Homozygous mutation of the LRRK2 ROC domain as a novel genetic model of parkinsonism

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Abstract

Background: Parkinson’s disease (PD) is one of the most important neurodegenerative disorders in elderly people. Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene are found in a large proportion of the patients with sporadic and familial PD. Mutations can occur at different locations in the LRRK2. Patients with LRRK2 ROC-COR mutations face an increased risk of typical motor symptoms of PD, along with cognitive decline. An animal model with a mono- genic LRRK2 gene mutation is a suitable model for exploring the pathophysiology of PD and identifying potential drug therapies. However, the effect of homozygous (HOM) LRRK2 in PD pathophysiology is unclear.

Methods: We established human LRRK2 (hLRRK2) R1441G HOM transgenic (Tg) mice to explore the phenotype and pathological features that are associated with hLRRK2 R1441G Tg mouse models and discuss the potential clinical relevance. The open field test (OFT) was performed to examine motor and nonmotor behaviors. A CatWalk analysis system was used to study gait function. [18F]FDOPA PET was used to investigate functional changes in the nigrostriatal pathway in vivo. Transmission electron microscopy was used to examine the morphological changes in mitochondria and lysosomes in the substantia nigra.

Results: The R1441G HOM Tg mice demonstrated gait disturbance and exhibited less anxiety-related behavior and exploratory behavior than mice with hLRRK2 at 12 months old. Additionally, [18F]FDOPA PET showed a reduction in FDOPA uptake in the striatum of the HOM Tg mice. Notably, there was significant lysosome and autophagosome accumulation in the cytoplasm of dopaminergic neurons in R1441G hemizygous (HEM) and HOM mice. Moreover, it was observed using transmission electron microscopy (TEM) that the mitochondria of R1441G Tg mice were smaller than those of hLRRK2 mice.

Conclusion: This animal provides a novel HOM hLRRK2 R1441G Tg mouse model that reproduces some phenotype of Parkinsonism in terms of both motor and behavioral dysfunction. There is an increased level of mitochondrial fission and no change in the fusion process in the group of HOM hLRRK2 R1441G Tg mouse. This mutant animal model of PD might be used to study the mechanisms of mitochondrial dysfunction and explore potential new drug targets.

Keywords: Anxiety, Fission, Gait, GTPase activity, Homozygous, LRRK2, Parkinsonism, PET, R1441G

Background

Mutations in leucine-rich repeat kinase 2 (LRRK2) are the one of the most common genetic causes of Parkinson’s disease (PD). Mutations can occur at various locations in LRRK2. To date, eight mutations in LRRK2, including N1437H, R1441 G/H/C, Y1699C, I2012T, G2019S, and I2020T, have been shown to be associated with PD.
LRRK2 (HOM) mutations in a large population of patients carrying homozygous with an autosomal dominant inheritance pattern, there is a large population of patients carrying homozygous (HOM) mutations in LRRK2 R1441G and G2019S worldwide. Patients with mutations in the LRRK2 gene show the phenotypic features of sporadic PD, including not only motor symptoms but also nonmotor symptoms such as depression, and cognitive impairment.

Mammalian LRRK2 is a 2527-residue protein, with a catalytic core domain, a kinase domain and a number of putative protein–protein interaction domains. The catalytic core domain consists of a Ras GTPase-like domain termed ROC (Ras of complex protein) followed by a COR (carboxy-terminal of Ras) domain immediately before the kinase domain. The regulation of LRRK2 kinase activity depends on the ROC domain of the dimer, and dimerization may depend on the COR domain as a molecular hinge. Various LRRK2 mutation in vitro and in vivo assays have been established and demonstrated that mutations in LRRK2 could be involved in the pathogenesis of PD through the autophagic–lysosomal pathway, intracellular trafficking, mitochondrial dysfunction, and the ubiquitin–proteasome system. Inhibitors of LRRK2 kinase have been introduced in clinical trials for symptomatic treatment of sporadic PD and monogenic PD patients with LRRK2 mutations.

Many animal models of LRRK2 heterozygous gene mutation have been established in the last decade. However, most mouse models do not show visible PD symptoms under normal conditions but are more susceptible to external stress. Only a few studies have focused on the mutation of the ROC-COR domain and investigated the underlying molecular pathways of neurodegeneration.

Herein, we generated HOM human hLRRK2 R1441G mice to evaluate motor function, gait analysis, anxiety and metabolic neuroimaging (small animal PET/CT imaging of [18F]FDOPA). Moreover, we further measured the GTPase activity and total protein expression of LRRK2 with and without phosphorylation and examined the ultrastructural changes in mitochondria and lysosomes in the midbrain. Next, we investigated the pathophysiology of the hLRRK2 R1441G mutation on the regulation of mitochondrial fission/fusion dynamics and the autophagy pathway in this animal model.

