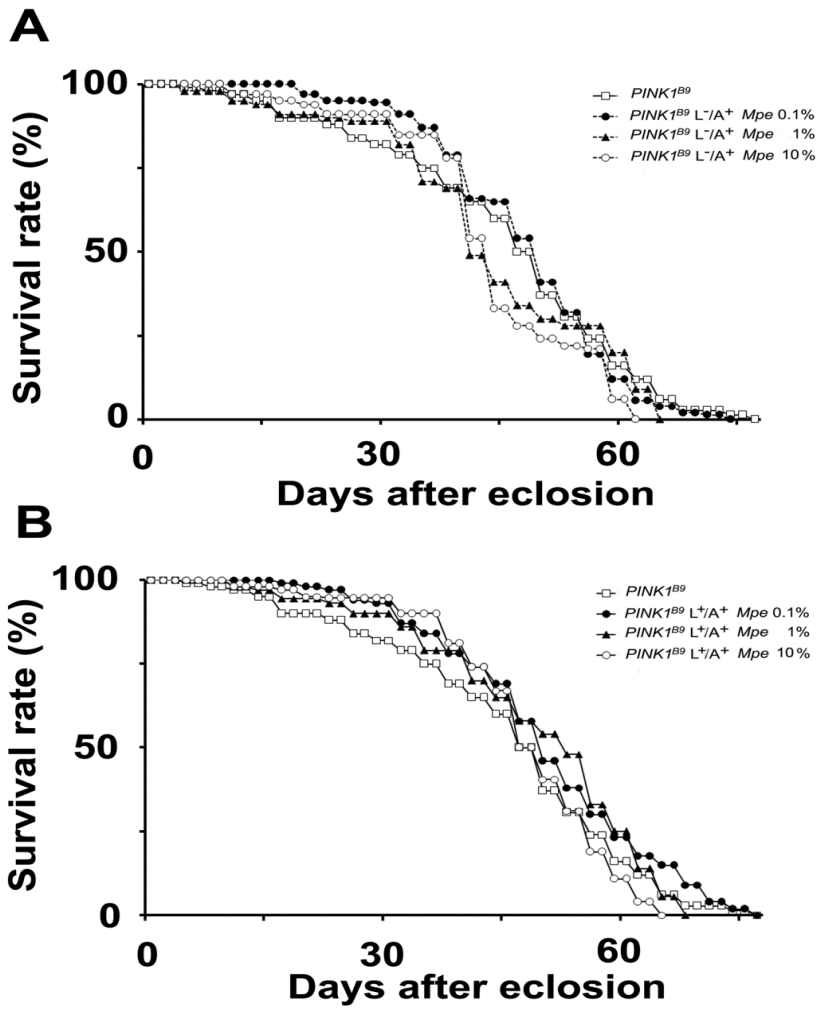

Figure S1. Effects of *Mpe* administered at different concentration on lifespan.
(A): Lifespan, expressed as % survival rates of untreated and *Mpe*-treated PINK1^{B9} at the 4 dose-step tested: 0, 0.1, 1 and 10% (w/w) only when adults (L^-/A^+).
(B): lifespan of untreated and *Mpe*-treated PINK1^{B9} at the 4 dose-step tested: 0, 0.1, 1 and 10% (w/w) only when adults (L^+/A^-).

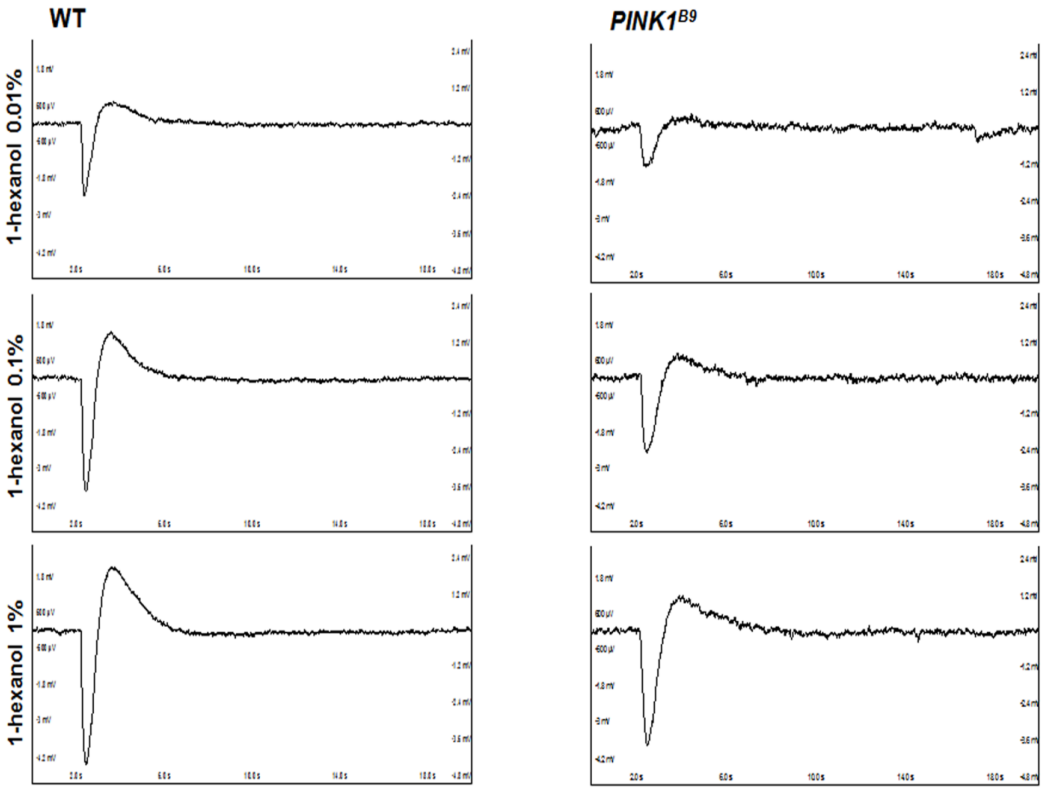

Figure S2. EAGs samples.

Dose-response relationships for olfactory stimulations in WT and PINK1^{B9} adult flies and their differences in signal amplitude and shape.

