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Progressive dopaminergic alterations and mitochondrial abnormalities in LRRK2 G2019S knock in mice

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Abstract

Mutations in the *LRRK2* gene represent the most common genetic cause of late onset Parkinson's disease. The physiological and pathological roles of *LRRK2* are yet to be fully determined but evidence points towards *LRRK2* mutations causing a gain in kinase function, impacting on neuronal maintenance, vesicular dynamics and neurotransmitter release. To explore the role of physiological levels of mutant *LRRK2*, we created knock in mice harboring the most common *LRRK2* mutation G2019S in their own genome. We have performed comprehensive dopaminergic, behavioral and neuropathological analyses in this model up to 24 months of age. We find elevated kinase activity in the brain of both heterozygous and homozygous mice. Although normal at 6 months, by 12 months of age, basal and pharmacologically induced extracellular release of dopamine is impaired in both heterozygous and homozygous mice, corroborating previous findings in transgenic models over-expressing mutant *LRRK2*. Via *in vivo* microdialysis measurement of basal and drug- evoked extracellular release of dopamine and its metabolites, our findings indicate that exocytotic release from the vesicular pool is impaired. Furthermore, profound mitochondrial abnormalities are evident in the striatum of older homozygous G2019S mice, which are consistent with mitochondrial fission arrest. We anticipate the G2019S will be a useful pre-clinical model for further evaluation of early mechanistic events in *LRRK2* pathogenesis and for second-hit approaches to model disease progression.

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Keywords

Parkinson's disease; gene-targeted mouse model; dopamine; microdialysis; mitochondria

INTRODUCTION

Mutations and genetic variability in the *LRRK2* gene represent the most common genetic cause of Parkinson's disease (PD). The frequency of the pathogenic mutations is rare at around 2% overall (Di Fonzo et al., 2006; Farrer et al., 2007), however the most common mutation G2019S is found in up to 40% of patients in certain ethnic populations (Kachergus et al., 2005; Ozelius et al., 2006; Ishihara et al., 2007). In addition to pathogenic mutations, common genetic variability in *LRRK2* is a risk factor for sporadic PD (Tan, 2006; Ross et al., 2008; Ross et al., 2011).

LRRK2 Parkinsonism has some unique features, including an age-dependent penetrance (Healy et al., 2008; Hulihan et al., 2008), with some aged carriers escaping disease (Kay et al., 2005) suggesting that disease manifestation is subject to other genetic or environmental modifiers, and potentially that the course of the disease may be altered by therapy. At the neuropathological level, *LRRK2* Parkinsonism typically resembles idiopathic PD, exhibiting dopamine neuronal loss with synucleinopathy. Exceptions do exist in some kindreds, with patients that carry the same mutations having differential pathologies, including neuronal loss only and filamentous tau inclusions (Zimprich et al., 2004). The presence of pathologies that overlap with other neurodegenerative diseases such as Alzheimer's disease and Progressive Supranuclear Palsy has led to speculation that *LRRK2* dysfunction may be upstream of several important neuronal signaling cascades relevant to other neurodegenerative diseases, and as such, a *LRRK2* based therapeutic may have wider applications than just *LRRK2* PD.

The physiological and pathological roles of *LRRK2* protein are not yet fully understood but it is generally accepted that it functions as a kinase, with an important role in neuronal maintenance, vesicular trafficking and neurotransmitter release in the brain. The overwhelming data from rodent models with near-physiologic levels transgenic expression suggest that mutant *LRRK2* impairs dopamine neurotransmission, in the absence of neuronal loss (Li et al., 2009; Li et al., 2010; Melrose et al., 2010; Zhou et al., 2011; Beccano-Kelly et al., 2014b; Liu et al., 2014; Tsika et al., 2014; Walker et al., 2014; Lee et al., 2015) whereas higher levels of expression of *LRRK2*, via heterologous promoters or viral delivery, leads to dopamine neuronal death in mice and rats (Lee et al., 2010; Dushonchet et al., 2011; Ramonet et al., 2011). Nigro-striatal dopamine alterations were not found in two previously reported gene-targeted *LRRK2* mutant models (Tong et al., 2009; Herzig et al., 2011). However, stimulated catecholamine release from adrenal chromaffin cells was reduced in the R1441C knockin mice, and mutant mice displayed differential responses to pharmacologically induced behaviors (Tong et al., 2009). G2019S knock in mice did not display altered dopamine drug-induced locomotor behaviors, but peripheral phenotypes were evident, including a moderate decrease in diastolic blood pressure and changes in mTOR signaling in the kidney (Herzig et al., 2011).

We have created a G2019S KI mouse model and performed an extensive dopaminergic and behavioral evaluation in heterozygous (HET) and homozygous (HOMO) animals. We show that both HET and HOMO G2019S mice have elevated kinase activity in the brain from a young age. Similar to the two previously described LRRK2 knock in models, we do not observe loss of dopamine neurons. However, by utilizing microdialysis to measure extracellular release of monoamines in freely moving mice, we are able to demonstrate a progressive dopaminergic phenotype in HET and HOMO G2019S KI mice, which is characterized by a decrease in basal and evoked dopamine release in the striatum. Additionally, by measuring extracellular dopamine metabolism, we also provide evidence that suggests that the amount of packaged dopamine available release by exocytosis is less. Finally, we reveal that HOMO KI mice display progressive changes in mitochondria which are consistent with arrested fission. We anticipate that this G2019S KI model will be useful for further studies of pre-clinical mechanism of Parkinson's disease and assessment of neuroprotective therapies.

METHODS

Animals

All animal procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised 1996.

Generation of G2019S KI mice

G2019S KI mice were generated at Ozgene PLC (Australia) utilizing a construct designed to replace two bases in *LRRK2* exon 41. The base 'G' at *LRRK2* cDNA position 6055 is conserved in mouse and human (as is the Gly amino acid codon). The 'G' transversion mutation to an 'A' in humans changes the codon at position 2019 from GGC (Gly) to AGC (Ser); however, in mouse, it changes the codon GGG (Gly) to AGG (Arg). Therefore, in order to change codon 2019 to Ser in the mouse, a two base mutagenesis was required to change GGG to AGC in exon 41 of the targeting construct. A floxed neomycin (neo) selectable cassette was placed immediately downstream of exon 41. Regions of 5'homology (4kb) and 3'homology (4.7kb) were used to drive the homologous recombination event by standard gene targeting techniques in C57BL/6 Bruce4 embryonic stem (ES) cells (Kontgen and Stewart, 1993). Following electroporation of the targeting construct, cells were selected for neo resistance. Targeted ES cells were confirmed by Southern blotting and PCR. Euploid, targeted ES cells were then microinjected into Balb/cJ blastocysts and re-implanted into pseudopregnant dams. Resultant chimeras were bred to C57BL/6J breeders to establish transmission. Black (i.e. those with the ES cell germline) progeny that were heterozygous for the gene-targeted allele were then bred to Cre recombinase "deleter" mice (Ozgene) on C57BL/6J background to allow excision of the floxed Neo selection cassette. Cre was then removed by breeding to C57BL/6J wild type mice. Resultant mice were then transferred to our colony and bred in HET x HET breedings. Single nucleotide polymorphism analysis with 124 evenly spaced markers covering the mouse genome indicated that the strain was congenic on C57BL/6 with no evidence of any contaminating strain, although it should be noted that *LRRK2* knockout mice (Hinkle et al., 2012b) produced in a similar manner via

gene targeting in Bruce 4 ES cells contain both C57BL/6J and C57BL/6N markers (<http://jaxmice.jax.org/strain/012444.html>). As no gender differences were noted, male and female mice were used in all experiments. For most experiments we included wild-type (WT), HET and HOMO mice however due to cost and space limitations, some experiments compared only HOMO and WT mice.

Routine genotyping was performed by a PCR-based strategy utilizing primers that cover the mutation and remaining 34 base loxP sequence following cre-excision of the selection cassette. The primers were (forward 5' CAGGTAGGAGAACAAGTTTAC'3, reverse 5' GGGAAAGCATTTAGTCTGAC'3) and yielded a 307bp band in WT, 383bp band in HOMO and both bands in HETs.

