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A novel mitochondrially-targeted apocynin derivative prevents hyposmia and loss of motor function in the leucine-rich repeat kinase 2 (LRRK2^{R1441G}) transgenic mouse model of Parkinson's disease

Brian P. Dranka^{1,2}, Alison Gifford¹, Donna McAllister¹, Jacek Zielonka¹, Joy Joseph¹, Crystal L. O'Hara³, Cheryl L. Stucky³, Anumantha G. Kanthasamy⁴, and Balaraman Kalyanaraman^{1,*}

¹Department of Biophysics, and Free Radical Research Center, Medical College of Wisconsin, Milwaukee, WI

³Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, WI

⁴Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA

Abstract

Recently, we demonstrated that dimeric apocynin prevented loss of motor function in the leucine-rich repeat kinase 2 (LRRK2^{R1441G}) transgenic (tg) mouse (treated with 200 mg/kg, three times per week) [B.P. Dranka et al., *Neurosci. Lett.* 549 (2013) 57-62]. Here we extend those studies by treating LRRK2^{R1441G} mice with an orally-available, mitochondrially-targeted apocynin derivative. We hypothesized that the increased mitochondrial permeability of Mito-apocynin, due to the triphenylphosphonium moiety, would allow improvement of Parkinson's disease (PD) symptoms at lower doses than those required for diapocynin. Tests of motor coordination (pole test, Rotor-Rod) revealed a significant deficit in coordinated motor function in LRRK2^{R1441G} mice by 15 months of age. Decreased performance on the pole test and Rotor-Rod in the LRRK2^{R1441G} mice was prevented with Mito-apocynin treatment (3 mg/kg, three times per week). Decreased olfactory function is an early indication of PD in human patients. LRRK2^{R1441G} tg mice displayed deficits in sense of smell in both the hidden treat test, and a radial arm maze test. Interestingly, treatment with Mito-apocynin prevented this hyposmia, and animals retained normal ability to identify either a scented treat or a food pellet as well as wild type littermates. Together, these data demonstrate that the mitochondria-targeted apocynin analog is effective in preventing early PD-like symptoms in the LRRK2^{R1441G} mouse model.

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*Corresponding author: Balaraman Kalyanaraman, Ph.D., Department of Biophysics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, Phone: 414-955-4000, Fax: 414-955-6512, balarama@mcw.edu.

²Present address: Seahorse Bioscience, 16 Esquire Road, North Billerica, MA 01862

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Keywords

Neurodegeneration; Parkinson's disease; Apocynin; Hyposmia

Introduction

Parkinson's disease (PD) patients exhibit early non-motor symptoms that are considered "preclinical", but are increasingly recognized for their diagnostic potential [1]. Hyposmia (diminished ability to smell and detect odors) is an early sign of PD [2,3] and other neurodegenerative diseases [4]. Approximately 50% of people with PD experience severe and chronic pain; shoulder, feet and knee pain is one of the key signs of PD onset and often precedes motor complaints [5-7]. We previously used the LRRK2^{R1441G} transgenic (tg) mouse model [8] to investigate PD-like symptoms. Early symptoms of PD included decreases in motor coordination (pole test, Rotor-Rod) [8]. Results showed that the decreased performance on the pole test and Rotor-Rod in the LRRK2^{R1441G} mice was prevented with diapocynin administered at high concentrations (200 mg/kg) [4,8,9]. Diapocynin is a dimeric molecule formed from oxidation of apocynin, a naturally-occurring methoxyphenol, which has been previously shown to protect dopaminergic neuronal cells in MPP⁺ cellular model of PD [10].

In this work, we synthesized Mito-apocynin-C₁₁ (apocynin conjugated to a mitochondria-targeting triphenylphosphonium cation moiety (TPP⁺) *via* an alkyl chain consisting of eleven carbon atoms (**Fig. 1**). The presence of a highly lipophilic and delocalized cationic moiety in Mito-apocynin-C₁₁ makes it more cell-permeable and selectively target mitochondria. Sequestration into mitochondria is facilitated by TPP⁺ conjugation to apocynin *via* long carbon-carbon side chains [8]. Results show that Mito-apocynin-C₁₁ administration in significantly lesser amounts markedly improved the coordinated motor function and olfactory function in LRRK2^{R1441G} mice.