Acknowledgments

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Section 2

Drug effects of *Withania somnifera* in a LRRK2 *Drosophila* model

Functional and Morphological Correlates in the Drosophila LRRK2 loss-of-function Model of Parkinson's Disease: Drug Effects ofWithania somnifera (Dunal) Administration

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Abstract

The common fruit fly *Drosophila melanogaster* (*Dm*) is a simple animal species that contributed significantly to the development of neurobiology whose leucine-rich repeat kinase 2 mutants (LRRK2) *loss-of-function* in the WD40 domain represent a very interesting tool to look into physiopathology of Parkinson's disease (PD). Accordingly, LRRK2 *Dm* have also the potential to contribute to reveal innovative therapeutic approaches to its treatment. *Withania somnifera* Dunal, a plant that grows spontaneously also in Mediterranean regions, is known in folk medicine for its anti-inflammatory and protective properties against neurodegeneration. The aim of this study was to evaluate the neuroprotective effects of its standardized root methanolic extract (*Wse*) on the LRRK2 *loss-of-function Dm* model of PD. To this end mutant and wild type (WT) flies were administered *Wse*, through diet, at different concentrations as larvae and adults (L^+/A^+) or as adults (L^-/A^+) only. LRRK2 mutants have a significantly reduced lifespan and compromised motor function and mitochondrial morphology compared to WT flies 1% *Wse*-enriched diet, administered to *Dm* LRRK2 as $L^-/A^+$and improved a) locomotor activity b) muscle electrophysiological response to stimuli and also c) protected against mitochondria degeneration. In contrast, the administration of *Wse* to *Dm* LRRK2 as L^+/A^+, no matter at which concentration, worsened lifespan and determined the appearance of increased endosomal activity in the thoracic ganglia. These results, while confirming that the LRRK2 *loss-of-function* in the WD40 domain represents a valid model of PD, reveal that under appropriate concentrations *Wse* can be usefully employed to counteract some deficits associated with the disease. However, a careful assessment of the risks, likely related to the impaired endosomal activity, is required.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder [1] affecting 2% of the population over 60 years with an increasing incidence over age 85 [2]. The progressive loss of dopaminergic neurons in the substantia nigra of the midbrain leads to a deficiency of dopamine causing the typical motor symptoms such as tremor, bradykinesia and rigidity [3][4]. Although the etiopathogenesis is not fully understood and most cases seem sporadic, genetic variables play a key role in the predisposition to PD onset with at least 5 to 10% of PD patients clearly associated with genetic factors [5]. Indeed, since the seminal paper of Polymeropoulos et al. [6], which identified the first mutation related to PD in the alpha-synuclein gene, other genes involved in the etiology of familial forms of parkinsonism have been discovered [7–15]. Among them, the identification of several leucine-rich repeat kinase 2 (LRRK2) gene mutations has opened a novel scenario in Parkinson's disease genetics [16]. In fact, the G2019S LRRK2 mutation is the most common in Caucasian patients occurring in 1–2% of sporadic cases of PD [17][18], while other mutations, such as the G2385R variants contribute to the susceptibility to develop PD especially in Chinese patients [19]. LRRK2 encodes for a protein with a number of independent domains that is expressed, although at a low level, in all tissues. In the brain it is found in the cortex, striatum, hippocampus, cerebellum, and at the level of the dopaminergic neurons in the *substantia nigra* [20–23]. Most mutations of this gene are associated with a late onset Parkinsonism [15]. Mutations of the gene LRRK2 that elicit the disease occur at the level of the functional domain Roc (R1441C and G), at the level of the COR (Y1699C and R1628P) and of MAPKKK domains (G2019S and I2020T) and in only one of the WD40 domains (G2385R) [11][15][24]. This latter is known to be crucial in several basic cell functions such as vesicle sorting during endocytosis and exocytosis of synaptic vesicles as well as vesicle-mediated transport and cytoskeleton assembly [25][26]. The role of the WD40 domain is suggested to be crucial in controlling the LRRK2-regulated kinase activity having a critical role in the self-interaction and autophosphorylation-mediated mechanisms of neuronal toxicity [27]. Accordingly, deletion of this domain has been shown *in-*

vitro to cause the reduction of the kinase activity that is restored over-expressing the gain of function mutation of the gene [28].

Translational animal models are particularly useful in studying neuronal dysfunction and investigating the etiology and molecular aspects of neurodegenerative diseases. Among the animal species that significantly contributed to the development of these studies, the *Drosophila melanogaster* (*Dm*) represents a simple, yet experimentally and translationally powerful, organism that contributed significantly not only to the development of neurobiology but also to the progress of knowledge on neurodegenerative diseases. Notably, most of the genes implicated in familial forms of PD have a counterpart in this insect [29], and *Dm* mutants of PD have been genetically engineered to model key features of the human condition and have been successfully used in studying PD pathogenesis and in exploring new strategies of disease treatment [30–33]. Previous studies on LRRK2 PD form using *Dm* mutants (dLRRK2) did not clarify the role of LRRK2 in Drosophila, both in mutants *gain-of-function* for the kinase domain [15][34] and *loss-of-function* ($LRRK^{ex1}$ mutant) [35–37].

Fully effective medications to treat neurodegenerative diseases are currently lacking and the discovery of novel drug targets for long-sought therapeutics is a great challenge for researchers and clinicians. The use of plant extracts is largely employed worldwide in traditional medicine, constituting the basis of health care in many societies, to treat disparate pathologies [38]. The well-known therapeutic properties of the medicinal plants have been investigated in various animal models and the observations of such investigations have served in many instances as the basis of new drugs development [39][40][33]. A common plant of the Indian flora, also found in Southern Europe, including Sardinia (Italy), is *Withania somnifera* (*Ws*) Dunal. Its roots, used in Ayurvedic medicine for many central nervous system disorders [41][42], are a valuable herbal medication and the recognized pharmacological effects of *Ws*, such as anti-oxidant, neuroprotection and functional recovery made it of prime interest also in the treatment of PD [43][44].

The aim of this paper was twofold: on one hand to confirm the validity of the $LRRK^{ex1}$mutant [35][37], from now on named *LRRK2 WD40 loss-of-function* ($LRRK2^{WD40}$), as animal model of parkinsonism in *Dm*; on the other hand, to investigate the antiparkinsonian potential of the standardized methanolic extract of *Wse* roots on this mutant, as compared to *Dm* wild type (WT, Canton-S). To this end we tested lifespan, climbing activity, electrophysiological muscle parameters and subcellular ultrastructure (mitochondria and lysosomes) of the neurons involved in the motor circuitry, as those present in the *Dm* thoracic ganglia.

Materials and Methods

Fly Strains

For these experiments we used adult wild type (WT; Canton -S) and $LRRK2^{WD40}$ mutant ($LRRK^{ex1}$, #34750, from Bloomington Stock Center) *Drosophila melanogaster* (*Dm*) males. After emergence from pupae, WT and LRRK2 mutant males were separated. WT and mutant flies were reared on a standard cornmeal-yeast-agar medium in controlled environmental conditions (24–25°C; 60% relative humidity; light/dark = 12/12 hours). In addition, groups of mutant and WT flies were reared on a standard medium supplemented with the standardized methanolic extract of *Withania somnifera* root (*Wse*) (gift of Natural Remedies Ltd, Bangalore, India) at three different concentrations (0.1, 1 and 10% w/w) whereas other independent groups of WT and mutant flies were reared with 0.01% (0.5 mM) L-3,4-dihydroxyphenylalanine (L-Dopa). *Wse* and L-Dopa were added once the mixture was stirred for 10 min and had cooled down sufficiently [45]. All treatments were performed in two combinations concerning their life cycle: as adults (L^-/A^+) or from larvae and adults (L^+/A^+). Standard genetic procedures were used during the study.