Reverse Transcriptase PCR

RNA was isolated from frozen striatum using TRIzol® (Invitrogen, Life Technologies, Grand Island, NY) according to manufacturer's instructions. cDNA was synthesized using Superscript II (Invitrogen). Real time PCR assays were performed in triplicate on a 384 well plate using an ABI 7900 detection system (Applied Biosystems, Life Technologies) to assess the relative level of murine *LRRK1* (Mm00713303_ml), *SNCA* (Mm00447333_ml) and *MAPT* (Mm00521988_ml) and *PRKN* (Mm00450186_ml). In all instances murine *GAPDH* (Mm99999915_ml) was used as the endogenous reference gene.

Antibodies

UDD3 raised to LRRK2 residues 100–500 was provided by Dr. Alessi (Univ. Dundee) and was used for immunoprecipitation. Antibody LRRK2 c41-2/MJFF2 (1:750 immunoblotting) recognizes amino acids 970 to 1000 was from Epitomics and was also used for western blotting. Tyrosine hydroxylase (TH) (Affinity Bioreagents, now ThermoFisher Scientific, Waltham, MA) was used to visualize dopamine neurons by immunohistochemistry (1:200). Detection of α -synuclein was with a mouse monoclonal to α -synuclein (1:3500, clone 42, immunohistochemistry) from BD Transduction Labs (San Jose, CA). Activated microglia were detected by Iba-1 (1:2000, Wako USA, Richmond, VA). Tau antibodies were CP-13 (1:1000 immunohistochemistry, 1:200 immunoblots), Tau-5 (1:500 immunoblots) and PHF-1 (1:500 immunoblots) all gifts from Dr. Peter Davies, Albert Einstein College of Medicine, 12E8 (1:10,000 immunohistochemistry) a gift from Dr. Peter Seubert, Elan Pharmaceuticals, and Tau-1 (1:1000 immunoblots) from Millipore (Billerica, MA). Neurofilament antibody was SMI-31 (1:50K, Sternberger Monoclonals, now Covance, Princeton, NJ). For mitochondrial proteins: Drp-1 (1: 1000, Novus Biologicals, Littleton, CO), pDrp-1 S616 (Cell Signaling, Danvers, MA), Fis-1 (1:1000, Enzo Life Science, Farmingdale, NY), Mitofusin-1 (Mfn-1) and 2 (Mfn-2) (both 1:500, Abcam, Cambridge, MA), Opa-1 (1:5000, Novus Biologicals), Parkin (1:5000, Abcam), PINK-1 (1:500, Novus Biologicals), translocase of outer mitochondrial membrane 20 (TOMM20 1/500 immunoblots and 1/200 immunofluorescence 1/500, Santa Cruz Biotechnology, Dallas, TX), inner membrane markers total OXPHOS and NDUFS3 (both Abcam), matrix markers SOD2 and cyclophilin F (both Abcam). Autophagy markers LC3 (1:5000, Novus Biologicals) and P62/SQSTM1 (Proteintech, Chicago, IL). GAPDH (1:15,000, Sigma), VDAC (1:1000) or

vinculin (1:50000, Sigma, St. Louis, MI) were used as a loading controls. VDAC was also used as a mitochondrial fraction marker.

Immunoblotting

Analysis of phosphate-buffer soluble LRRK2 and tau protein (N=4–6 half brains or N=3 striata were used per genotype per age) was performed in lysates prepared in 50mM Na₂PO₄, 10mM Na₄P₂O₇·10H₂O, 20mM NaF, 2mM EGTA, 2mM EDTA, 2mM Na₃VO₄ with 1mM phenyl-methyl-sulfonyl fluoride (PMSF), 1/100 dilution of protease inhibitor cocktail and Phosphatase I and II inhibitors (all Sigma) and 2mM DTT added just before homogenization, as previously described (Melrose et al., 2010); with slight variation for LRRK2 immunoblots, in that the sample was brought to 1x lithium dodecyl sulfate loading buffer (LDS) in 10 volumes of phosphate homogenization buffer and 10μL of sample was loaded per well. For the phosphate buffer insoluble fraction, the pellet was sonicated in 10x volumes of 1x LDS diluted in the phosphate buffer and 10μL of sample was loaded per well. For immunoblots to mitochondrial and autophagy markers (N=3 per group), hemi-brains were homogenized in RIPA buffer (50mM Tris pH 8.0, 150mM NaCl, 1mM EDTA, 1mM EGTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton-X 100, 1x Complete Protease inhibitor (Roche, Indianapolis, IN) protein, 1mM PMSF and 1mM phosphatase inhibitors I and II (Sigma). For mitochondrial fractionation, striatum and hippocampi (N=2–3 per genotype) were homogenized in 10mM Tris-HCl pH 7.4 containing 1mM EDTA, 0.2M D-Mannitol, 0.05M sucrose, 0.5mM Na₃VO₄, 1x Complete Protease inhibitor (Roche) using a teflon pestle. Crude nuclei were removed by centrifugation at 500xg, then the supernatant centrifuges at 11,000xg for 10 minutes at 4°C, yielding the heavy mitochondrial pellet (HM) and a light mitochondrial pellet (LM). Lysates were electrophoresed by SDS-PAGE using 8%, 8–16% or 14% Tris-Glycine gels (Invitrogen) and transferred onto PVDF. Membranes were incubated with primary antibodies overnight at 4°C followed by HRP-conjugated secondary antibodies (1:15000; Jackson ImmunoResearch Laboratories, West Grove, PA). Bands were visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore) on X-ray film. For densitometric analysis Image J software version 1.63 was used.

Immunoprecipitation and Kinase Assays

UDD3 antibody was coupled to protein-A sepharose beads at a 1μg to 1μl bead ratio by incubation at 4°C rotating. Following incubation, beads were centrifuged for 1 minute at 13,000x g, the supernatant removed and the beads washed four times in phosphate-buffered saline (PBS) to remove unbound antibody. Clarified lysates were incubated at a ratio of 10ml antibody bound beads to 10 mg brain tissue, for 1 hour at 4°C. The complexes were then collected by centrifugation at 13,000x g for 2 minutes before repeated washing (3x) in lysis buffer plus 500mM NaCl. For direct elution, 4x LDS loading buffer was then added to the beads and the mixture incubated at 100°C for 10 minutes. The eluent was then diluted 2x and collected by centrifugation through a 0.22μm Spinex column. β-mercaptoethanol was then added to the eluent to 1% and sample incubated for 5 minutes at 70°C before immunoblotting. Beads were resuspended in buffer A (50mM Tris pH 7.5, 1 mM EGTA, 270mM sucrose, 1mM benzamide, 2mM PMSF). For kinase assays, 10mg of brain tissue (N=3–4 per genotype for each age point) was used per 10μl of antibody bound beads.

Peptide kinase assays were set up in a total volume of 50 μ l with immunoprecipitated LRRK2 in 50mM Tris/HCl, pH 7.5, 0.1mM EGTA, 10 mM MgCl₂ and 0.1mM [γ -³²P] ATP (~300–500 cpm/pmol, Perkin Elmer, Waltham, MA) in the presence of 20 μ M of the Nictide peptide substrate (RLGWRRFYTLRRARQGNTKQR (Nichols et al., 2009). Assays were carried out at 30°C for 30 minutes shaking. Reactions were terminated by applying 30 μ l of the reaction mixture onto P81 phosphocellulose paper and immersing in 50mM phosphoric acid. After extensive washing, the radioactivity in the reaction products was quantified by Cerenkov counting. Kinase assay data was analyzed using a 2-way Analysis of Variance (ANOVA), with age and genotype as factors, and Tukey's post-hoc comparisons.

Stereology

Brains from 18–20 month old littermate WT (N=5) and HOMO (N=3) mice were post-fixed in 4% paraformaldehyde (PFA) for 24 hours followed by 30% sucrose cryoprotection for 48 hours. Brains were sectioned exhaustively at 50 μ m thickness using a freezing sledge microtome. For dopamine neuron estimates, after a random start, every third section was stained free floating with TH antibody. Free floating immunostaining was performed utilizing the VECTASTAIN® ABC System (Vector laboratories, Burlingame, CA). Sections were mounted onto glass slides, allowed to dry overnight, lightly counterstained with cresyl-violet and then dehydrated and cover slipped. Quantification was performed at high magnification (400X) using the optical fractionator number probe in Stereo Investigator software (MicroBrightField, Williston, VT). The Gundersen co-efficient of error (CE) value was 0.09 or less for each estimate. Data was plotted as mean $\pm$ SEM and statistically analyzed by Student's T-test.