Materials and Methods

Mice

A colony of LRRK2^{R1441G} (FVB/N-Tg(LRRK2*R1441G)135Cjli/J) and wild type litter mates was established from commercially available breeders as previously described [8,9]. Male mice were housed on a 12 h light/dark cycle with ad libitum access to food and water unless otherwise noted. All experiments were performed in accordance with the Guide for Care and use of Laboratory Animals and approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. At 12 weeks of age, mice began receiving 3 mg/kg Mito-apocynin C₁₁ or saline, 3x/wk, *via* oral gavage. This treatment scheme continued for the duration of the experiment, and mice were sacrificed at 15 months of age (**Fig. 1A**).

Mito-apocynin-C₁₁ synthesis

The novel compound Mito-apocynin-C₁₁ (**Fig. 1B**) was synthesized by modifying the previously described protocol for Mito-Q synthesis [11,12]. **Figure 2** shows the key steps

involved in the synthesis. The identity of the product was verified by mass spectrometry (Shimadzu LC-MS 8030 mass spectrometer) and purity determined by HPLC with absorption detection (Agilent 1100, equipped with Kinetex C₁₈ column) (**Fig. 1C**). The detailed synthetic procedure is described in the **Supplemental Materials**.

Open field measurements

Total ambulatory function was monitored over 20 minutes in individual mice using a 40 × 40 cm open field photobeam system (San Diego Instruments; San Diego, CA). Any beam break in the first photobeam level (i.e., excluding rearing) was counted and summed for the total movement for each mouse. A second level of photobeams was used to concomitantly track rearing. A heat map of position within the open field apparatus was generated using XY coordinate data in R. Data were normalized to percentage of time spent at each coordinate for each mouse, and then averaged. A 2 × 2 binning strategy was used for the data display.

Motor coordination behavioral analysis

Coordination was assessed using a Rotor-Rod (San Diego Instruments; San Diego, CA). Mice were placed on a horizontal bar (3.175 cm diameter) which rotates. The speed of rotation was increased from 0 to 8 rpm in 10 sec, from 8 to 10 rpm in the next 5 sec, maintained at 10 rpm for 5 sec, and then increased from 10 to 11 rpm over the next 5 sec.

Von Frey filament analysis

To avoid sensitization and interactions between assays, these tests were all performed on different days. The experimenter was blinded to genotype for each assay. Prior to all three experiments, mice were placed in small plastic cages on top of a wire mesh and were allowed to acclimate for an hour.

Up-down test—The glabrous skin of the bilateral hind paws was stimulated with calibrated von Frey filaments using the up-down method as described by Chaplan [13] to determine the 50% response threshold.

Light touch assay—Mice were then subjected to a light touch dynamic assay as described by Garrison et al. [14]. In brief, a puffed out cotton swab was used to stroke the glabrous skin of the bilateral hind paw. Positive responses consisted of paw lifts, flutters, or licks. This was repeated five times on each paw.

Needle test—To measure responses to a definitively noxious stimulus, a spinal needle was used to poke the glabrous skin of both hind paws. The glabrous skin of each plantar hind paw was probed 10 times to determine a percent response. Positive responses were characterized by fluttering, holding paw in the air, and licking (**Suppl. Fig.1**).

Hyposmia testing

Sense of smell was tested in mice fasted overnight (a minimum of 12 h). Mice were then placed individually in a novel cage with clean bedding. Each cage contained both a buried chow pellet and a fruit-scented cereal treat. Animals were allowed to seek out the treat

independently, and 3 trials were averaged for each mouse to determine the time-to-treat. Trials were video recorded and then timed by an observer blinded to the treatment group. Mice were not conditioned for the “buried treat/olfactory” test.

Statistical analysis

Motor function data were acquired using a San Diego Instruments Photobeam Activity System (PAS) Open Field apparatus or Rotor-Rod (San Diego Instruments; San Diego, CA) [15]. One-way ANOVA was used for significance tests unless otherwise specified in the legend. $p < 0.05$ was considered statistically significant.