Survival curves

With the aim of selecting the optimal *Wse'*s concentration to perform the whole study, *Dm* were grown on standard diet supplemented with different concentrations of *Wse* at 25°C. Cohorts of 60 flies (6 flies/tube) from each experimental group (i.e. *Wse*-untreated and *Wse*-treated WT, *Wse*-untreated and *Wse*-treated $LRRK2^{WD40}$) were monitored every 2 days for their survival. Mortality was analyzed using Kaplan-Meier survival curves and the statistical comparisons were made with a Gehan-Breslow-Wilcoxon test. All experiments were done in triplicate.

Climbing assay

The climbing assay (negative geotaxis assay) was used to assess locomotor ability [46]. Climbing data were obtained from different age groups (I: 3–6; II: 10–15; III: 20–25 days old) of untreated-WT, *Wse*-untreated and *Wse*-treated $LRRK2^{WD40}$ mutants. Cohorts of 30 flies from each experimental group were subjected to the assay. Flies were placed individually in a vertically-positioned plastic tube (length 10 cm; diameter 1.5 cm) and tapped to the bottom. Climbing time (s) was recorded upon crossing a line drawn at 6 cm from the bottom. The number of flies that could climb unto, or above, this line within 10 seconds was recorded and expressed as percentage of total flies. Data were expressed as average ± standard error of the mean (SEM) from three experiment replications. Statistically significant differences ($p<0.05$) among WT, *Wse*-untreated and *Wse*-treated $LRRK2^{WD40}$ were indicated. The statistical evaluation was made with a one-way analysis of variance (ANOVA) followed by LSD post-hoc test.

Electrophysiological recordings

At the time of the experiments, flies from group II were anesthetized by using CO_2 and carefully anchored to a wax support ventral side down, as previously reported [47][48] and placed underneath a stereomicroscope. In details, two tungsten stimulating electrodes, connected to a stimulator (Master 8, A.M.P.I,

Jerusalem, IL) and a stimulus isolation unit (DS2A, Digitimer Ltd., Hertfordshire, UK) were placed into both eyes of the fly in order to activate the Giant Fiber System (GFS). Stimulus intensity and duration were adjusted in every single experiment until the muscle response was detected; maximal stimulation intensity was not greater than 10 V, and stimulus duration was not greater than 0.5 ms. A ground tungsten wire was placed into the fly abdomen. A borosilicate recording electrode, shaped by a puller (P97, Sutter Instruments, Novato, CA) with a resistance of 40-50MΩ when filled with 3M KCl, was placed into the right or left backside of the fly in order to record Post Synaptic Potentials (PSPs) from the Dorsal Longitudinal Muscle fibers (DLMs). PSPs were recorded with an Axopatch 2-B amplifier (Axon Instruments, Foster City, CA), filtered at 0.5 kHz and digitized at 1 kHz. PSPs were recorded in bridge mode, measured using peak and event detection software pCLAMP 8.2 (Axon Instruments, Foster City, CA) and analyzed off-line by pCLAMP fit software (Axon Instruments, Foster City, CA). All recordings were obtained from at least 10 different flies belonging to each experimental group (i.e. WT, *Wse*-untreated and *Wse*-treated $LRRK2^{WD40}$). Experimenters were blind to the treatment.

Additional electrophysiological experiments were performed by applying a protocol consisting in a single GFS stimulation, delivered every 20 s, followed by PSPs recording. In this different set of experiments, the "frequency of following" was determined by delivering trains of 10 stimuli at frequencies of 100 Hz (with 10 ms between stimuli) or 200 Hz (with 5 ms between stimuli). Data are expressed as mean + SEM and one or two-way ANOVA followed by Tukey's or Bonferroni's post-hoc test ($p<0.05$), were used in order to determine significant differences between groups.

Electron microscopy analysis

Drosophilae WT, *Wse*-untreated and *Wse*-treated at 1% (L^-/A^+) and 10% (L^+/A^+) $LRRK2^{WD40}$from group II were anesthetized with CO_2 before brains and thoracic ganglia being rapidly dissected out and fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. After several rinsing in the

same buffer, the samples were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 h and stained overnight at 4°C in aqueous 0.5% uranyl acetate solution. Then the samples were washed several times in distilled water, dehydrated in a graded ethanol series and then embedded in SPURR resin. Roughly 70 nm thick sections, corresponding to portions of the thoracic ganglia and antennal lobes (ALs; homologous to olfactory bulbs in vertebrates), were cut with a Diatomediamond knife on a Leica EM UC6 ultramicrotome. (Leica Microsystems, Germany). Images were obtained with a FEI Tecnai G2 F20 (FEI Company, The Netherlands) transmission electron microscope equipped with a Shotky field emission gun operating at an acceleration voltage of 80 kV and recorded with a 2k x 2k Ultrascan Gatan CCD camera (Gatan, USA).

Results

Effects of *Wse* on the lifespan of LRRK2^{WD40}

Fig 1A shows that LRRK2^{WD40} mutants exhibit a significantly shorter life span than WT controls. To evaluate a possible toxic effect, *Wse* was tested at different concentrations (0.1, 1 and 10% w/w in their standard diet) as L^-/A^+ onto WT insects. In this respect, no significant effects were detected at any *Wse* concentration but 10% which significantly reduces the duration of life (Fig 1B) as compared to untreated WT controls.

To evaluate the influence of the extract of *Wse* on the duration of life of the LRRK2^{WD40} mutants that, as reported above, demonstrated a reduced life span in respect to untreated- WT, they were treated with *Wse* at the same concentrations as L^-/A^+ (Fig 1C) or as L^+/A^+ (Fig 1D). As shown by the Kaplan-Meier survival curves, administration of *Wse* induces a statistically significant increase, even if by a different extent, in the lifespan of mutants LRRK2^{WD40}, when the insects were fed in the adult stage only at 0.1% and especially 1% concentrations ($p<0.05$ Breslow-Gehan-Wilcoxon test). This restoring effect was lost when insects were treated at10% *Wse* L^-/A^+ (Fig 1C), and at any concentration when administrated to larvae and adults (L^+/A^+) LRRK2^{WD40}(Fig 1D). The overall results are in accordance with the hypothesis that *Wse* accumulation, due to high concentration and/or long period administration, can induce a possible toxic effect.