High performance liquid chromatography (HPLC)

HPLC with electrochemical detection was performed as previously described (Melrose et al., 2010) in striatal tissue punches from frozen brains from littermate WT (N=9) and HOMO (N=4) mice aged 6 months and 18 months (N=4 per genotype). The amounts of monoamines/metabolites in the tissue samples were determined by comparing peak area values with those obtained from external standards run on the same day. Neurochemical concentrations were determined by normalizing samples to protein concentrations obtained from the pellets (BCA method). Data was plotted (mean $\pm$ SEM) and statistically analyzed by 2 way ANOVA (age and genotype as factors) followed by Tukey's Post hoc comparisons.

Microdialysis

WT, HET and HOMO littermates (n=8–11 per genotype, per age) aged 6 and 12 months were anesthetized with isoflurane. Guide cannulae (CMA Microdialysis, Sweden) were surgically implanted into the striatum using a standard stereotaxic frame (Kopf Instruments, Tujunga, CA) utilizing coordinates (from Bregma anterior-posterior +0.1cm, lateral-medial +0.2cm, dorso-ventral –0.2cm) according to the Mouse Brain Atlas (Paxinos and Franklin, 2001). Mice were allowed to recover for at least 24 hours. Microdialysis experiments were carried out on conscious, freely moving mice with surgically implanted guide cannulae. On the day of the experiment, the stylet in the guide cannula was replaced with the microdialysis probe (CMA/7 with 2 mm membrane, CMA Microdialysis). The probe was

perfused at 2 μ l/min with artificial cerebrospinal fluid (aCSF; 145mM NaCl, 1.2mM CaCl₂, 3mM KCl, 1.0mM MgCl₂) for a two hour equilibration period before collection. Dialysate samples were automatically collected every 20 minutes into vials containing 0.8M phosphoric acid (final concentration 50mM) to retard oxidation of monoamines. Four baseline collections were taken at 20-min intervals, and a subcutaneous injection of 2 mg/kg amphetamine was given 9 min into the fourth collection. Following amphetamine treatment, six subsequent samples were collected every 20 min for a further 120 min. Samples were analyzed by HPLC for dopamine and metabolite content. Data was plotted (mean $\pm$ SEM) and statistically analyzed by Analysis of Variance followed by either Bonferroni's or Tukey's Post Comparison test (see individual results for full details of tests applied).

Pathological analysis

Brains from at least six mice from each genotype were analyzed per time point (~3, 6, 12, 18 months). Kidney, lung and heart were also analyzed from 18 month old mice by basic histology only. Formalin fixed, paraffin embedded tissue sections were dewaxed in xylene and rehydrated in descending alcohols and water. For antigen retrieval in paraffin sections, tissue was pressure cooked (10 minutes) in distilled water (all antibodies, except α -synuclein and TOMM20). Appropriate disease/tissue positive controls were included for each antibody (diffuse Lewy body disease for α -synuclein, Alzheimer for tau antibodies, Alzheimer/vascular dementia for Iba-1). DAB colormetric immunohistochemistry was performed using the Dako Autostainer (Dako, Carpinteria, CA). Tissue was quenched for endogenous peroxidases in 0.03% H₂O₂ and blocked in Dako All-purpose blocking solution for 30 minutes. Primary antibody was incubated for 45 min at room temperature. All secondary antibodies were from the Envision⁺ System Labeled Polymer HRP (Dako), followed with DAB substrate (Dako). Sections were lightly counter stained in Gills 3 hematoxylin. Standard haemotoxylin and eosin histological staining was also used to assess gross morphology. Immunofluorescence histochemistry for TOMM20 was performed on paraffin sections, using goat-anti rabbit conjugated Alexa antibody (Life Technologies). Antibodies were incubated in Dako antibody diluent. Primary antibody was incubated overnight. Sections were incubated in sudan black prior to coverslipping. All images taken with Plan Apochromat 63/1.4 Oil DIC M27 lens on a Zeiss Super Resolution microscope (SIM), Elyra PS.1). Figure images shown are from the super-resolution channel and are single planes from z-stacks.

Electron microscopy (EM)

WT, HET and HOMO mice (N=2 per genotype) aged 15 months and N=3 HOMO and WT mouse aged 23 months were perfused with 4% paraformaldehyde, brains were removed and post-fixed in Trump's solution overnight. The next day, striatum was dissected from each brain and subjected to EM staining as described previously (Trushina et al., 2012). Striatal tissue was incubated in 1% osmium tetroxide, dehydrated in a graded series of ethanol and embedded in Quetol 651 (Ted Pella, Inc, Redding, CA). Thin sections (0.09–0.1 μ m) were cut parallel to the ventral surface using a diamond knife (Diatome, Hatfield, PA US) and an Ultracut E microtome (Reichert-Jung, Wien, Austria). Sections were collected on copper grids, post-stained with lead citrate and viewed at ~80 kV with a JEOL 1400 transmission electron microscope (JEOL USA, Peabody, MA).

Quantification of EM micrographs was performed as previously described (Trushina et al., 2012), in 10 random images from the striatum from each mouse. Mitochondria residing in the neuropils were counted and measured.

Behavioral Analysis

Mice were acclimated in the test room under normal lighting conditions for 1 hour prior to testing. The cohort (N=10–13 per genotype, all littermates) consisted of males and females and was tested at both 6 months and 12 months.

Open Field Assessment (OFA)—The OFA test was used to examine general locomotor activity and anxiety. For the test the mouse was placed facing one side of a brightly lit open field arena (150 Lux) and ANYMaze software (Stoelting Co., Wood Dale, IL) was used to track exploratory behavior over 15 minutes (5 minute bins).

Rotarod—Motor co-ordination was measured using an automated rotarod system (Rotamex-5 Columbus instruments). The spindle dimensions were 3.0cm × 9.5cm and the speed of the rod was set to 4–40rpm acceleration, increasing 1rpm every 5 seconds. The equipment was equipped with a sensor that automatically stops the timer if the mice cling and roll around on the rod. Mice were trained for 3 days, each day for 4 consecutive trials, allowing 10 minutes rest between trail.

Gait Dynamics—Mouse gait dynamics were obtained using a motorized treadmill (with a transparent belt and digital video camera mounted underneath) by ventral plane videography and analyzed with DigiGait® Version 9 software (Mouse Specifics, Inc. Quincy, MA). Each mouse was individually placed in the treadmill compartment for a few seconds and then the belt was turned on at a low speed (4 cm/sec) just prior to testing. The motor speed was then set to 24 cm/sec, collecting an average 4 seconds of videography to obtain at least 12 or 15 sequential step images, respectively. Mice that did not have stride regularity indices (alternate step sequences) at 100% were still included in the study to evaluate inter-limb coordination.

Each individual gait signal per limb consists of a stance duration (time in contact with surface) and swing duration (time not in contact with surface) which together are the stride duration. Stride frequency is calculated by measuring the number of strides over time. Stride length is calculated by dividing the belt speed over the stride frequency. Paw angles and step angles at full stance are determined by software geometry calculations (fitting ellipses to the paws) of ellipse centers, major axes and vertices. For analysis, the left and right gait measurements were combined, as where the forelimb and hindlimb data.

Tests were carried out beginning with the least invasive to most invasive. For 6 months time-point this was OFA→NOR→EPM→LDE→rotarod→gait→CF. At the 12 months time-point OFA→NOR→EPM→LDE→rotarod→gait→MWM. The experimental paradigm was chosen to minimize the use of animals and determine any progressive alterations, albeit with the caveat that CF is an invasive test and hypothetically could impact the subsequent emotional testing (but not motor or MWM) six months later.

Experimental details of additional behavioral testing (novel object, conditioned fear, light-dark exploration and Morris Water Maze) are provided in the supplement. Analysis of the ANYmaze-generated data, CF and MWM data was performed by ANOVA followed by Fisher's Least Significant Difference (LSD) multiple comparisons. Rotarod and DigiGait data were analyzed by ANOVA followed by Tukey's post-hoc comparisons.