Results

Mito-apocynin-C₁₁ prevents loss of coordinated motor function

Total ambulatory function was assessed as described in an open field tracking system (**Fig. 3A**). Total movements were not different between genotypes consistent with a previous cohort studied in our laboratory [8]. Importantly, chronic treatment with Mito-apocynin-C₁₁ did not negatively affect motor function. We also examined preference for specific areas of the open field apparatus. All groups displayed a preference for the corners of the enclosure; however, there was no difference in this preference between genotypes or treatment groups (**Fig. 3B**).

Fine motor coordination was assessed next by using the Rotor-Rod test. Saline-treated LRRK2^{R1441G} mice performed more poorly on this test than wild type mice (**Fig. 3C**). Interestingly, the ability to remain on the spinning rod was retained in LRRK2^{R1441G} mice orally gavaged with Mito-apocynin-C₁₁. To rule out the potential contribution of sensory neuron loss in the LRRK2^{R1441G} tg mice, a comprehensive set of tests for mechanical sensation was performed using calibrated von Frey filaments. The average force required to elicit a behavioral response in control mice is the same as in the LRRK2^{R1441G} tg mice (**Fig. 4A**). The percentage of responses was also not different between genotypes when the stimulus force was held constant (**Fig. 4B**). An alternative stimulus to test sensation of light touch was also tested with the dynamic light touch stimulus. Similar to the von Frey filaments, there was no difference in response between genotypes (**Fig. 4C**). This suggests that the LRRK2^{R1441G} tg mice exhibit neither sensory loss nor obvious mechanical pain behavior. However, given the recent evidence linking mitochondria dysfunction with neuropathic pain [16-18], it would be more prudent to use another animal model of PD (e.g., Mito-Park) [19] to demonstrate pain behavior and mechanisms.

LRRK2^{R1441G} tg mice have hyposmia which is prevented with Mito-apocynin-C₁₁ treatment

We previously reported that control saline-treated LRRK2^{R1441G} tg mice qualitatively display a loss of home cage motor function, despite the lack of differences in the open field test. One potential explanation for this disparity is that the exploratory behavior which these mice typically demonstrate may be diminished if they have a loss of smell. Hyposmia is a common feature of early stage PD in human patients [20]. In a previous study, we suggested that the LRRK2^{R1441G} tg mouse could be a good model for this early stage disease owing to the low gross motor defects, but declining fine motor function. We thus examined sense of

smell in our cohort of mice treated with Mito-apocynin- C_{11} . As shown in **Supplemental Video 1**, and summarized in **Figure 5**, $LRRK2^{R1441G}$ tg mice at 15 months of age had a dramatically increased time required to locate either a chow pellet or a fruit-scented cereal treat buried under fresh bedding in a novel cage. Average time required to find the treat is shown in **Figure 5A**. Time-stamped video stills are of mice which closely approximated the mean (**Fig. 5B**). Notably, long-term treatment with Mito-apocynin- C_{11} prevented this decrease, with performance remaining near the level of the wild type mice.

Discussion

Early identification of PD is currently the only method to improve morbidity and long term survival of patients. The future of PD patient care includes coupling an understanding of non-motor symptoms to new biomarkers that will enable early disease detection. Early detection of PD symptoms will afford better patient care and more effective drug therapy [20].

Scent testing remains one of the most effective methods for early detection of PD symptoms in high-risk patients. The University of Pennsylvania Scent Identification Test has been validated in multiple clinical trials to offer diagnostic value for the early detection of PD [20]. However, animal models which recapitulate this early loss of smell in disease progression have not yet been developed. Previously, we reported that the $LRRK2^{R1441G}$ tg mouse did not have significant motor deficits as previously identified [8,9], however, these mice did have differences in coordinated motor function – thought to precede over PD motor dysfunction. We postulated that these mice may then be a good model for early PD symptoms. In keeping with this hypothesis, we reported here that $LRRK2^{R1441G}$ tg mice have increased latency in the time to identify a scented treat in a novel environment. Importantly, this loss was prevented by long-term treatment with an orally available mitochondrially-targeted apocynin derivative. Mitigation of hyposmia by Mito-apocynin is clearly one of the major findings of this study.