Methods

Mice

All animal experimental procedures were approved by the Committee on Animal Research of National Taiwan University and carried out in accordance with the guidelines of the Committee. All mice were bred in the Laboratory Animal Center, National Taiwan University College of Medicine. All animals were housed in groups at 20–25 °C with 60% relative humidity, a 12/12 h light/dark cycle, and free access to food and water. Hemizygous (HEM) wild-type (WT) hLRRK2 mice (FVB/N-Tg (LRRK2)1Cjli/J, no. 009610) and HEM hLRRK2 R1441G transgenic (Tg) mice (FVB/N-Tg (LRRK2*R1441G)135Cjli/J, no. 009604) were purchased from the Jackson Laboratory and bred on an FVB/N background (Jackson stock number. 001800). HOM hLRRK2 R1441G Tg mice were generated by crossing HOM hLRRK2 R1441G Tg mice with HOM hLRRK2 R1441G Tg mice. The HEM wild type hLRRK2 was used as an ideal control. HEM and HOM hLRRK2 R1441G Tg mice were used as comparison groups. Genomic DNA was obtained from mouse ear biopsies and amplified by polymerase chain reaction (PCR) and quantitative PCR (qPCR) using sequencing primers designed as described below. PD is found more frequently in men than in women, and only male mice were used for experiments in this study.

Genotyping HOM

Genomic DNA was extracted from 5 mm ear punch biopsies by lysing the tissue in 10 mg/ml protease K (in 50 mM Tris pH 8.0, 2 mM NaCl, 10 mM EDTA, 1% SDS) at 65 °C overnight followed by salt extraction and ethanol precipitation. The mice were genotyped using genomic DNA from the ear biopsy tissue was used to identify the mouse genotype. The following primers were used for PCR amplification of hLRRK2: 5′-TGA TTCTCGTTGGCAGCAT-3′ and 5′-GCCAAAGCA TCAGATTCTTC-3′. PCR was conducted in a 20 μl reaction volume containing approximately 1 μl genomic DNA, 0.5 μl of each 100 μM primer, and 10 μl 2× Master Mix (Promega). The thermocycler conditions were set to 94 °C for 2 min 30 s, then 35 cycles of 94 °C for 30 s, 68 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 2 min. To identify the genotype of the mouse model as either HOM or HEM for hLRRK2, the other sets of primers were used for real-time qPCR: For the hLRRK2 gene, the primer and probe sequences were as follows:
forward primer 5′-GCATTAGAGATTTATCCCTG
GAA-3′, reverse primer 5′-GTACTGACCTTGGTCATC
TGGATA-3′, and probe 5′-FAM-ATGGATTACGTG
CTTCACACCTGCA-BHQ1-3′. FAM is a fluorescent
reporter and BHQ1 is a quencher fluorophore. The apoli-
poprotein B gene (apoB) was used as an internal control
to normalize variations in the amount of input DNA. The
product size was 73 bp. The primer and probe sequences
were as follows: forward primer 5′-CACGTGGGCTCC
AGCATT-3′, reverse primer 5′-TCACCACTTATTCT
GCTTTTG-3′, and probe 5′-Cy5-CCAATGGTCGGG
CCTGCTCAA-BHQ2-3′. Cy5 was a second fluorescent
reporter. qPCR was conducted in a 20 μl reaction volume
containing approximately 50 ng genomic DNA, 0.8 μl of
each 10 μM primer and 0.3 μl of each 10 μM probe, and
10 μl 2 × qPCR Master Mix (Promega). The thermocycler
conditions were set to 95 °C for 2 min, followed by 40
cycles of 95 °C for 15 s, and 60 °C for 1 min.

Behavior assay
All the behavioral tests on all mice were performed at the
same time of day.

Spontaneous locomotor activity
All procedures were performed in the dark as an adap-
tation to typical mouse behavior. Mice were placed in a
corner of an open-field apparatus (16 × 16 × 15 inches,
San Diego Instruments, San Diego, CA, USA) consist-
ing of a Plexiglas cages white floors and translucent walls.
Locomotor activity was monitored using a 16 × 16 array
of photobeams (beam interval, 1 inch). The sampling rate
was once per second. The patterns of beam breaks were
computed (Photobeam Activity System—Open Field,
San Diego Instruments, San Diego, CA, USA) to obtain
parameters of locomotor activity. Data were collected for
30 min and each time in a 10-min period over three con-
secutive periods.

Spontaneous gait analysis
All procedures were performed in the dark (except
for light emitted from the nearby computer screen) to
enhance the contrast of the paw print images. The Cat-
Walk system (Noldus Information Technology, Wage-
ningen, Netherlands) was used to analyze the gait of
unforced moving mice. CatWalk includes a hardware
system with a glass walkway plate, illuminated with green
light that is reflected within the glass; at points of contact,
the light is reflected toward a high-speed video camera.
CatWalk software 10.0 was used for quantitative assess-
ment of animal footprints. A successful run was defined
as a complete run along the tracks without any interrup-
tion or hesitation. Mice that failed the CatWalk training
were excluded from the study. The average number of 5
replicate crossings made by each mouse was recorded.
Mice were subjected to computer-assisted CatWalk test-
ing every month for 1 year.