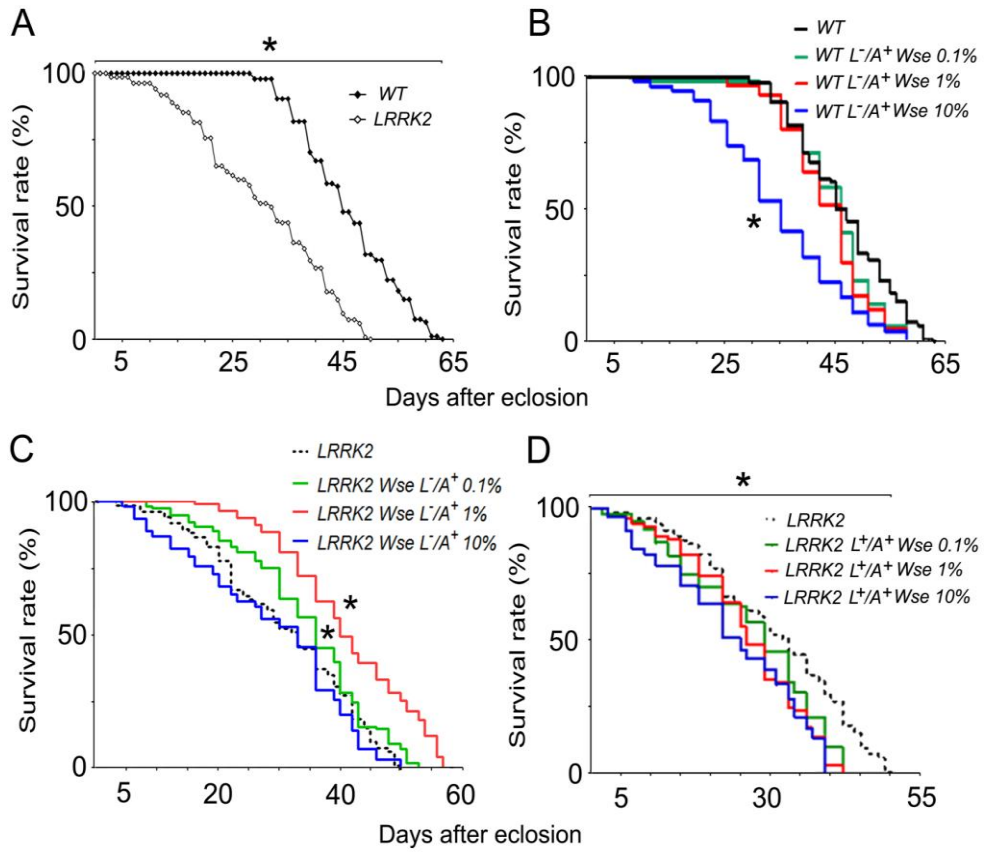

Figure 1. Effects of *Wse* on lifespan.

(A): Lifespan, expressed as % survival rates, of wild type (WT) and LRRK2 flies. (B): Lifespan of untreated WT compared to treated WT, only when adults (L^-/A^+), with *Wse*, 0.1%, 1% and 10%. (C) Lifespan of untreated LRRK2 mutants compared to treated LRRK2 mutants, only when adults (L^-/A^+), with Withania somnifera extract (*Wse*), 0.1%, 1% and 10%. (D) Lifespan of untreated LRRK2 mutants compared to treated LRRK2 mutants, from their larval stage to the end of their life-cycle (L^+/A^+), with *Wse*, 0.1%, 1% and 10%. *indicates $p<0.05$ at Kaplan-Meier survival curves (Gehan-Breslow–Wilcoxon—Graph Pad Prism 5.01), (A) untreated LRRK2 compared to untreated WT, (B) untreated WT compared to treated WT and (C-D) untreated LRRK2 compared to treated LRRK2.

Effect of *Wse* on the locomotor ability of LRRK2^{WD40}

According to results obtained following *Wse* administration paralleled with life span we decided to test *Wse* at 1% w/w effects on the climbing activity (negative geotaxis) of mutants. Fig 2A shows a significant increase in the climbing time in the threeage groups tested (I: 3–6; II: 10–15; III: 20–25 days old) of LRRK2^{WD40} as compared to subjects of the WT group ($p<0.001$) with a tendency to deterioration of the motor performance with aging. The exposure of LRRK2^{WD40} to 1% w/w *Wse* as L^-/A^+, induces, in groups I and II, the recovery of motor disability showing a significant decrease of time to climb compared to untreated mutants; a similar result was also found in insects of groups I-II that were fed 1% *Wse* from larvae and adults (L^+/A^+). On the other hand, *Wse* administration both to L^-/A^+ and L^+/A^+ failed to significantly ameliorate motor behavior in group III aged flies with respect to untreated mutants. L^-/A^+ flies treated with *Wse* showed a clear tendency toward rescue.

Moreover, as in zebrafish LRRK2 *loss-of-function*-WD40, another PD model in which a significant rescue of motor impairment after L-Dopa treatment was obtained [49] we also tested L-Dopa at 0.01% (0.5 mM) concentration in the feeding diet of both L^-/A^+ and L^+/A^+ mutant flies. The results presented in Fig 2B show that in *Dm* mutants the administration of L-Dopa rescued the impairment of climbing activity only in insects of group I, while worsening the performance in groups II-III.

We also considered the percentages of flies that were able to complete the test and the results are shown in S1 Fig. In this respect, results confirm the rescue of insects of groups I-II, treated with *Wse* both as L^-/A^+ and L^+/A^+, increase with respect to untreated ones. It is noteworthy that the percentage of insects of group II that completed the test was 75.2% in WT, 55.6% in untreated mutants, 80.6% in L^-/A^+ and 69.5% in L^+/A^+ *Wse*-treated mutants. In groupIII, the percentage of mutant insects achieving the target was the same no matter the treatment (being 40.9%, 43.4% and 37.9%, respectively) while more than 52% of WT insects accomplished the task, according to the evaluation criterion (10 sec).

The percentages of flies that were able to complete the test after L-dopa administration are shown in S1B Fig and demonstrate that the worsening was positively correlated to age and treatment duration. Thus, the effects of *Wse* as well as those of L-Dopa administration decrease with age but that of L-Dopa was drammatic. In fact, group III of L-Dopa-treated flies as L^+/A^+ the percentage achieving the target was only 15%.

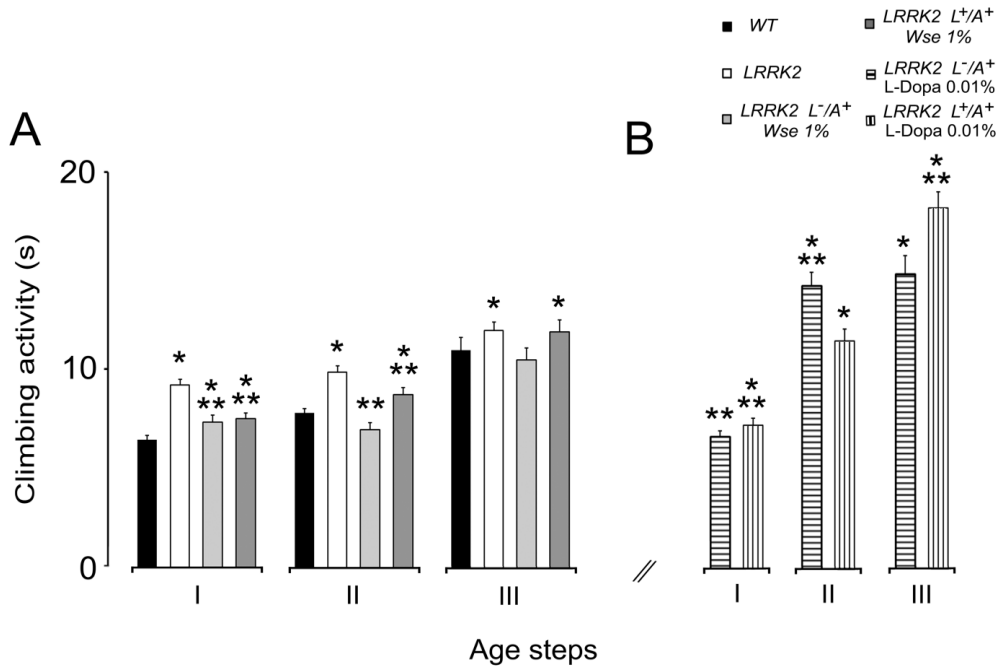

Figure 2. Effects of *Wse* on climbing activity.