Mitochondria are clearly recognized as a source of reactive oxygen species (ROS) in PD patients [12]. Therapeutic strategies to limit oxidant production are common, though this approach has not resulted in dramatic improvements in patient health. Recent studies demonstrated that mitochondrially-targeted antioxidants were protective in a pharmacologic mouse model of PD [12]; however, the mechanism of this protection is still poorly defined. One explanation for this disparity is that the time of intervention has been too late in clinical trials. Indeed, here we show that a novel mitochondrially-targeted apocynin derivative can prevent early PD symptoms with no side effects in these mice. Mito-apocynin exerts neuroprotective effects at much lower concentrations (≈ 3 mg/kg) as compared to diapocynin, 200 mg/kg). Thus the potential for a long-term chronic treatment with relatively non-toxic mitochondria-targeted drug at the earliest detection of the disease is feasible.

Our data demonstrate the potential for prevention of early PD symptoms through the use of low-dose mitochondria-targeted agents. However, the mechanism of neuroprotection in the $LRRK2^{R1441G}$ model remains unclear. As we previously reported, there does not appear to be significant neurodegeneration in the substantia nigra of the $LRRK2^{R1441G}$ mice at 16

months of age [8]. One open question is whether there is early neurodegeneration in the olfactory bulb of these mice which explains the hyposmia described here. Although activation of microglia is documented in PD patients and animal models [21,22], it is not clear whether glial activation is responsible for these effects. Concomitant with glial activation, NADPH oxidase activity (e.g., NOX-2 isoform) is increased in PD brains [23]. Preliminary experiments using MPTP model indicated diminished neuroinflammation in mito-apocynin treated mice (not shown). The anti-neuroinflammatory effects of Mito-apocynin (not shown) and other apocynin analogs in MPTP mouse model of PD have been reported [24]. Mito-apocynin attenuates MPTP-induced expression of gp91phox. Both diapocynin and Mito-apocynin inhibit ROS/reactive nitrogen species (RNS)-mediated nitration and oxidative damage to proteins in MPTP mouse model of PD [24]. Although Mito-apocynin affords neuroprotection in Mito-Park and LRRK2^{R1441G} mouse model, the exact mechanism of protection is presently not known. The neurotoxic role of NOX2 and other NOX enzymes in genetic mice models still remains to be established. Whether ROS-derived from NOX-2 or mitochondria is involved in olfactory dysfunction still remains to be determined. AMP-activated protein kinase (AMPK) functions as a cellular energy sensor and serves as a signaling molecule in neurons and AMPK activation induces changes in energy-sensing mechanism [25]. Mitochondria-targeted agents (Mito-apocynin) could activate AMPK signaling and restore olfactory dysfunction. At this stage, these aspects remain speculative and future studies will attempt to address these mechanistic possibilities. Nonetheless, the data presented here demonstrate the importance of this powerful new approach to early PD symptom prevention in a mouse model. Based on our findings, we conclude that LRRK2^{R1441G} tg mice may be more suitable for the study of early, non-motor PD-like symptoms (e.g., hyposmia) as opposed to the gross motor defects detected later in PD progression, and that mitochondria-targeted natural product derivative (Mito-apocynin) could be used to prevent early non-motor symptoms. In contrast to the present findings, it was reported in a recent publication that the overexpression of the R1441G mutated form of the human LRRK2 gene did not cause significant change in olfactory function in the transgenic mice compared to non-transgenic control mice [26]. Reasons for this difference are not currently known. However, ongoing research in our laboratories also indicate that mito-apocynin treatment attenuates a wide range of PD-like symptoms in the Mito-Park mice model of PD [19].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

LRRK2 leucine-rich repeat kinase 2

PD	Parkinson's disease
RNS	reactive nitrogen species
ROS	reactive oxygen species

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Highlights

- LRRK2^{R1441G} mice are hyposmic.
- Mito-apocynin C₁₁ prevents hyposmia and corrects deficits in motor function.
- No difference in response to mechanical sensitivity was found.