[18F]FDOPA micro PET
[18F]FDOPA was prepared and synthesized at the
Department of Nuclear Medicine at National Taiwan
University Hospital. All PET scans were acquired on
an Argus PET/CT (SEDecal) with a spatial resolution
of 1.1 mm, a transaxial field of view (FOV) of 68 cm
and an axial FOV of 4.7 cm. Animals were anesthetized
and maintained with a mixture of 1.5% isoflurane and
nitrous oxide:oxygen (7:3). Static scans were acquired
30 min after a single bolus injection of [18F]FDOPA
(12.9 ± 1.47 MBq in 0.1 ml saline) via the tail vein in 3D
mode for 60 min. Briefly, images were reconstructed
using the 2D ordered-subset expectation maximization
(OSEM) algorithm with radians and scatter corrections
and without attenuation. There were a total of 61
slices of reconstructed images, each with a matrix size of
175 × 175. The correction and images were analyzed in
PMOD software (version 3.2, PMOD Technologies), and
the volumes of interest (VOIs) were drawn over the right
and left striatum and cerebellum (CB) in irregular shapes.
To ensure proper VOI placement, PET images were
coregistered with a mouse MRI template. The quantita-
tive analysis of [18F]FDOPA uptake in brain regions was
first shown as a standardized uptake value (SUVR) by the
formula: SUV measured tissue activity [Bq/ml]/(injected
dose [Bq]/body weight [g]). The SUVR ratio (SUVR) of
[18F]FDOPA was calculated by (the sum of right and left
striatum uptake − cerebellar uptake)/cerebellar uptake
with cerebellum as the reference region.

Determination of GTPase (guanosine triphosphatase)
levels
GTPase activity in tissue sample lysates was determined
using an ATPase/GTPase ELIPA Biochem Kit (Cytoskel-
eleton, Denver, CO, USA) that measures the amount of
inorganic phosphate (Pi) generated during hydrolysis
on a real-time basis, according to the manufacturer’s
instructions.

Transmission electron microscopy (TEM)
For TEM analysis, mice were deeply anesthetized by
intraperitoneal injection of sodium pentobarbital and
transcardially perfused with ice-cold 0.9% saline and 4%
paraformaldehyde (PFA) in 0.1M phosphate buffered
saline (PBS, pH 7.4). The substantia nigra pars compacta
(SNC) of mice were then cut into 1 mm3 squares and
postfixed in Trump’s solution (4% formaldehyde + 0.1%
glutaraldehyde in 0.1M phosphate buffer) for 1 h at room
temperature. Subsequently, the samples were kept in the
2% glutaraldehyde in 0.1M Na-cacodylate buffer (pH 7.4) overnight. Tissue was fixed in 1% osmium tetroxide and 1% aqueous uranyl acetate, dehydrated in a graded series of ethanol, and embedded in Embed 812/Araldite (EMS, Hatfield, PA). Thin sections (0.1 μm) were collected on copper grids, poststained with lead citrate and viewed at 80 kV with a JEOL 1400 transmission electron microscope (JEOL USA, Peabody, MA) or TEM H-600 (HITACHI, Tokyo, Japan).

**Tissue dissection**
Mice were euthanized with CO₂, followed by decapitation. The tissues were quickly isolated and cooled in ice-cold saline. The brains were placed on an ice-cold brain matrix (Stoelting) for further dissection of the indicated brain areas such as the SNc and striatum. For western blotting (WB), the tissues were immediately frozen and stored at −80 °C until assayed.

**Fractionation of mitochondria**
Briefly, fresh brains were obtained within 1 h of sacrifice and kept on ice. A Mitochondria Isolation Kit (Sigma-Aldrich) was used for mitochondrial fractionation, and the procedures followed the manufacturer’s instructions. The purified mitochondria were stored at −70 °C or subjected to the next analysis.

**Protein extraction**
Total proteins were prepared from whole brain, SNc, and striatum tissues for western blotting analysis. Frozen tissue samples were homogenized with a microhomogenizer in ice-cold CelLytic™ MT Cell Lysis Reagent (Sigma-Aldrich) containing 1× Protease Inhibitor Cocktail (Sigma-Aldrich) and 1× PhosSTOP™ (Roche Diagnostics Ltd.). After tissue disruption, homogenates were centrifuged at 12,000 ×g for 10 min to pellet the tissue debris. The supernatants were transferred to a clean test tube.