RESULTS

Generation of G2019S knock in mice

The targeting strategy for generation of *LRRK2* G2019S KI mice is shown in Fig. 1a. Homozygous mice received from Ozgene PLC were bred to Jackson C57BL6/J mice and subsequent heterozygous offspring were bred together to obtain WT, HET and HOMO KI animals. Sequencing of cDNA was performed to confirm the presence of the G2019S mutation at the RNA level in the mouse genome (Fig. 1b). To determine if *LRRK2* mRNA levels were equivalent across genotypes we used quantitative Taqman assays, using cDNA made from RNA extracted from striatum, the area of highest *LRRK2* expression. Expression of *LRRK2* cDNA levels were equivalent for *LRRK2*, the paralog *LRRK1*, and other Parkinson's genes *SNCA* and *MAPT* (Fig. 1c). Hemi-brain and striatal lysates probed with *LRRK2* MJFF2 antibody, revealed similar levels of protein expression level in WT, HET and HOMO mice (Fig. 1d, e) at different aged time points. *LRRK2* HET and HOMO mice were fertile and appeared to be healthy from birth with normal body weights. HET x HET breedings yielded Mendelian ratios in line with expected inheritance and survival (from over 80 litters 26 % WT, 49 % HET, 25 % HOMO).

Dopaminergic system analysis

LRRK2 protein is well documented to be found at highest levels in the striatum in mammals (Galter et al., 2006; Melrose et al., 2006; Taymans et al., 2006; Higashi et al., 2007a; Higashi et al., 2007b; Melrose et al., 2007; Westerlund et al., 2008a; Westerlund et al., 2008b; Mandemakers et al., 2012; Davies et al., 2013a; Giesert et al., 2013; West et al., 2014). Therefore we reasoned that the G2019S mutation might impact on the functional integrity of the nigro-striatal dopaminergic pathway. To assess dopamine neuronal number in the substantia nigra, we performed unbiased stereological counting in mice aged 18–20 months and found that dopamine neuronal numbers were equivalent between WT and HOMO mice (Fig. 2a). On visual inspection TH immunohistochemistry in nigral and striatal sections from WT and HOMO mice was comparable (Fig. 2b). Next we analyzed axonal neurochemistry in striatal lysates from WT and HOMO mice aged 6 and 18 months. There was significant age effect on dopamine (DA) levels ($P < 0.001$) with decreases at 18 months in both WT ($p < 0.05$) and HOMO ($p < 0.01$) compared to 6 months, but no effect of genotype (Fig. 2c). The levels of metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were also significantly affected by age ($P < 0.0001$) with a significant decline in both WT ($p < 0.0001$) and HOMO ($p < 0.0001$) between 6 and 18 months, but no genotype effect (Fig. 2d). Metabolite homovanillic acid (HVA) was also affected by age ($P < 0.0001$) with a decline from 6 to 18 months in both WT ($p < 0.001$) and HOMO ($p < 0.01$), whereas genotype effect just missed significance ($P = 0.06$, Fig. 2e). The DOPAC/DA ratio can be used as an indicator of the rate of DA turnover (synthesis and metabolism) when DA levels are not altered by the

drug treatments (Bannon and Roth, 1983) and the HVA/DA ratio can be used as an indicator of the sum of DA metabolism and release (Westerink, 1985). No age or genotype effects were evident for DOPAC/DA (Fig. 2f), however for HVA/DA there was a significant effect of age ($P<0.01$) and genotype ($P<0.001$) and a significant interaction between age and genotype ($P<0.001$). Individual comparisons revealed that the decline in HVA/DA in WT from 6 to 18 months was significant ($p<0.001$) but not for the HOMO mice (Fig. 2g). Accordingly, at 18 months comparisons between WT and HOMO mice revealed a significant difference ($p<0.001$), suggesting that the sum of DA metabolism and release is altered in HOMO mice.

To measure real-time monoamine neurotransmitter release in the striatum, i.e. extracellular release of dopamine and its metabolites, we performed *in vivo* microdialysis in awake, freely moving animals. Dopamine was measured at baseline levels as well as after pharmacological challenge with amphetamine. In mice aged 6 months of age, there was no significant effect of genotype for dopamine baseline nor for evoked levels (Fig. 3a–c) or for metabolites (data not shown). However, in older mice aged 12 months old a significant difference between average levels of basal dopamine of WT, HET and HOMO KI mice ($P<0.01$) with dopamine levels over 50% less in HET and HOMO compared to WT ($p<0.01$, Fig. 3d). To assess basal and stimulated release, dopamine levels were plotted over time. While the overall effect of genotype missed significance ($P=0.07$), post-hoc tests at individual time points revealed the levels of dopamine 20 minutes after injection of amphetamine were significantly lower for both HET and HOMO mice (<0.001 , Fig. 3e). To examine the response to amphetamine challenge as a percentage, post-injection DA levels were normalized to individual DA baselines, and percentage response plotted over time (Fig. 3f). Genotype as a variable was not significant, but a significantly higher percentage response to amphetamine challenge was evident in HET ($p<0.05$) and HOMO ($p<0.001$) mice, despite having lower baseline levels and releasing on average less dopamine after challenge. This likely suggests that reverse transport of dopamine, facilitated by the dopamine transporter, is intact.

Although the average levels of basal DA release in HET and HOMO mice were lower compared to WT, the average level of extracellular metabolites HVA and DOPAC, remained equivalent across the genotype, i.e. no parallel decline in HET and HOMOS (Fig. 4a, b). On comparison of mean of the individual sample extracellular DOPAC/DA and HVA/DA ratios at baseline (Fig. 4c, d) we found that WT mice the average DOPAC/DA and HVA/DA were ~400 respectively, whereas in HET and HOMO mice the ratios were almost double at ~700–800 ($p<0.01$). Metabolic ratios following amphetamine stimulation were not different between genotypes (Supplemental Figure 1).

Behavioral Analysis

In the OFA there was no overall effect of genotype at 6 months of age for any of the parameters tested (Fig 5a–f), however individual post-hoc comparisons revealed that the total distance traveled was slightly increased in 6 month-old HOMO mice compared to WT and HET mice ($p<0.05$, Fig. 5a). On average the time to the 1st entry of the center zone was lower in the HOMO mice but did not reach significance. At 12 months of age (Fig. 6), the distance traveled was not significantly different across the genotypes (Fig. 6a), but there

were modest differences in the first segment of the test (0–300s) in OFA center exploration behavior between HOMO and WT mice, with HOMO mice making more entries into the center zone compared to WT mice (Fig. 6b, $p<0.05$), spending more time in the center (Fig. 6c, $p<0.01$) but exhibiting more frequent freezing bouts (Fig. 6d, $p<0.05$) and increased amount of time spent freezing (Fig. 6e, $p<0.05$). In the second and last segments no differences were observed across genotype for any of the parameters.

Motor testing for gait dynamics in the 6 month cohort (Table 1, upper), revealed a modest effect of genotype was evident for stride length, stride frequency and stride duration ($P<0.05$), with post-hoc comparisons revealing that HETS exhibited a slightly better performance. At 12 months (Table 1, lower), no effect of genotype differences were detected for any of the parameters.

Rotarod testing for co-ordination and balance (Fig. 7) revealed that an overall effect of genotype ($P=0.0027$) in the 6 months cohort, post-hoc tests revealing that on day 3 and 4 the HOMO mice had enhanced performance compared to both WT and HET mice ($p<0.01$). At 12 months there was no effect of genotype.

Overall genotype differences were not observed for emotional behaviors (NOR, LDE or EPM) at either age (Supplemental Figure 2) nor for tests associated with learning (CF and MWM) (Supplemental 3 and 4 respectively).