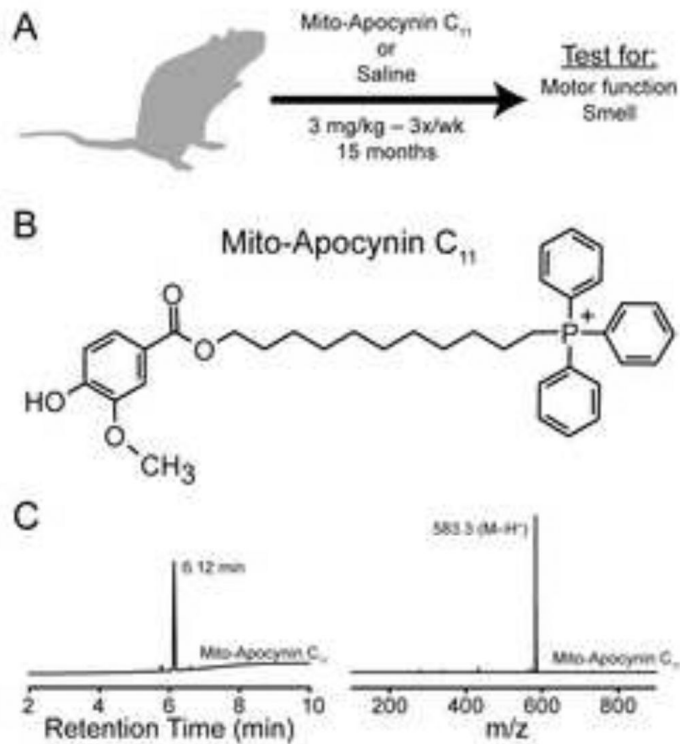


Figure 1. Experimental design

(A) LRRK2^{R1441G} tg or wild type littermates were orally gavaged with 3 mg/kg Mito-apocynin-C₁₁ 3x/wk from 3 months of age until 15 months. Animals were tested for gross motor function and hyposmia as described. (B) Chemical structure of Mito-apocynin-C₁₁. (C) Compound identity and sample purity were confirmed by mass spectrometry (*right panel*) and HPLC ($\lambda_{\text{obs}} = 250 \text{ nm}$, *left panel*), respectively. The mass peak (583.3) shown in the right panel corresponds to molecular ion of Mito-apocynin-C₁₁ cation.