**Western blotting**
The protein concentration of brain capillary membrane samples was determined using a Bradford protein assay. Normalized brain capillary membrane samples were separated and transferred using the NuPAGE electrophoresis and blotting system (Invitrogen). After protein transfer, the blotting membranes were incubated overnight with primary antibody (Fis1, Genetex; Drp1 (D6C7), Cell Signaling; LC3B, Genetex; LRRK2 [MIF (c41-2)], Abcam; SQSTM1/p62 [EPR4844], Abcam). After antibody incubation, the membrane was washed and incubated with the corresponding HRP-conjugated secondary antibody (1:5,000; Genetex). Proteins were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore), and protein bands were visualized with a VisionWorks™LS Analysis Software (UVP Inc.) and analyzed with ImageJ as an arbitrary optical density unit.

**Statistical analysis**
We performed all statistical analyses in GraphPad Prism. We analyzed the effect of one variable on more than two groups using a one-way ANOVA and pairwise t-tests with a Bonferroni correction, Dunn’s test, or Holm–Sidak post hoc analysis. We analyzed the effect of two variables using a two-way ANOVA and pairwise t tests with a Bonferroni correction, Dunn’s test, or Holm–Sidak post hoc analysis. The statistical significance threshold was p < 0.05 for all tests.*< 0.05, **< 0.01, ***< 0.001, ****< 0.0001.

**Results**
**Poor growth and abnormal GTPase and Ser935 expression**
Earlier studies reported that HEM hLRRK2 R1441G Tg mice induce age-dependent, levodopa-responsive slowness of movement associated with diminished dopamine release and axonal pathology of nigrostriatal dopaminergic projection [33]. However, these phenotypes could not always be replicated in independent studies. Compared to the non-Tg mice, previous studies have reported that HEM hLRRK2 R1441G mice significantly decreased motor function and non-motor behavior at 15 months old [13] or 20 months old [7]. Therefore, we generated HOM LRRK2 R1441G mice. We used real-time PCR analyses to identify hLRRK2 R1441G HOM mice. As shown in Fig. 1A, the expression of exogenously introduced hLRRK2 mRNA was elevated in HOM mice. The mRNA of HOM hLRRK2 R1441G Tg mice is doubled when it was compared to the hLRRK2 and HEM hLRRK2 R1441G Tg mice. The immunoblots showed a significant difference in protein expression between non-Tg or hLRRK2 R1441G mice and HOM hLRRK2 R1441G mice in the brain at 3 months of age (Fig. 1B). When food and water were available ad libitum, both R1441G HEM and HOM mice grew slower (Fig. 1C).

R1441G mutation suppresses GTPase activity and promotes GTP binding which in turn mediates a three to fourfold increase in LRRK2 kinase activity [49, 51]. We next collected SNc from 12-month-old mice to assess the effect of R1441G mutation on the expression of GTP hydrolysis. As shown in Fig. 1D, compared with that in hLRRK2 mice, GTPase activity was significantly reduced in HEM hLRRK2 R1441G mice, whereas significantly increased GTPase activity was found in HOM hLRRK2 R1441G mice. Ser935, located prior to the leucine-rich repeat domain on the LRRK2 enzyme, is the most common site at which phosphorylation is measured [14]. We decided to investigate Ser935 phosphorylation to assess
the LRRK2 phosphorylation. The data revealed that Ser935 phosphorylation was abolished in R1441G HOM mice (Fig. 1E).

**HOM mice have an abnormal motor and nonmotor behaviors**

We then analyzed age-matched non-transgenic (non-Tg), hLRRK2, R1441G HEM and HOM male mice at 9, and 12 months of behavioral tests, including spontaneous locomotor activity levels (Fig. 2A–D), anxiety-related emotional (Fig. 2E–G) and exploratory behaviors (Fig. 2H), and gait analysis (Fig. 3).

First, the quantity of locomotion as assessed in the open field test the (OFT) was affected in HOM group, with decreased distance traveled (Fig. 2A, n = 5 per group) and average speed (Fig. 2B, n = 5 per group). When the Tg mice were placed in an open field, the ambulatory activity of 12-month-old R1441G HOM mice decreased significantly compared to 12-month-old hLRRK2, R1441G HEM mice (Fig. 2C, n = 5 per group).

To investigate whether HEM and HOM hLRRK2 R1441G mice display nonmotor behaviors, we studied anxiety-related emotional and exploratory behaviors in freely moving HEM and HOM hLRRK2 R1441G mice,
hLRRK2 mice and non-Tg mice using the OFT. The OFT is also widely used to assess the emotionality in rodents [48]. Compared to the non-Tg and hLRRK2 mice, we found that the HOM group also significantly decreased the time spent in the peripheral (Fig. 2F, n = 5 per group) and central (Fig. 2E, n = 5 per group) zones of the maze statistically analyzed. Rearing is a common measure of activity and exploratory behavior used in the OFT. Moreover, R1441G HOM mice exhibited less exploratory rearing behavior in the dark phase than R1441G HEM mice at 12 months old (Fig. 2G, n = 5 per group). However, contrary to HOM hLRRK2 R1441G mice, 12-month-old HEM hLRRK2 R1441G mice had slightly increased rearing activity compared to the non-Tg mice. The data were consistent with a previous study [33].