(A-B): Climbing activity of LRRK2 adult males treated with *Wse* 1% as compared with WT and untreated LRRK2 (A) and climbing activity of LRRK2 adult males treated with L-Dopa 0.01% (0.5mM) as compared with WT and untreated LRRK2 (B). Values are average ± SEM. * indicates $p<0.05$ at one-way ANOVA followed by LSD post hoc test as compared to WT; ** indicates $p<0.05$ at one-way ANOVA followed by LSD post hoc test as compared to LRRK2.

Effects of *Wse* on the kinetic properties of evoked PSPs recorded from DLM in LRRK2^{WD40}

In order to detect potential changes in the function of the DLM neuromuscular junction of LRRK2^{WD40} flies, from group II, we first evaluated the basal kinetic properties of evoked PSPs (ePSPs) recorded from the DLM after GFS electrical stimulation. More precisely, we evaluated the response latency, that is the time between stimulation of the GFS and subsequent muscle PSP peak, and PSP peak amplitude, that is the maximal muscle depolarization from baseline value. Fig 3 shows that the basal properties of ePSPs recorded from DLM muscle of WT animals results in a latency of 1.84 ± 0.1 ms and in an averaged amplitude of 19 ± 3 mV.

Notably, LRRK2^{WD40} mutation results in a significant decrease (21%, $p< 0.05$) of ePSPs latency when compared to WT animals (Fig 3A and 3B). Such effect was no longer apparent in LRRK2 (L^-/A^+) flies that were treated with *Wse* 1%. Surprisingly, latency in LRRK2 treated flies was significantly higher with respect to both WT as well as untreated LRRK2 animals. No significant change was detected in PSP peak amplitude among flies from the different experimental groups (Fig 3A and 3C).

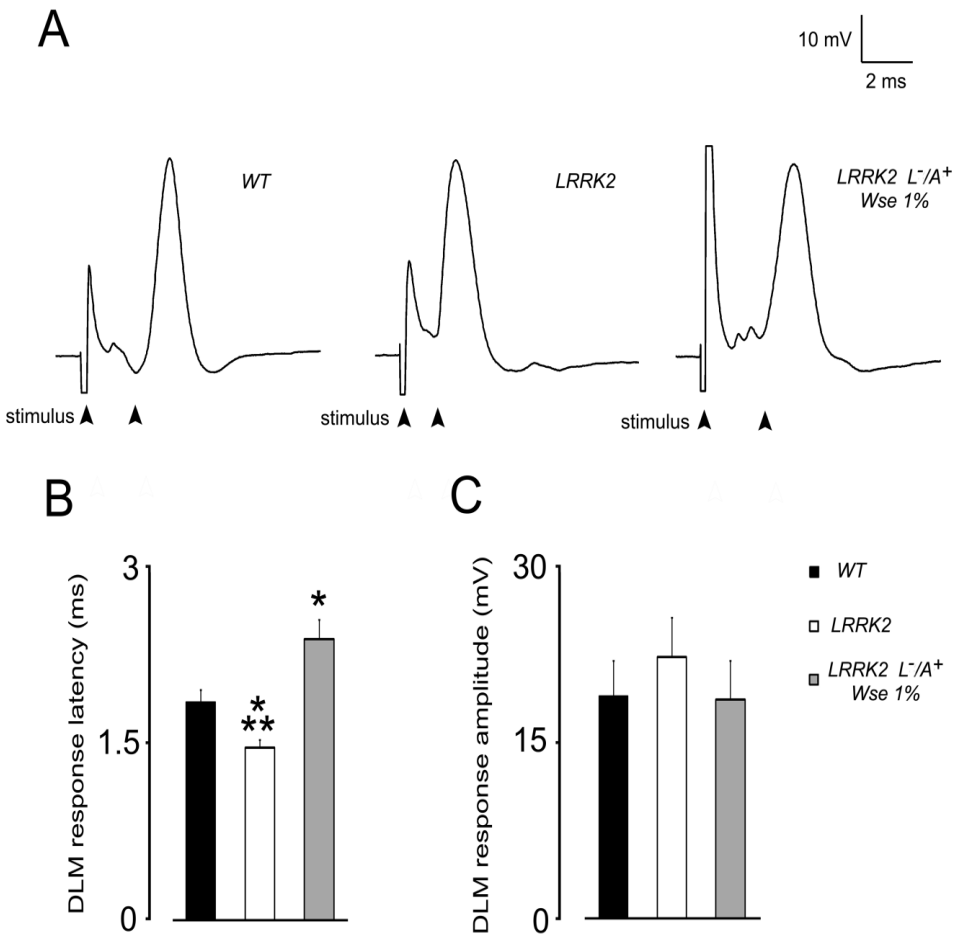

Figure 3. Effect of LRRK2 gene mutation and treatment with *Wse* 1% (L^-/A^+) on PSP latency and amplitude recorded from Drosophila DLM.

(A): Representative traces obtained from three different flies in which PSP latency is calculated as the time (ms) from stimulus application to the peak of PSP (black arrows). (B, C): Bar graphs represent the mean ± SEM of PSP latency (ms) and amplitude (mV) recorded from flies of the indicated experimental groups. *indicates $p< 0.05$ compared to WT, **indicate $p<0.05$ compared to treated LRRK2; one-way ANOVA, followed by Bonferroni post-hoc test.

Effects of *Wse* on the PSP responses to high frequency stimulation of GFS of LRRK2^{WD40}

We then tested flies by recording the "frequency of following" which consisted in applying a train of 10 stimuli at different frequencies (100 or 200 Hz) to GFS. As previously reported [48], in WT flies, a train of 10 stimulations at 100 Hz induced repetitive responses of DLM with minimal decrement of PSP amplitude as compared to the first PSP (Fig 4A and 4B).

The response to 100 Hz stimulation in LRRK2^{WD40} was not different from that observed in WT (Fig 4A and 4B). In contrast, the response to 100 Hz in *Wse*-treated LRRK2^{WD40} (L^-/A^+) flies revealed a significant decrement of PSP amplitude when compared to the first PSP (Fig 4A and 4B). At the higher frequency of electrical stimulation, the DLM responses of WT started to decrease in amplitude after the 2nd PSP with 200 Hz stimulations (Fig 4A and 4C).