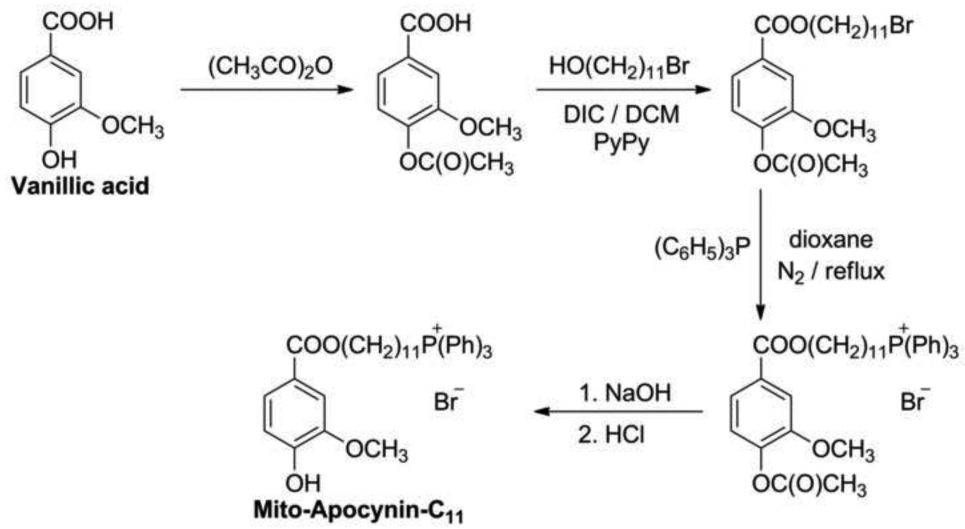


Figure 2. The synthetic pathway involved in the preparation of Mito-apocynin-C₁₁

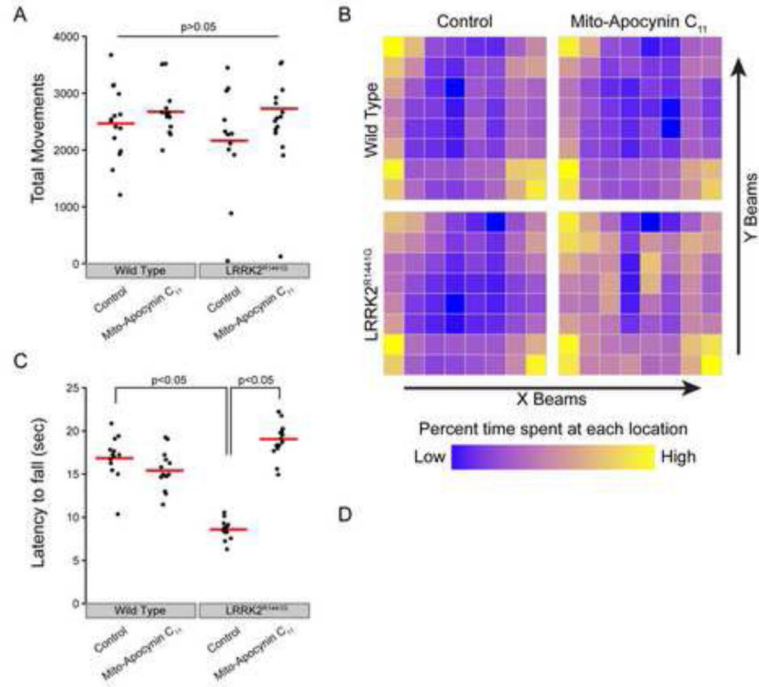


Figure 3. Gross motor function and foot sensory function is not altered in *LRRK2^{R1441G}* tg mice (A) Total movement in an open field photobeam tracking apparatus did not identify any deficit in gross motor function. Mouse movement patterns were also not different between genotypes or treatments. (B) Heat maps were generated using the percentage time each animal occupied a specific set of coordinates. (C) Fine motor coordination comparison between mice of different genotypes and treatments as monitored by measurements of latency to fall.

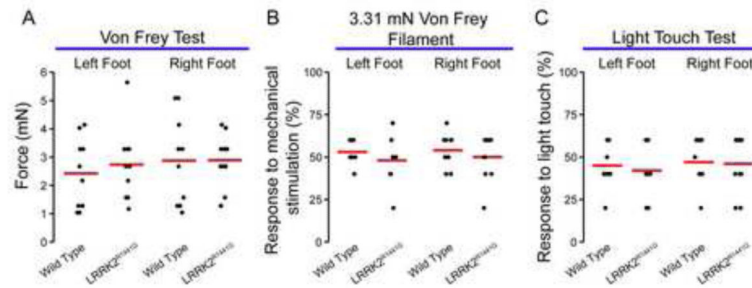


Figure 4. Assessment of response to mechanical sensitivity using Von Frey filament analysis Control and LRRK2 mice were subjected to mechanical sensation testing using an up-down von Frey test (repeated stimulation with a 3.31 mN filament), a light-touch assay, and a needle assay. Behavioral testing for mechanical sensitivity of control and LRRK2 mice revealed no significant difference in any of the tests performed (A-C). Individual mice are represented by each black dot. The red bar represents the mean. Significance was assessed by two-tailed t-test, with $p < 0.05$ noted as significantly different. (Mann Whitney U test for non-parametric von Frey data, and Chi-square test for percentage responses). $n = 10$ mice per group.

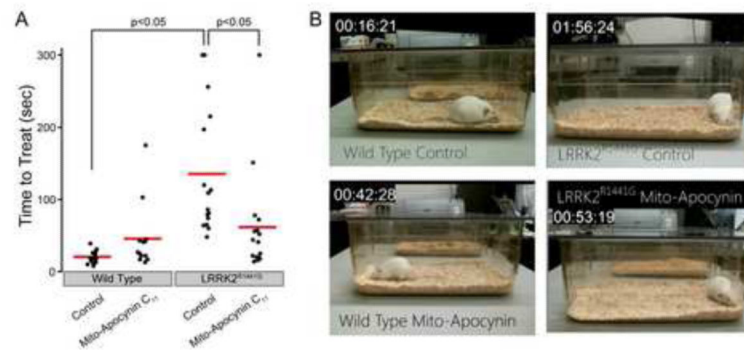


Figure 5. Figure 5: Mito-apocynin-C₁₁ improves time-to-treat performance in LRRK2^{R1441G} tg mice

(A) The time required to identify either a chow pellet or a fruit cereal treat was monitored in mice in a novel cage with clean bedding. Individual mice are represented by the black dots and the mean is shown as the red bar. (B) Video still images were selected to represent the mouse which had the time-to-treat closest to the group mean. Time stamps indicate the elapsed time required to locate the treat.