Gait impairments are the most commonly observed clinical manifestation in PD patients [38]. To investigate whether HEM and HOM hLRRK2 R1441G mice display the gait impairments, we compare 12-month-old heterozygote and homozygote hLRRK2 R1441G, hLRRK2 and non-Tg mice in the CatWalk system. The CatWalk system captures a substantial number of gait parameters, both dynamic and static. A significant difference was noted between the hLRRK2 group and the HEM and HOM groups (Fig. 3, n = 5 per group). The gait activity as assessed in the CatWalk system with swing duration did not significantly change in all group (Fig. 3A, n = 5 per group). In contrast to the swing duration, swing velocity (cm/s) was shorter in the hLRRK2 HOM group (Fig. 3B, n = 5). The stance duration is the time during which the paw is in contact with the glass plate. As shown in Fig. 3C, 12-month-old R1441G HOM mice showed significantly longer stance duration than 12-month-old non-Tg (n = 5) and hLRRK2 mice (n = 5).
In contrast, Fig. 3D shows that cadence was shorter in R1441G HOM mice than in hLRRK2 mice (Mean Diff = 11.66, p < 0.05, n = 5 per group). The stride length displays the distance between successive placements of the same paw. For stride length, HOM mice had a significantly shorter stride length than hLRRK2 mice (Fig. 3E, Mean Diff = 0.1005, p < 0.01, n = 5 per group). For the base of support (BOS) of the hind limbs (Fig. 3F), it was noted that the HOM group placed significantly more units of pressure on the hind paws than the non-Tg groups (Fig. 3F, n = 5) and HEM mice (Fig. 3E, n = 5). Altogether, these results suggest that R1441G HOM mice exhibited a change in the quality of gait and decreased locomotor activity.

**PET assessment of dopamine depletion in the striatum**

[18F]FDOPA studies are the most widely and routinely used PET tracer for studying striatal changes in PD.
patients. When the depletion of dopamine in the striatum was measured by using PET imaging, all mice exhibited $^{[18F]}$FDOPA uptake in the striatum (Fig. 4A–D). Figure 4A–D shows coregistered $^{[18F]}$FDOPA PET images with computed tomography (CT), showing coronal brain slices of representative animals from the non-Tg (Fig. 4A), hLRRK2 (Fig. 4B), hLRRK2 R1441G HEM (Fig. 4C) and hLRRK2 R1441G HOM (Fig. 4D) groups. Brain uptake of $^{[18F]}$FDOPA in both non-Tg, and hLRRK2 groups followed a similar pattern in the striatum. However, the image signals were lower in the hLRRK2 R1441G HEM (Fig. 4C) and hLRRK2 R1441G HOM (Fig. 4D) groups. As Fig. 4E shows, the average SUV for $^{[18F]}$FDOPA in hLRRK2 R1441G HEM and HOM mice was significantly lower than that in hLRRK2 mice (n = 5–6 per group).

**Mutant-hLRRK2 altered function and mitochondrial morphology in the brain**

To determine if the hLRRK2 R1441G HEM and HOM mutation can generate the effect of mitochondrial morphology, TEM images were collected from the SN of hLRRK2, R1441G HEM and HOM mice at 12 months of age. The results of TEM image analysis of mitochondrial size are shown in Fig. 5A. Mitochondria of R1441G HEM and HOM mice shrank and were smaller in size compared to hLRRK2 mice.

We next investigated the effect of R144G1 on the expression levels of mitochondrial fission (i.e., Drp1 and Fis1) and fusion proteins (i.e., OPA1, Mfn1, and Mfn2) in brain tissue. Compared with WT hLRRK2 mice, a significant increase in Drp1 (Fig. 5D, n = 3 per group) in R1441G HOM mice and Fis1 levels (Fig. 5E, n = 3 per group) was observed in R1441G HEM mice, while OPA1, Mfn1 and Mfn2 levels remained unchanged (data not shown). These findings suggest an increased level of mitochondrial fission and no change in the fusion process.

**The lysosome morphology was defective in SNc regions of Tg mice with hLRRK2 R1441G by TEM**

We further characterized the lysosome morphology in hLRRK2 R1441G mutant Tg mice. TEM was used to observe the effect of the hLRRK2 R1441G mutant on the lysosome morphology. In hLRRK2 mice, lysosomes appeared as dense, spherical, membrane-enclosed vesicles (Fig. 6A). However, we observed abnormal lysosomes in R1441G HOM mice. First, we found enlarged membrane-bound vesicles that were filled with membranous and granular contents. In addition, we found the formation of closed autophagosomes fused with lysosomes.