The same protocol of recording at 200 Hz performed in untreated LRRK2^{WD40} flies showed that DLM responded to each of the 10 stimulations whose amplitude of PSPs was only slightly diminished (Fig 4A and 4C). In treated LRRK2^{WD40} (L^-/A^+) insects stimulations at 200 Hz elicited DLM PSPs which, similarly to WT flies, had amplitudes that decreased with respect to the first PSP. Two-ways ANOVA revealed a significant effect of the untreated-LRRK2^{WD40} group compared to WT when responding to the 200-Hz stimulation ($P<0.05$)

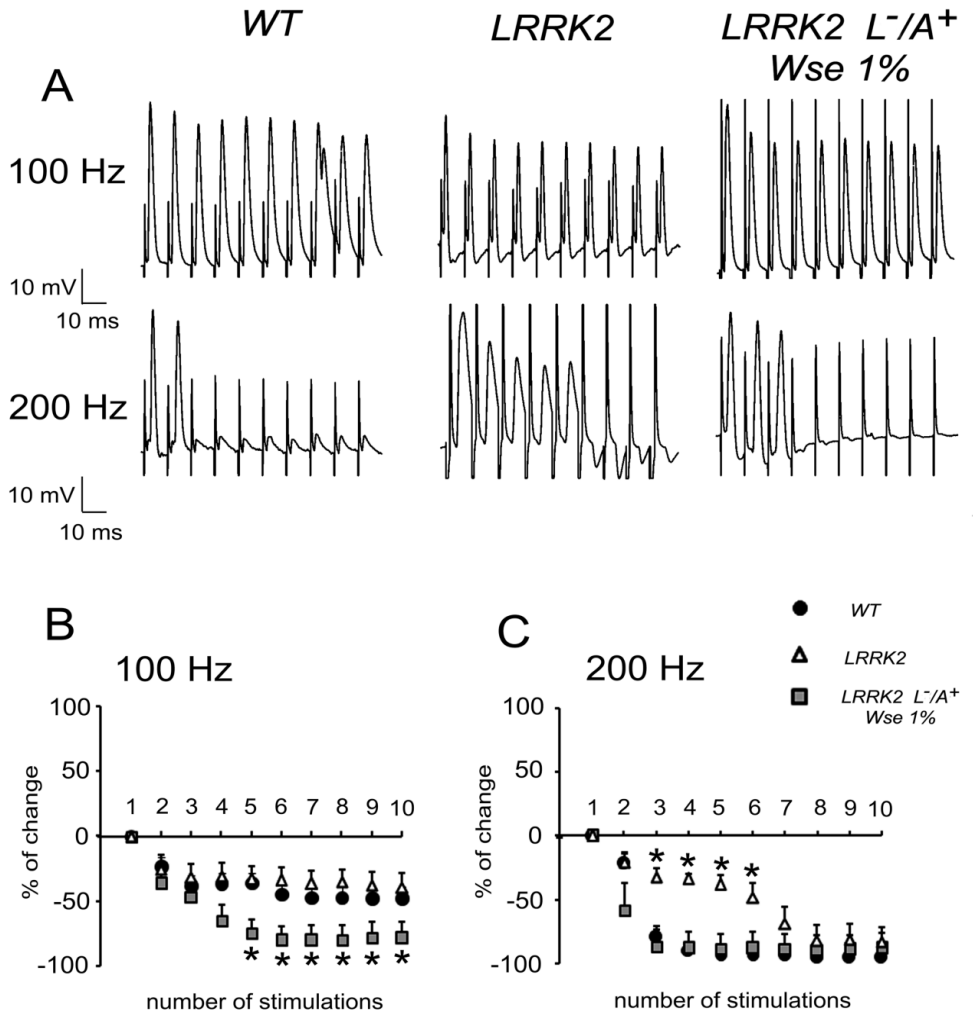

Figure 4. Effect of LRRK2 gene mutation and treatment with *Wse* on the "frequency of following" recorded in Drosophila DLM.

(A): Representative traces obtained from three different flies in which PSPs were evoked in response to 10 stimulations at 100 (top) or 200 Hz (bottom). (B,C): Scatter plot graphs showing the changes in PSP amplitude following stimulation at 100 (B) or 200 Hz (C). All values are expressed as the mean ± SEM of the % relative to the amplitude of the first PSP. *indicates $p < 0.05$ compared to WT and *Wse*-untreated LRRK2 (B) and compared to WT and *Wse*-treated LRRK2 (C), two-way ANOVA.

Effects of *Wse* on the subcellular morphology of LRRK2^{WD40}

Fig 5 shows representative transmission electron microscopy images of thoracic ganglia and antennal lobes (ALs) of untreated *Dm* LRRK2 mutants (A) and of 1% and 10% *Wse*-treated, as L^-/A^+ (B and C) and as L^+/A^+ (D-F), insects. In mitochondria of the thoracic ganglia of LRRK2 mutants, we observed regions with several damaged, swollen, and with clearly fragmented cristae, that we failed to find in the corresponding regions after treatment with 1% *Wse* (in Fig 5 compare A with B and C).

However, after treatment with 10% *Wse* L^+/A^+, we observed, in the corresponding regions of the thoracic ganglia, numerous altered mitochondria with a granular, irregularly shaped electron-dense material in their matrix (Fig 5D and 5E). Moreover after the same treatment we observed, in *Drosophila* LRRK2^{WD40}ALs numerous late endosomes/ phagosomes vacuoles inside presynaptic terminals and dendrites (Fig 5F).