Kinase Activity Assays

The G2019S mutation is known to cause elevated kinase activity *in vitro* and in G2019S murine BAC mice (Li et al., 2007; Greggio et al., 2009). Until recently the ability to immunoprecipitate endogenous LRRK2 from brain tissue was hampered by the lack of specific high affinity antibodies, and was only possible in epitope tagged transgenic models. Using protocols developed with newer monoclonal antibodies (Davies et al., 2013b), we assayed kinase activity in immunoprecipitated lysates prepared from hemi-brains from WT, HET and KI mice aged 4, 10–12 and 16–18 months. We chose to examine three aged time-points because we reasoned that the kinase activity may alter with age, which we speculated may correlate with the onset of the dopamine phenotype. Immunoprecipitates were assayed for kinase activity using the Nictide peptide (Nichols et al., 2009) and kinase activity was normalized for the amount of LRRK2 added to the assay, as quantified by densitometric analysis of western blots from the IP beads. A statistically significant effect was observed for age ($P<0.05$) and genotype ($P<0.0001$) but no interaction between the variables (Fig. 8a). Post hoc comparisons did not reveal significant age-related differences in any of the genotypes. Consistent with the hypothesis that kinase activity is enhanced by the G2019S mutation, highly significant differences between the genotypes were found at each age with HETS having ~1.8-fold more activity and HOMOS having ~4-fold activity compared to WT (see graphs in Fig. 8a for individual p-value comparisons).

While kinase assays did not reveal any differential age-related differences in kinase activity, we asked whether the solubility or levels of LRRK2 altered with time in G2019S KI mice compared with their WT littermates. Phosphate buffer-solubilized (PS) and Laemmli buffer-solubilized (LS) hemi-brain lysates, prepared from WT, HET and HOMO mice aged 3 and

12 months were immunoblotted for LRRK2 using c41-2 antibody (Fig. 8b for representative examples). Equal volumes of PS and LS lysate were loaded onto the gels to allow calculation of PS versus LS ratios. In addition total LRRK2 in the PS fraction was normalized to GAPDH, and LS fraction to VDAC (Davies et al., 2013b) and values compared across genotypes. Density quantification followed by statistical analysis (Fig. 8c–h) revealed that LRRK2 levels were found to be equivalent in WT, HET and HOMO mice in both the PS and LS fractions at both 3 and 12 months and PS/LS ratios were accordingly similar, suggesting LRRK2 levels and solubility within these experimental fractions, remain stable at least up to 12 months,

Neuropathology

Gross morphological analysis of brain and peripheral organs was performed on sections stained with hameotoxylin and eosin (H&E) taken from WT, HET and HOMO mice aged up to up to 18–20 months of age. Abnormal morphology has been reported in the periphery of LRRK2 deficient rodents (Tong et al., 2010; Herzig et al., 2011; Hinkle et al., 2012a; Tong et al., 2012; Baptista et al., 2013; Ness et al., 2013). Unlike our LRRK2 homozygous knockout mice (Hinkle et al., 2012a) we did not observe any visible discoloration, pigmentation, overt morphological or inflammatory changes in the kidney of HET or HOMO mice up to 20 months (Supplemental Figure 5). Lung tissue morphology appeared normal in aged HET and HOMO mice, with no evidence of vacuolization of pneumocytes previously reported for LRRK2 knockout mice (Herzig et al., 2011). Like lung and kidney tissue, LRRK2 levels are also known to high in heart tissue and LRRK2 knockout mice exhibit diastolic hypertension (Herzig et al., 2011), thus we also examined the hearts of aged WT, HET and HOMO mice, however no abnormalities were noted. H&E stained brain sections did not reveal any overt neuropathological alterations, however we additionally examined brains from young (3–6 months) middle aged (10–14 months) and aged (18+ months) WT, HET and HOMO mice via immunohistochemistry for a variety of markers associated with Parkinson's disease pathology including alpha-synuclein, tau, neurofilament and Iba-1. No differences were observed in alpha-synuclein, neurofilament or Iba-1 (data not shown) however in some brain regions in the oldest mutant animals, tau CP-13 pSer202 immunoreactivity appeared to be increased in intensity, with some positive puncta, increased neuropil immunoreactivity, cytoplasmic staining, as well as occasional positive neurites, which were not found in WT mice (Fig. 9 and 10 show coronal sections at the striatal and mid-brain level). At the striatal level there was no positive staining in the caudate/putamen region, however the corpus callosum had visibly heavier tau staining in both HET and HOMO G2019S mice and slightly increased cortical tau immunoreactivity. At the level of the mid-brain there was little tau reactivity in the nigra in any genotype. Immunostaining intensity was increased in some nuclear groups, particularly the thalamic parafascicular nuclei, the sub-thalamic nucleus, pre-mammillary nuclei and the lateral hypothalamus in HET mice. The posterior amygdaloid nucleus was also more noticeably stained in HOMO mice. Interestingly, the thalamic nuclear groups, the amygdaloid nucleus and the lateral hypothalamus are all areas containing cells with high LRRK2 expression (see Supplemental Figure 6). To determine if we could detect quantitative differences, we performed immunoblotting in hemi-brain lysates with tau antibodies tau-5 (total tau), CP-13 (pSer202), PHF-1 (pSer396/404) and tau-1 (non-phosphorylated axonal tau) in lysates from LRRK2

WT, HET and HOMO KI mice aged 12 months (Fig. 11). We found that total tau levels were equivalent across the genotypes, as were non-phosphorylated axonal tau levels (tau-1). In line with the immunohistochemical findings, phospho-tau at pSer202/Thr205 was found to differ significant between the genotypes ($P=0.02$) and individual post-hoc Tukey's comparisons (taking into account both bands, which represent a combination of murine tau 4-repeat isoforms phosphorylated to different extents) revealed a modest but significant increase in HOMO versus WT mice ($p<0.05$). Tau phosphorylated at pSer396/404 was equivalent across the three genotypes.

Ultrastructure Analysis

Given the lack of overt PD related pathology, we reasoned that more subtle changes may be evident at the ultra-structural level. We performed EM examination of striatal sections in mice aged 15 (WT, HET and HOMO) and 23 months (HOMO and WT only) (Fig. 12). In 15 month old HET mice, mitochondria were found to have altered distribution, clustering in the neuropils, possibly as a result of abnormal organelle trafficking (Fig. 12b). A few HET mitochondria were found to have altered shape and cristae morphology. The changes in mitochondria in 15 month-old HOMO mice were more pronounced (Fig. 12c, d), with evidence of abnormal shape that resembled beads-on-the-string, possibly indicative of fission or fusion abnormalities (Trushina et al., 2012) and the number of swollen mitochondria with altered cristae was also increased. By 23 months of age, these changes became more profound in HOMO mice including the presence of cristae swelling, disorganization and severely condensed mitochondria (Fig. 12e and f, WT and HOMO respectively). We performed quantitative analysis in the striatum of the 23 months old WT and HOMO samples, by counting the number of mitochondria in the neuropils, measuring the length of both mitochondria and neuropils and noting abnormal mitochondria (Table 2). We found that the number of mitochondria per neuropil length was significantly reduced in the HOMO mice compared to WT ($p<0.001$) and mitochondria in HOMO were around 10% longer ($p<0.01$).

To determine if the morphological changes were restricted the striatum, we examined morphology in two additional areas in the same sample – the cortex and the hippocampus – the former being rich in LRRK2 expression, the latter having relatively low expression compared to the striatum and cortex (Fig. 13). Some mitochondria with disorganized cristae were noted in hippocampus and cortex, with the cortex being more affected than the hippocampus, but changes were not to the extent of the striatum. Both cortical and hippocampal regions in HOMO mice appeared to have a higher abundance of lipid droplets and lipofuscin accumulation, compared with the control age matched WT mice.

To further examine the “beads-on-a-string” morphological changes and rule out fixation artefacts, we performed immunofluorescence histochemistry on paraffin sections taken from the striatum of aged WT, HET and HOMO mice with outer mitochondrial membrane marker TOMM20 (Fig. 14). Z-stacks were collected by confocal microscopy. Abnormal ‘threads’ connecting mitochondria could be observed in HET G2019S mice and to a greater extent in HOMO G2019S mice, consistent with EM findings.