To separately analyze the impact of LRRK2 R1441G on autophagic pathways, we first monitored the levels of microtubule-associated protein light chain 3 (LC3), a well-established marker for macroautophagy. Meanwhile, the levels of LC3 and p62/SQSTM1 weren’t statistically significant in mice expressing hLRRK2 R1441G HEM and HOM (Fig. 6C and D).
Discussion

Regarding the ideal control for a mutant mouse model overexpressing LRRK2 Tg, a large proportion of previous PD studies in Tg mutant mice have used non-Tg mice as controls [7]. However, Tg mice are generated by inserting a foreign gene into the genome. When an exogenous gene is added to the mouse genome, it often leads to phenotypic changes. For example, bacterial artificial chromosome (BAC) LRRK2 WT Tg mice were recently reported to show enhanced motor performance and striatal dopamine transmission compared to non-Tg mice [31]. Therefore, we believe that the ideal controls for mutant Tg mice...
are Tg animals expressing the WT allele at comparable levels to the mutant mice to control for the effects of overexpression by itself [10]. Accordingly, we used HEM hLRRK2 WT Tg mice as the ideal controls and HEM and HOM hLRRK2 R1441G Tg mice as comparison groups. This is the major difference between our study in the current Tg mouse model and previous studies in Tg mice overexpressing hLRRK2 with the R1441G mutation.

Fig. 6 LRRK2 R1441G HOM mice accumulated more autophagosomes in the SNc. A Transmission electron microscopic images of autophagosomes from the SN of 12-month-old hLRRK2 and R1441G transgenic mice. Selected regions in images at different magnifications (I, 10,000×; II, 20,000×). Arrows indicate autolysosomes, and asterisks indicate autophagosomes. Markers of autophagy (LC3 and p62) in the SNc of hLRRK2 and R1441G transgenic mice were determined by western blotting. p62 undergoes degradation at the early phase of autophagy. p62 in mitochondria serves as an adapter for autophagosome recognition. The data appear to downregulate the autophagy process, as observed by the increasing LC3-II conversion and the accumulation of p62, a marker of autophagic degradation.
In the present study, we found that hLRRK2 mice showed hyperactivity and enhanced performance in motor function tests compared with non-Tg mice (Figs. 2 and 3). The results we find are consistent with previous results [31]. Compared with age-matched hLRRK2 Tg mice, the hLRRK2 R1441G HOM Tg mice expressed obvious age-dependent motor deficits at 12 months of age in our study (Figs. 2 and 3). Grossly, the mice did not show outward abnormalities or muscle wasting. In the OFT for the assessment of spontaneous movement and anxiety, the mice with HOM hLRRK2 R1441G showed decreased ambulatory and fine activities. Moreover, this group of mice also revealed a reduction in time spent in the central and peripheral zones of the maze (Fig. 2E–G), supporting the coexistence of anxiety [48]. The motor dysfunction is consistent with the findings of hLRRK2 R1441G Tg mice, which showed age-dependent motor disability evaluated by the administration of apomorphine and drug-induced rotational behavior [33]. Regarding rearing behavior in the OFT, there was a decreased frequency of rearing, indicating impaired vigilance and/or exploration [54]. The motor dysfunction of the hLRRK2 R1441G Tg mice mimics the general bradykinesia of PD in humans. Impaired rearing behavior has been observed in mice with dopamine depletion in 6-hydroxydopamine mice [61]. In the gait analysis by the CatWalk system (Fig. 3), the decreased swing velocity, stride length, and cadence in 12 month-old hR1441G HOM mice were similar to the characteristics of the gait pattern, small steps, slow and shuffling gait-observed in the PD patients. The above findings in the present study demonstrated both motor and cognitive dysfunction in the hLRRK2 R1441G Tg mouse, which resembles the motor and nonmotor symptoms in human PD [4].

By the current criteria, a neuropathologic diagnosis of sporadic PD requires both neuronal loss in the substantia nigra pars compacta (SNpc) and the presence of α-synuclein [43]. However, several reports of autopsy in cases with LRRK2 G2019S or R1441G mutations linked to PD may challenge these criteria [1, 16, 18, 37]. Patients with a family history of parkinsonism and LRRK2 G2019S mutation present with characteristic motor features, drug response and clinical course but no α-synuclein positive inclusions in the dopaminergic neurons and neurites in the substantia nigra [1]. Our data showed no difference in phospho-Ser129 α-synuclein in the substantia nigra of LRRK2 R1441G Tg mice with or without the LRRK2 G2019S mutation by western blot. The immunobiochemical examination of TH-positive neurons in the substantia nigra did not show a significant reduction in the neuronal number between hLRRK2 and hLRRK2 R1441G Tg mice (Additional file 1: Fig. S1). However, the [18F]FDOPA PET study showed significantly decreased uptake of ligand in the striatum in both HEM and HOM hLRRK2 R1441G Tg mice (Fig. 4E). This finding suggests that the dysfunction of nerve terminals in the nigrostriatal system occurs prior to dopamine neuron loss in this hLRRK2 R1441G Tg mouse model, which is consistent with previous studies in the BAC Tg mouse model expressing the human disease-causing LRRK2 (R1441G) mutant [32]. In this BAC Tg mouse model, axonal degeneration presented with spheroids in the medial forebrain bundle and striatum at 2–4 and 9 months. In other genetic or toxic models of parkinsonism such as adeno-associated virus (AAV) A53T or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice, the results showed retrograde axonal degeneration before the change in dopamine neurons in the substantia nigra [39, 46]. Furthermore, there is also evidence in the human study of PD indicating that axonal degeneration is an early and predominant feature. The dopamine transporter (DAT) and vesicular monoamine transporter (VMAT) study showed 50–70% decreased uptake when the DA neuron loss was 30%. Taken together, the evidence suggests that axonal degeneration and synaptic changes occur prior to DA neuron death in the present Tg mouse models and human PD study.