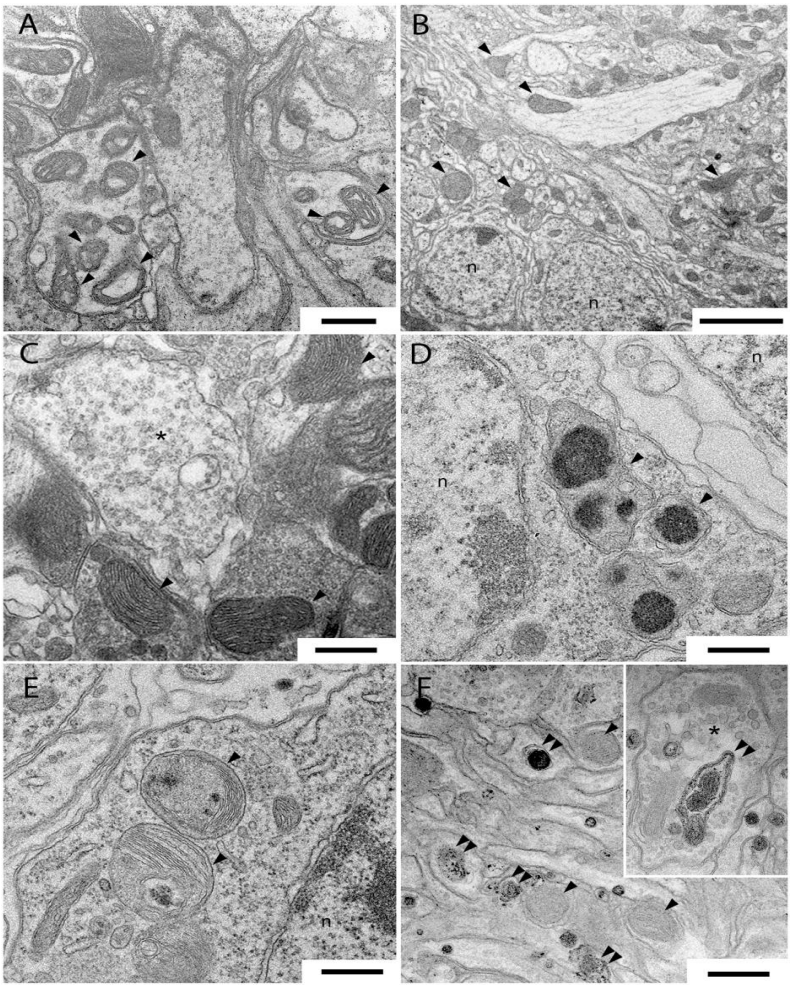

Figure 5. Samples of transmission electron microscopy images of thoracic ganglia and antennal lobes in Drosophila LRRK2 mutant (A) and after treatment with 1% in L^-/A^+ insects (B, C) and 10% L^+/A^+ (D-F) extract of *Wse*.

(A): abnormal mitochondria in the thoracic ganglia neuropil of Drosophila LRRK2. (B, C): conventional mitochondria in thoracic ganglia of Drosophila LRRK2 after treatment with 1% *Wse* L^-/A^+ imaged at low (B) and higher magnification (C). (D, E): abnormal mitochondria in Drosophila LRRK2 thoracic ganglia cell bodies after treatment with 10% *Wse* L^+/A^+. Note the irregular electron-dense substance clearly recognizable inside the mitochondria. (F and Inset): numerous endosomes are present inside the antennal lobes neurites of Drosophila LRRK2 after treatment with 10% *Wse*. Scale bars are 0.5 μm except in B that is 2.5 μm.

Discussion

One of the aims of the present study was to validate the use of LRRK2^{WD40} as a model of PD. In this respect, these mutant flies show reduced lifespan, and motor impairments (face validity) and mitochondrial dysfunctions (construct validity) that characterize Parkinsonism. Furthermore, this study was aimed at evaluating the action of the standardized extract of the roots of *Withania somnifera (Wse*) and its possible neuroprotective effects on the Parkinson's genetic model of *Drosophila melanogaster* LRRK2^{WD40}. Although almost all of the mutations in LRRK2 have a number of related features, these mutants object of the present study lack, in particular, the WD40 domain responsible for coding a protein chaperone known to be involved in a number of cellular functions such as cytoskeletal, neurotransmitter vesicular pathway and lyso-endosomal activities [25] The results presented here show that the addition of 1% *Wse* to standard diet of only LRRK2^{WD40} adults (L^-/A^+), but not of L^+/A^+, significantly **a)** increases their lifespan compared to untreated controls and **b)** improves their locomotor abilities and **c)**affects evoked electrophysiological parameters. Furthermore, in thoracic ganglia, under electron microscopy observation, we found that *Wse* administration dramatically rescued the mutation-related loss of mitochondrial structural integrity. Interestingly, *Wse* chronic administration to flies as L^+/A^+, no matter the concentration, induces a worsening of symptoms associated with parkinsonism and a further decrease of lifespan as compared to WT controls as well as to untreated LRRK2^{WD40} (Fig 1B)

The flight muscle degeneration accompanied by defects in motor activity [50–52] detected in our study is probably related to dysfunction of dopaminergic neurons. Accordingly, in a zebrafish model LRRK2 *loss-of-function* in the WD40 domain, it was previously reported a rescue of motor impairment following L-Dopa administration in the early larval stage from days post fecundation (DPF) 5 to 6 [49]. Notably, although this and our model of LRRK2 *loss-of-function* differ in a number of factors such as animal species, life period and duration of L-Dopa administration, the present results also demonstrate an improvement of motor

deficit (climbing activity) in the mutants of the group I treated as L^-/A^+. However, extension of the treatment to flies of group II and III did not rescue the mutation-dependent impairment but elicited a worsening in both L^-/A^+ and L^+/A^+ treated flies (Fig 2B).

The observed rescue of impaired motor ability by *Wse* administration to $LRRK2^{WD40}$ *Dm* while confirming the condition of mutation-dependent impaired motility, as shown in tests of climbing (Fig 2), also supports the suggestion that *Wse*'s effects might be attributable to increased neurotransmission [53][54]that would result in a better locomotion. Electrophysiological data showed that mutation of the LRRK2 gene was associated with a significant decrease in PSP latency when compared to WT animals, an effect that was no longer apparent in LRRK2 (L^-/A^+) flies that were treated with *Wse* 1%. However, no significant change of PSP peak amplitude was detected among flies from the different experimental groups suggesting that in $LRRK2^{WD40}$ mutants there is a higher probability of (but not necessarily an optimally coordinated) muscle contraction compared with WT without changes in muscle contraction *per se*. Surprisingly, *Wse* treatment was able to revert the effect of mutation making the response latencies recorded in LRRK2 (L^-/A^+) treated flies much higher as compared with both untreated LRRK2 and WT flies. The decrease in PSP latency together with the decreased responsiveness to high frequency stimulation observed in untreated-$LRRK2^{WD40}$ flies appears to well correlate with the motility impairment observed in these flies. As for the possible mechanism, Augustin and colleagues [48] reported that recording the "frequency of following", a GFS train stimulation at 200 Hz induced in WT a significant decrement of PSP amplitude relative to the first PSP because the intermediary synapses do not have sufficient time to recover between stimuli. Conversely, a stimulation train at 200 Hz performed in untreated $LRRK2^{WD40}$ flies showed that, relative to the first PSP, the amplitude of PSPs was only slightly diminished, starting from the second response, and treatment with 1% *Wse* made the responses similar to those observed in WT. Thus, the effect of *Wse* on the functional changes associated with the mutation clearly discloses a beneficial aspect of this treatment. At this time, we cannot

explain in deep details the abnormal effect of *Wse* treatment in LRRK2 flies (i.e. increased PSP latency and exacerbated effect on 100 Hz response vs WT), and this might at least in part be justified recalling the complexity of the projection pathway from the brain to the thoracic ganglion, where axons form electrical synapses with interneurons and the latter form chemical synapses on each motor neuron innervating the DLMs [55][56]. However, mutation of $LRRK2^{WD40}$ may be correlated with a significant impairment in neurotransmitter release from presynaptic terminals [25][57].