Immunoblotting for mitochondrial and autophagy markers

To measure changes that may be occurring in the expression of mitochondrial proteins, we performed quantitative immunoblotting on half-brain mitochondrial extracts prepared from mice aged 15 months with various markers of different mitochondrial compartments, fission and fusion proteins, autophagy markers and the OXPHOS assembly complex (Fig. 15a, b). A significant difference between the mean levels of outer mitochondrial membrane marker TOMM20 ($P=0.018$) was observed, with around a 20% increase in both HET and HOMO mice compared to WT mice ($p < 0.05$ for both). Similarly, genotype effects were significant for both fission markers Drp-1 and Fis-1 ($P=0.02$ and $P=0.002$ respectively). Fis-1 levels were over 45% lower in HOMO mice versus WT and HET mice ($p < 0.01$) and Drp-1 levels were around 30% lower in HOMO compared to WT mice ($p < 0.05$). A genotype effect of PINK-1 ($P=0.024$) revealed PINK-1 levels were reduced in WT versus HET mice ($p < 0.05$) but not HOMO mice. Parkin levels and fusion proteins Mfn-1, Mfn-2 and Opa-1 were not found to be significantly affected by genotype. A significant effect of genotype was observed for autophagy marker LC3-II ($P=0.0035$), post-hoc comparisons revealing significantly increased levels in HOMO mice compared to HET and WT mice ($p < 0.01$). P62 levels were not different between WT and mutant mice, nor were mitochondrial matrix markers SOD2 and cyclophilin F, although there was a significant difference between HET and HOMO mice for cyclophilin F, since mean levels in HET mice were higher than both WT and HOMO mice. Inner membrane marker NDUFS3 levels did not differ between genotypes, however assembly levels of the inner membrane OXPHOS assembly complex were statistically different in mutant mice compared to WT. Differential assembly levels can indicate alterations in mitochondrial oxidative phosphorylation bioenergetics. Both complex V marker (ATP5A subunit) and complex III marker (UQCRC2) marker NDUFS3 were significantly higher in HET mice compared to WT and HOMO mice. Complex I subunit marker (NDUFB8) levels were significantly lower in HOMO mice compared to both WT and HET mice ($p < 0.001$).

We next asked if quantitative fission protein changes were evident outside of the striatum, thus total lysates prepared from hippocampus of aged WT, HET and HOMO mice were probed with antibodies to Drp-1 and its activated phosphorylated form pDrp-1 616 (Fig. 16a). pDrp1 616 levels were normalized to Drp1, and were found to be significantly affected by genotype ($P=0.011$), with HET and HOMO levels being significantly lower than WT controls ($p < 0.05$).

Finally, to further determine if differential levels of fission protein Drp-1 were found between the cytoplasm and the mitochondria, we performed fractionation to enrich mitochondria from striatum of WT, HET and HOMO mice (Fig. 16b). While not quantitative, the immunoblots indicate that levels of both Drp-1 and the activated pDrp-1 616 are reduced in the mitochondrial fraction of HOMO mice.

DISCUSSION

Herein, we demonstrate that mice expressing just one or two copies of mutant G2019S LRRK2 in their own genome have significantly elevated kinase activity and progressive dopaminergic alterations. To our knowledge, this is the first report showing that kinase

activity is increased in a gene-targeted LRRK2 mouse model and the first comprehensive *in vivo* investigation of extracellular striatal dopamine release of dopamine and its metabolites in a LRRK2 knock-in model.

Using brain microdialysis to measure extracellular basal and evoked neurotransmitter release, we find that dopamine and its metabolite levels are unaltered at 6 months but by ~12 months of age, basal DA and amphetamine-stimulated is decreased in both HET and HOMO mice. A major advantage of *in vivo* microdialysis over voltammetry measurements is the ability to detect neurotransmitters and their metabolites simultaneously (Arbuthnott et al., 1990; Darvesh et al., 2011). We find that extracellular metabolite:DA ratios are almost double in G2019S mice (~600) compared to the values obtained for WT animals (~300). Based on the literature, extracellular metabolite: DA ratios are generally in the range of 100–300 in rodents (Sharp et al., 1986; Arbuthnott et al., 1990) but it has previously been shown that levels of basal or pharmacologically induced release of DA does not necessarily correlate with either DOPAC or HVA levels (Sharp et al., 1986; Zetterstrom et al., 1986; Zetterstrom et al., 1988), although DOPAC and HVA (converted from DOPAC by catechol-O-methyltransferase or COMT) levels are significantly correlated (Sharp et al., 1986), which we found to be true (data not shown). This dissociation is explained because a major portion of DOPAC originates from the intraneuronal metabolism of DA that has *not* been released or taken up, but instead the synthesized DA that is supplied and bundled for exocytotic release (Zetterstrom et al., 1988; Arbuthnott et al., 1990). In view of the fact that extracellular DOPAC metabolism appears to be occurring normally, the number of TH neurons is normal and the total amount of axonal striatal DA is similar across WT, HET and HOMO up to 18 months of age, our microdialysis data indirectly suggests that synthesis is intact in the G2019S KI LRRK2 mice. It seems likely instead that in mutant G2019S mice, either less of the newly synthesized dopamine is packaged into vesicles and available for release (i.e. remaining in the cytosol); or alternatively it may be packaged, but exocytotic release process is impaired, or perhaps a combination of both. Based *on in vitro* studies, amphetamine has long been thought to deplete vesicular stores and redistribute vesicular catecholamine to the cytosol, thereby acting on vesicular and membrane transporters and effecting “vesicular” and “cytosolic” pools of monoamines (Sulzer et al., 2005). The flood of cytosolic DA is thought to be released in a carrier-mediated fashion, via reversal of the action of the dopamine transporter. However, *in vivo* actions of amphetamine in freely moving animals may be different to *in vitro* preparations or from *in vivo* recordings in anesthetized rodents. A recent voltammetric study using freely moving rats demonstrated that amphetamine actually promotes exocytotic release (Daberkow et al., 2013). In our model, if total axonal levels of DA are normal, and assuming that carrier mediated transport is intact, one would expect if there was less DA in the vesicles (and more in the cytosol), upon vesicular-to-cytosolic redistribution and efflux, the extracellular DA levels would be similar. However, if amphetamine also acts on exocytotic release and that is impaired functionally, then we would expect to see less overall extracellular DA release, as is the case. Interestingly, the percentage response (normalized to individual baseline DA) was greater in mutant mice, which indicates that reverse transport (i.e. carrier dopamine transport of the available cytosolic DA) is intact in the mutant mice. Taken together, we speculate that exocytosis from the vesicular DA pool is impaired by mutant LRRK2. This finding is

consistent with emerging data from several model systems implicating LRRK2 as a pivotal player in regulating synaptic function. In culture models it is proposed that mutant LRRK2 impairs dopamine neurotransmission via influencing synaptic structure and function through effects on presynaptic proteins, for example via impaired phosphorylation of presynaptic proteins (Piccoli et al., 2011; Belluzzi et al., 2012). LRRK2 has also been shown to regulate synaptogenesis and dopamine receptor activation through modulation of PKA activity (Parisiadou et al., 2014). In rat PC12 cells, expression of G2019S mutant causes an increase in extracellular dopamine, an increase in growth hormone release from SHSY5Y cells, and increased dopamine receptor expression both SHSY5Y cells and in the striatum of LRRK2 G2019S transgenic mice (Migheli et al., 2013). In LRRK2 knockout rats and mice, exocytosis is impaired from lysosome-related storage organelles are impaired in alveolar type-II epithelial cells (Herzig et al., 2011; Miklavc et al., 2014). Recent data in drosophila have shown LRRK2 mediated phosphorylation has profound effects on membrane tubulation and association, and on synaptic vesicle endocytosis (Matta et al., 2012) and LRRK2 has also been proposed to regulate endocytic membrane trafficking in an Rab7-dependent manner (Gomez-Suaga et al., 2014). The endocytic impeding effects of mutant G2019S can be rescued with LRRK2-IN-1 inhibitor in mutant drosophila (Matta et al., 2012) and acute treatment with LRRK2 inhibitors reduced the frequency of spontaneous currents, the rate of synaptic vesicle trafficking and the release of neurotransmitter from isolated synaptosomes (Cirnaru et al., 2014).

In line with normal DA neuronal counts in aged G2019S KI mice, we did not find evidence of loss of *total* striatal dopamine but we did note a significant increase in the metabolite HVA levels and in the HVA/DA turnover ratio, an indicator of the sum of dopamine metabolism and release. HVA is the minor metabolite in rodents, but the major metabolite in humans and is produced outside of the terminal by post-synaptic via conversion of DOPAC via COMT or 3-methoxytyramine via mono-amine oxidase. Increased striatal DA turnover has been found in various rodent models, as well as in early stage sporadic PD patients and asymptomatic LRRK2 carriers (Sossi et al., 2002; Zigmond et al., 2002; Sossi et al., 2004; Galter et al., 2010; Sossi et al., 2010; Sterky et al., 2012). In general, increased turnover generally reflects increased impulse flow and it has been suggested that this is a compensatory increase in activity of remaining DA neurons and may help to explain why up to 80% of striatal DA can be lost before the onset of clinical motor symptoms (Sossi et al., 2010).