In this study, we investigated the changes in GTPase activity in both R1441G HEM and HOM mutation mice; we found that there was decreased GTPase activity in the HEM mutation but increased GTPase activity in the HOM mutation. This is consistent with previous in vitro studies that showed that the rate of GTP hydrolysis was reduced in cellular modes transfected with the HEM mutants of R1441C/G and Y1699C [23, 29, 32]. However, none of the previous studies investigated the effect of HOM mutation on GTPase activity. The present study is the first to demonstrate that hLRRK2 R1441G HOM mice have higher GTP hydrolysis activity than hLRRK2 mice (Fig. 1D). To explore the source of the increased GTPase activity, we confirmed that it was yielded from mitochondrial fission protein in the Tg mice. Our results showed increased Drp1 expression on mitochondrial fragments of R1441G HOM mice (Fig. 5). However, we did not find a change in mitochondrial fusion proteins (Opa-1, Mfn-1, and Mfn-2, Fig. 5A). These findings are compatible with the EM study, which revealed shrinkage and small size phenomena in SN mitochondria in R1441G HOM mice by TEM (Fig. 5A). In studies on murine primary neurons and human neuroblastoma, the interaction between endogenous LRRK2 and the fission regulator Drp1 increased Drp1 phosphorylation and mitochondrial fission [42, 60]. This LRRK2- and Drp1-dependent mitochondrial fragmentation is enhanced by overexpression of WT and R1441C LRRK2 but can be reversed by inhibiting Drp1 or increasing fusion [52, 60].
Studies have shown that the phosphorylation of Drp1 at Ser935 causes fission. Notably, increased Ser616 phosphorylation has been observed in patients with sporadic PD [8, 47].

The autophagy–lysosomal pathway is another important mechanism for the pathophysiology of PD with LRRK2. According to our results, lysosomes are well resolved in hLRRK2 mice. In contrast, lysosomes were enlarged and clustered in age-matched R1441G HOM mice (Fig. 6A). Meanwhile, the levels of LC3 and p62/SQSTM1 were not statistically significant (Fig. 6C and D). This finding isn’t consistent with a previous study showing an accumulation of autophagic vacuoles, with increased levels of p62 as a marker of autophagy in HEK-293 cells with mutations in the GTPase domain (e.g., R1441C) [3]. Furthermore, the autophagy–lysosomal pathway is also impaired in the absence of LRRK2, involving lipofuscin granule accumulation and altered levels of LC3-II and p62 [57]. An investigation of the regulation of the tissue specificity of LRRK2 expression by autophagy showed the age-dependent accumulation of autophage vacuoles in the cortex and striatum of R1441C and G2019S Tg mice, suggesting that LRRK2 expression is regulated by autophagy specifically in neuronal somas and axial processes from the cortex and striatum [44]. Regarding the finding of obvious change in lysosomal morphology were seen in the SNC of HOM hLRRK2 R1441G mice by TEM, it is notable that there was no statistically significant difference in autophagy markers in the present study. However, to make it clear, future studies are precious by increasing the number of studied animals which could either reduce the standard errors of mean value or alleviate the variation of the autophagy process at different stages in HOM hLRRK2 R1441G Tg mice.

LRRK2 is constitutively phosphorylated at Ser935, which responds to LRRK2 kinase inhibition [11]. Ser935 phosphorylation is decreased by the PD-linked mutations R1441C and Y1699C [40], while these pathogenic variants show increased kinase activity toward Rab GTPases [51]. An intriguing aspect of our study was the complete loss of the Ser935 phosphorylation site has been described by a previous study in the LRRK2 Ser910Ala/Ser935Ala double knockin mice [65]. In this double knockin mouse line, no anxiety or motor dysfunction obverted at 9 months of age. Therefore, we surmised that the abnormal phenotype of the HOM hLRRK2 R1441G Tg mice in our study might not be caused by the loss of Ser935 phosphorylation. This phosphorylation site did not have a significant effect on the total protein level of LRRK2 in the different strains of Tg mice (Fig. 1B).