The impaired motility shown by the LRRK2 mutants is paralleled by the presence of scattered abnormal mitochondria in their thoracic ganglia, an observation corroborated by other studies that suggest the involvement of $LRRK2^{WD40}$ in mitochondrial homeostasis, responsible of mitochondrial degradation[58][59]. Intriguingly, the conventional mitochondrial morphology of $LRRK2^{WD40}$ flies observed after treatment with 1% *Withania* extract, and paralleled by an improvement in their motor capacity, suggests that *Wse* may also act suppressing mitochondrial dysfunction, as has been recently demonstrated for a green tea-derived catechin, epigallocatechin gallate (EGCG) [59] and as well as already demonstrated in the case of the mutant $PINK1^{B9}$ treated with the standardized seeds extract of another plant,*Mucuna pruriens* [33].

In conclusion, based on our results we can infer that the LRRK2 *loss-of-function* in the WD^{40}domain is a plausible model that recapitulates some of the essential features of Parkinsonism and that the extract of *Ws* can be usefully employed to counteract some deficits associated with this condition.
However, as demonstrated by Poddighe et al., [33] after *Mucuna pruriens* administration to *Dm* $PINK1^{B9}$ mutant model of PD, the use of a whole herbal extract requires careful assessment. In fact, the effects of *Wse* on $LRRK2^{WD40}$ might also be related to age (group I *vs*III), length of exposure (L^-/A^+ *vs* L^+/A^+) and *Wse* (0.1% *vs* 1% *vs* 10%) concentrations as suggested by the observation of its effects on climbing (Fig 2A and 2C) as well as on life duration (Fig 1C and 1D). Indeed, the negative effect of 10% *Wse* both on WT (Fig 1B) and on the *loss-of-function* $LRRK2^{WD40}$ mutant indicates that one or more components of the extract,

when administered chronically and at a concentration higher than optimal, may have toxic effects. This conclusion is supported by the observation that chronic administration of *Wse* to flies as L^+/A^+, no matter the concentration, and also at 10% to L^-/A^+, induces a worsening of symptoms associated with parkinsonism and a further reduction of lifespan as compared to WT controls and untreated $LRRK2^{WD40}$. This observation also indicates that *Wse* shows a concentration threshold, below which it does not work; b) has an optimal value for its effects; but c) whose effects at higher concentrations and/or after longer exposures became toxic. As discussed above, this suggests that *Wse* exerts its effects -as a drug- following a hormesis-like dose-response curve [60] and further highlights the need to assess the proper concentration of *Wse*. In this regard, the presence of numerous large sized lysosomes observed exclusively in the ALs of *Drosophila* $LRRK2^{WD40}$ treated with 10% *Wse*, corroborates its toxic effect, since lysosomes increases in number and size are one of the more common cause of degenerative brain disorders [61].

Supporting Information

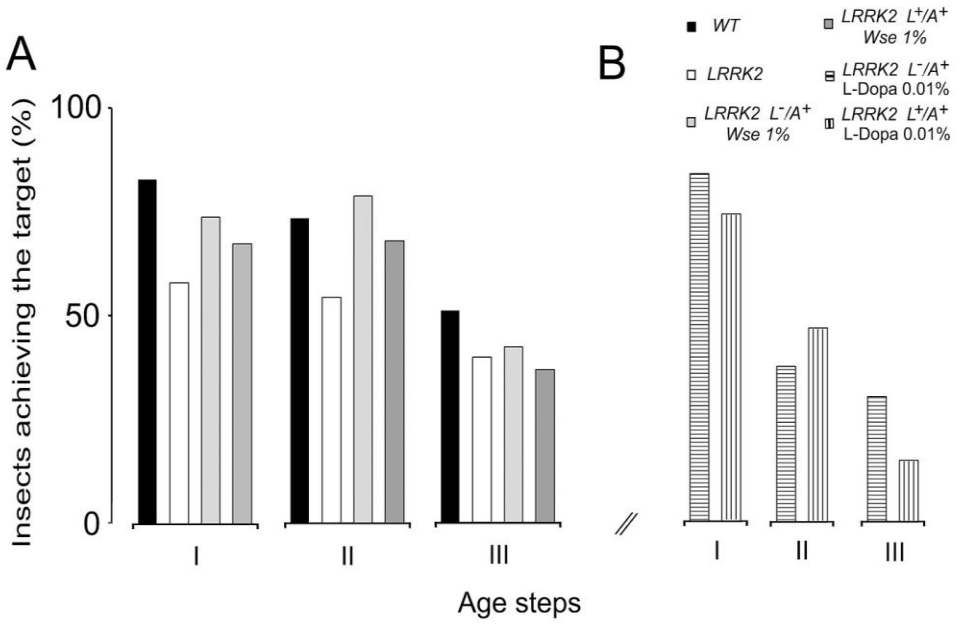

Figure S1 Percentages of insects able to achieve the test.

(A-B): Percentages of adult males WT, LRRK2, *Wse* 1% treated LRRK2 (A) and L-Dopa 0.01% (0.5mM) treated LRRK2 (B), that could climb unto, or above, the line drawn at 6 cm from the bottom of the tube within 10 seconds.Treatments were administered to flies both only when adults (L^-/A^+) and from their larval stage to the end of their life-cycle (L^+/A^+), and their effects were assayed at three different age steps (I: 3–6; II: 10–15; III: 20–25 days) of flies' life-span. Values are average ± SEM. * indicates $p<0.05$ at one-way ANOVA followed by LSD post hoc test as compared to WT; ** indicates $p<0.05$ at one-way ANOVA followed by LSD post hoc test as compared to LRRK2.

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Ph.D research outputs

Publications

Poddighe S, **De Rose F**, Marotta R, Ruffilli R, Fanti M, et al. (2014) *Mucuna pruriens* (Velvet bean) Rescues Motor, Olfactory, Mitochondrial and Synaptic Impairment in PINK1^{B9} Drosophila melanogaster Genetic Model of Parkinson's **Disease. PLoS ONE 9(10): e110802. doi:10.1371/journal.pone.0110802**

Solari P, Stoffolano JG Jr., **De Rose F**, Tomassini Barbarossa I, Liscia A (2015) The chemosensitivity of labellar sugar receptor in female *Phormia regina* is paralleled with ovary maturation: Effects of serotonin. **Journal of Insect Physiology 82: 38–45. doi:10.1016/j.jinsphys.2015.08.007 0022-1910**

De Rose F, Marotta R, Poddighe S, Talani G, Catelani T, Setzu MD, Solla P, Marrosu F, Sanna E, Kasture S, Acquas E, Liscia A (2016) Functional and morphological correlates in the Drosophila LRRK2 *loss-of-function* model of Parkinson's disease: drug effects of *Withania somnifera* (Dunal) administration. **PLoS ONE 11(1): e0146140. doi:10.1371/journal.pone.0146140**

De Rose F, Corda V, Solari P, Sacchetti P, Belcari A, Fanti M, Poddighe S, Kasture S, Solla P, Marrosu F, Liscia A Non-motor symptoms in a Drosophila model of Parkinson's disease: olfactory dysfunction in LRRK2 *loss-of-function* mutants **(*Manuscript Submitted to PLos ONE*)**

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