Not surprising, given the lack of dopamine neuronal loss, our behavioral battery did not determine any overt motor phenotype in the G2019S mutant mice. In fact the younger HET mice exhibited slightly enhanced gait dynamics and younger HOMO mice out-performed WT and HET mice on the rotarod. Along with the increased distance traveled by HOMO mice in the OFA at 6 months, which could reflect hyperactivity, it is a possibility that there is early behavioral ‘over-compensation’ to progressive neurochemical changes that occur in response to the mutation, which confer a temporary superior behavioral performance. Interestingly, a study utilizing the Herzig G2019S knockin mouse (Herzig et al., 2011), proposed that the G2019S mutation confers a hyperkinetic phenotype that is resistant to age-related motor decline (Longo et al., 2014). At the older time-point we did note some subtle

differences in open-field behaviors in HOMO mice in the initial testing segment, which may reflect an initial misinterpretation of open spaces. However, we must interpret these results with caution since a limitation of our behavioral paradigm was that the mice tested at 12 months were subjected to invasive fear conditioning analysis prior at 6 months. While this is not likely to impinge on subsequent motor testing (or water maze, which is considered more invasive), emotional behavioral testing data (i.e. OFA, NOR, EPM, LDE) potentially could be confounded, although we speculate this is unlikely after 6 months.

Despite the later onset of the dopamine release phenotype in the G2019S KI mice, there were no age related changes in kinase activity, or in LRRK2 levels in the G2019S KI mice. Nevertheless, it is important to note that kinase activity was measured in *ex vivo* lysates, in immunoprecipitated LRRK2 with a synthetic substrate, thus may not fully reflect the physiological situation. The cellular environment may be important for LRRK2's physiological role (Lewis and Manzoni, 2012) and kinase activity *in vivo* will be subject to variations depending on the LRRK2 levels, precise localization and the aging environment. At this time there is no known robust *in vivo* substrate/assay to allow *in vivo* quantification of kinase activity. It is possible that it is the cumulative effect of aberrant kinase activity over time that contributes to the progressive dopaminergic deficits. Testing if dopamine release deficits can be rescued by kinase inhibition will address this question. It is also conceivable that the release deficits are independent of kinase activity given that the importance of the other functional domains of LRRK2. In cultured neurons LRRK2 toxicity was found to be independent of kinase activity but instead linked to LRRK2 levels and its interaction with alpha-synuclein (Skibinski et al., 2014). Furthermore, LRRK2 participates as a scaffold in canonical Wnt signaling, which is linked to neuronal survival; and changes in LRRK2 expression levels affect pathway activity (Berwick and Harvey, 2012). The lack of difference in LRRK2 expression levels between mutant and WT mice differs from a previous study that reported reduced striatal LRRK2 levels in the striatum of G2019S homozygous knock in mice compared to wild-type controls (Herzig et al., 2012). The differences may be explained by differential lysate preparation and LRRK2 antibody used (MJFF2 monoclonal versus the Novartis in-house rabbit LRRK2 antibody), the use of half-brain versus isolated striatum, or reflect insufficient animal numbers to detect subtle differences.

In terms of neuropathology, no overt changes in line with classic PD hallmarks or evidence of aggregated protein deposits were observed. Previously, we have reported changes in phospho-tau and tau species regulation in our G2019S BAC model (Melrose et al., 2010). In agreement, we find in the brains of 18 month+ HET and HOMO G2019S mice an increased intensity of phospho-tau signal, accumulation in the cytoplasm, positive puncta and some immunopositive neurites, which was substantiated by quantitative immunoblotting. While the changes at the pSer202 epitope are subtle compared to the G2019S BAC model, they nevertheless further support a link between tau and LRRK2. Indeed, a recent study has shown that LRRK2 phosphorylates novel tau epitopes *in vivo* (pThr149 and pThr153) and epitope pSer199/202/Thr205, as well as promoting tauopathy in mutant human tau P301L mice (Bailey et al., 2013). Unfortunately, we were unable to assess the novel LRRK2

modulated phospho-tau epitope antibodies in the G2019S mice, because neither reacts with endogenous mouse tau.

The striking mitochondrial abnormalities in distribution, size and structure, in the HOMO G2019S mice substantiates and further extends on previous findings linking LRRK2 to mitochondria deficits, previously been reported for LRRK2 G2019S mouse models (MacLeod et al., 2006; Lin et al., 2009; Ramonet et al., 2011), in human LRRK2 patient material (Mortiboys et al., 2010; Angeles et al., 2011; Cooper et al., 2012; Papkovskaia et al., 2012; Sanders et al., 2014) and in cellular systems (Ng et al., 2009; Saha et al., 2009; Wang et al., 2011; Niu et al., 2012; Wang et al., 2012; Cherra et al., 2013; Hindle et al., 2013; Su and Qi, 2013; Saez-Atienzar et al., 2014). The significant reduction in the number of mitochondria per neuropil in the striatum of G2019S HOMO KI mice and the ~10% increase in length suggests that the mitochondria are adjusting to changes in their surroundings. One explanation could be that biogenesis is altered, and the produce of healthy mitochondria is impaired, so instead the damaged mitochondria increase in length, blocking fission and degradation through mitophagy to maintain residual function.

The altered assembly levels of the OXPHOS complex in the G2019S mutant mice could support the notion that mitochondrial bioenergetics is impaired in this model. The presence of abnormally shaped mitochondria that resemble “beads-on-the-string” where swollen round-shaped areas with perturbed cristae are connected with very narrow membranous segments, is associated with Drp-1-related fission arrest, and is observed in the brains from transgenic animal models of Alzheimer’s Disease (Trushina et al., 2012). Mitochondrial morphology alters dynamically by coordinated fission and fusion, and perturbing this dynamic leads to cellular dysfunction. The corresponding changes in the levels of Drp-1 and Fis-1 in HOMO brain, the elevation of mitochondrial membrane marker TOMM20 and reduction of phospho Drp-1 activation and recruitment to mitochondria all point towards fission arrest. *In vitro* data has previously shown that LRRK2 regulates mitochondrial dynamics via interaction with Drp-1 (Wang et al., 2011; Niu et al., 2012; Wang et al., 2012). Interestingly, steady state levels of mature short form of Opa-1, but not other dynamin related GTPases (Dmn1, Drp1, Mfn-2) were significantly reduced in frontal cortex from G2019S patient brains, and tended to be lower in idiopathic PD brains (Stafa et al., 2014). The same study did not however reveal differences in steady state levels of OPA-1 or other dynamin-related GTPases in cerebral cortex of G2019S transgenic mice, which previously were shown to display abnormal mitochondrial morphology (Ramonet et al., 2011). While, we did not detect a statistically significant reduction in Opa-1 in the G2019S mutant mice, on average levels were lower in the shorter form of Opa-1 in HOMO versus WT mice. Future studies with larger cohorts of G2019S animals at different age time-points are needed to assess the temporal course of mitochondrial phenotypes and to define the underlying molecular mechanisms.

While the morphological effects were most pronounced in the striatum, where LRRK2 expression is the highest, we also observed mitochondrial changes, albeit more subtle, in the cortex and the hippocampus. Levels of pDrp-1 were also reduced in the hippocampus, suggesting fission abnormalities even in areas of lower LRRK2 expression (unfortunately we did not have any corresponding cortical lysates to analyze). This suggests that the effects

of the G2019S mutation are not necessarily dopamine neuron specific, but they could be more vulnerable since the long nigro-striatal projections have high energy demands. High numbers of mitochondria are known to reside in pre-synaptic terminals so there is a high probability that mitochondria regulate/maintain synaptic function and neurotransmission (Keating, 2008; Du et al., 2010). Recently published *ex vivo* voltammetry studies performed in cortical cultures from the this G2019S mice revealed that glutamate release was markedly elevated, in the absence of any change to synapse density, indicating that physiological levels of G2019S LRRK2 elevate probability of release (Beccano-Kelly et al., 2014a). Measuring *in vivo* release of other neurotransmitters for example 5-HT in the hippocampus would be informative.