There are several limitations in the present study. First, the BAC Tg mice inserted with HEM hLRRK2 WT (FVB/N-Tg(LRRK2)1Cjli/J, no. 009610) and HEM hLRRK2 R1441G (FVB/N-Tg(LRRK2*R1441G)135Cjli/J, no. 009604) genomes [32] were used in this study. BACs have been used to some degree of success with mice while studying neurological diseases such as PD [63]. BACs are preferred for these kinds of genetic studies because they accommodate much larger sequences without the risk of rearrangement and are therefore more stable than other types of cloning vectors [5]. However, the enhancement of expression may result in the random integration of multiple copies of the transgene [63]. One of the most frequently described issues in BAC transgenesis consists of unwanted effects caused by integration of the large transgene in another gene. For example, this scenario can lead to a functional knockout of the affected gene or cause a change in its expression pattern [63]. This may lead to misinterpretation regarding the effect of transgene expression. A recent study in HEM hLRRK2 R1441G Tg mice (FVB/N-Tg(LRRK2*R1441G)135Cjli/J) showed that hLRRK2 R1441G integrated into mouse chromosome 1, causing a 436 bp deletion in an intron of the Khdrbs2 (KH domain containing, RNA binding, signal transduction associated 2) gene [20]. However, the deletion in introns did not affect protein sequences. Therefore, we propose that the risk of transgene inserted into the random genomic locus in the homogenous overexpression Tg mice is very low in this study. Second, we did not use GTPase inhibitors as a pharmacological treatment for motor dysfunction in these Tg mice [22]. Meanwhile, the changes in kinase activity in association with the increased GTPase activity in the SN were not measured in this study. Finally, whether the loss of Ser935 phosphorylation can cause the delocalization of LRRK2 in the soma and axon in this animal model also requires further elucidation.

Conclusions

We observed the alternations in the phenotype, functional image and morphological changes of the mitochondria and autophagosomes between the ideal control group (hLRRK2) and HOM hLRRK2 R1441G (Figs. 1, 2, 3, 4, 5 and 6). There were also several differences in the manifestations between the non-Tg and HOM hLRRK2 R1441G groups, including body weight, anxiety behavior,
motor dysfunction and GTPase activity. Taken together, the evidence suggests that HOM hLRRK2 R1441G over-expressing Tg mice are a novel genetic model of PD that could be used for the study of the pathophysiology and new targets of pharmacological therapy. However, given the limitation of the data presented in this study, it needs further studies to elucidate the usefulness of HOM hLRRK2 mice for pre-clinical modeling of PD.

**Abbreviations**

AAV: Adeno-associated virus; apoB: Apolipoprotein B; BAC: Bacterial artificial chromosome; BOS: Base of support; CB: Cerebellum; DAT: Dopamine transporter; FOV: Field of view; GTPase: Guanosine triphosphatase; HEM: Hemizygous; hLRRK2: Human LRRK2; HOM: Homozygous; LRRK2: Leucine-rich repeat kinase 2; MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; non-Tg; Non-transgenic; OFT: Open field test; PD: Parkinson’s disease; PFA: Paraformaldehyde; Pi: Inorganic phosphate; S: Striatum; SNc: Substantia nigra pars compacta; SUV: Standardized uptake value; SUVR: SUV ratio; TEM: Translate electron microscope; TM: Transgenic; VMAT: Vesicular monoamine transporter; VOF: Volumes of interest; WB: Western blotting; WT: Wild-type.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12929-022-00844-9.

**Additional file 1: Figure S1.** Immunohistochemical staining of TH-positive neurons in the midbrain of adult HOM hLRRK2 R1441G (12 months old) did not show a significant difference in the positive neurons between groups with or without genetic mutation.

**Acknowledgements**

We thank the staff of the Second Core Laboratory, Department of Medical Research, National Taiwan University Hospital for technical support. We also thank the Department of Nuclear Medicine, National Taiwan University Hospital, for providing adequate equipment for PET/CT experiments and image analysis. Finally, we thank the Department of Veterinary Medicine, Colleges of Veterinary Medicine, National Taiwan University, Department of Pathology, National Taiwan University Hospital and TMU Core Facility for providing technical support on operating the translate electron microscope.

**Author contributions**

MLC and RMW designed the study and wrote the manuscript. MLC performed the research and analyzed the data. MLC wrote the first draft of the paper; RMW edited and finalized the manuscript. Both authors read and approved the final manuscript.

**Funding**

This work was funded by the National Science Council (NSC98-2628-B-002-072-MY3); National Taiwan University (106R8805C); and the Ministry of Science and Technology, Taiwan (MOST102-2314-B-002-111-MY3).

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its additional files.

**Declarations**

**Ethics approval and consent to participate**

All animal experimental procedures were approved by the Committee on Animal Research of National Taiwan University and carried out in accordance with the guidelines of the Committee.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Received: 17 April 2022 Accepted: 5 August 2022**

**Published online:** 14 August 2022

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