Whether mitochondrial degradation is impacted in this model is unclear at this point. The number of autophagosomes, as evidenced by LC3-II levels, are elevated in the HOMO mice but the adaptor protein p62 (also known as SQSTM1), a selective substrate for autophagy that is required for the formation of ubiquitinated protein aggregates, is unchanged. Since the dynamic nature of the autophagy pathway makes interpretation of LC3-II levels difficult, future studies to measure autophagic flux will require measurement of these responses following administration of autophagy inducers and inhibitors. There is certainly convincing evidence linking LRRK2 and autophagy/mitophagy (Ramonet et al., 2011; Tong et al., 2012; Cherra et al., 2013; Hindle et al., 2013; Orenstein et al., 2013; Schapansky et al., 2014) with regulating roles for LRRK2 involving mTor signaling (Ferree et al., 2012; Manzoni et al., 2013; Schapansky et al., 2014, Herzig, 2011 #75; Yakhine-Diop et al., 2014), calcium homeostasis (Cherra et al., 2013) and MEK signaling (Hindle et al., 2013). This involvement of LRRK2 in autophagic pathways goes along with the role of LRRK2 regulating synaptic via vesicle trafficking and neurotransmitter release. In mice, inhibition of mTOR signaling induces formation of autophagic vacuoles in prejunctional dopaminergic axons and alters presynaptic structure and evoked dopamine release (Hernandez et al., 2012). Furthermore, presynaptic LRRK2 and alpha-synuclein accumulation was observed in Atg7 autophagy-deficient mice (Friedman et al., 2012).

As a final note, a perplexing aspect to this model is the opposing effects for certain parameters measured in HETS versus HOMOS, or an effect observed in one but seemingly absent in the other. For example, the reduction of PINK-1 in HETS. The data is difficult to reconcile for a gain-of-function mechanism, especially given the apparent incremental increase in kinase activity from one to two copies of the mutant allele [if indeed kinase is the toxic mediating mechanism]. One potential explanation could lie in the self-interaction of LRRK2 – via autophosphorylation and formation of dimers (Deng et al., 2008; Greggio et al., 2008; Greggio et al., 2009; Liao et al., 2014), leading to differential stoichiometry between WT-mutant and mutant-mutant pairing, which may impact structure and enzymatic activation. The sub-cellular localization of LRRK2 influences kinase activity (Berger et al., 2010) and LRRK2 mutations influence enzymatic kinase and GTPase activity and the propensity to form dimeric structures (Greggio et al., 2009; Sen et al., 2009; Webber et al., 2011). Intracellular degradation of wild-type LRRK2 is promoted by formation of heterodimers with the I2020T mutant (Ohta et al., 2013) and human co-transfected I2020T but not with human WT LRRK2, decreased the protein level of mouse LRRK2 (Miyajima et

al., 2015). Altered protein-protein interactions and post-translational modifications likely contribute to a disparity in the regulation of LRRK2 function, via aberrant modulation of membrane association and complex assembly. This may be even more complex if LRRK2 has region specific or cell-specific roles. It is pertinent to note here that in LRRK2-PD human patients the *clinical* phenotypes between heterozygote and homozygotes carriers are described as similar (Ishihara et al., 2006).

In summary, our data suggests that expression of G2019S LRRK2 in mice at endogenous levels via gene-targeting strategy is sufficient to perturb striatal dopamine neurotransmission and mitochondrial dynamics, in the absence of dopamine neuronal loss or behavioral changes. The alterations in this model may mirror pre-clinical changes in human Parkinson's disease. We anticipate future studies using this G2019S knock in model will be useful for the continued deciphering of the complex and enigmatic role of LRRK2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Angeles DC, et al. Mutations in LRRK2 increase phosphorylation of peroxiredoxin 3 exacerbating oxidative stress-induced neuronal death. *Hum Mutat.* 2011; 32:1390–7. [PubMed: 21850687]
- Arbuthnott GW, et al. Dopamine release and metabolism in the rat striatum: an analysis by 'in vivo' brain microdialysis. *Pharmacol Ther.* 1990; 48:281–93. [PubMed: 2128125]
- Bailey RM, et al. LRRK2 phosphorylates novel tau epitopes and promotes tauopathy. *Acta Neuropathol.* 2013
- Bannon MJ, Roth RH. Pharmacology of mesocortical dopamine neurons. *Pharmacol Rev.* 1983; 35:53–68. [PubMed: 6138783]
- Baptista MA, et al. Loss of leucine-rich repeat kinase 2 (LRRK2) in rats leads to progressive abnormal phenotypes in peripheral organs. *PLoS One.* 2013; 8:e80705. [PubMed: 24244710]
- Beccano-Kelly DA, et al. Synaptic function is modulated by LRRK2 and glutamate release is increased in cortical neurons of G2019S LRRK2 knock-in mice. *Front Cell Neurosci.* 2014a; 8:301. [PubMed: 25309331]
- Beccano-Kelly DA, et al. LRRK2 overexpression alters glutamatergic presynaptic plasticity, striatal dopamine tone, postsynaptic signal transduction, motor activity and memory. *Hum Mol Genet.* 2014b
- Belluzzi E, et al. Presynaptic dysfunction in Parkinson's disease: a focus on LRRK2. *Biochem Soc Trans.* 2012; 40:1111–6. [PubMed: 22988874]
- Berger Z, et al. Membrane localization of LRRK2 is associated with increased formation of the highly active LRRK2 dimer and changes in its phosphorylation. *Biochemistry.* 2010; 49:5511–23. [PubMed: 20515039]
- Berwick DC, Harvey K. LRRK2 functions as a Wnt signaling scaffold, bridging cytosolic proteins and membrane-localized LRP6. *Hum Mol Genet.* 2012; 21:4966–79. [PubMed: 22899650]

- Cherra SJ 3rd, et al. Mutant LRRK2 elicits calcium imbalance and depletion of dendritic mitochondria in neurons. *Am J Pathol.* 2013; 182:474–84. [PubMed: 23231918]
- Cirnar MD, et al. LRRK2 kinase activity regulates synaptic vesicle trafficking and neurotransmitter release through modulation of LRRK2 macro-molecular complex. *Front Mol Neurosci.* 2014; 7:49. [PubMed: 24904275]
- Cooper O, et al. Pharmacological rescue of mitochondrial deficits in iPSC-derived neural cells from patients with familial Parkinson's disease. *Sci Transl Med.* 2012; 4:141ra90.
- Daberkow DP, et al. Amphetamine paradoxically augments exocytotic dopamine release and phasic dopamine signals. *J Neurosci.* 2013; 33:452–63. [PubMed: 23303926]
- Darvesh AS, et al. In vivo brain microdialysis: advances in neuropsychopharmacology and drug discovery. *Expert Opin Drug Discov.* 2011; 6:109–127. [PubMed: 21532928]
- Davies P, et al. Comprehensive characterization and optimization of anti-LRRK2 (leucine-rich repeat kinase 2) monoclonal antibodies. *Biochem J.* 2013a; 453:101–13. [PubMed: 23560750]
- Davies P, et al. Comprehensive Characterization and Optimization of Leucine Rich Repeat Kinase 2 (LRRK2) Monoclonal Antibodies. *Biochem J.* 2013b
- Deng J, et al. Structure of the ROC domain from the Parkinson's disease-associated leucine-rich repeat kinase 2 reveals a dimeric GTPase. *Proc Natl Acad Sci U S A.* 2008; 105:1499–504. [PubMed: 18230735]
- Di Fonzo A, et al. A common missense variant in the LRRK2 gene, Gly2385Arg, associated with Parkinson's disease risk in Taiwan. *Neurogenetics.* 2006; 7:133–8. [PubMed: 16633828]
- Du H, et al. Early deficits in synaptic mitochondria in an Alzheimer's disease mouse model. *Proc Natl Acad Sci U S A.* 2010; 107:18670–5. [PubMed: 20937894]
- Dusonchet J, et al. A rat model of progressive nigral neurodegeneration induced by the Parkinson's disease-associated G2019S mutation in LRRK2. *J Neurosci.* 2011; 31:907–12. [PubMed: 21248115]
- Farrer MJ, et al. Lrrk2 G2385R is an ancestral risk factor for Parkinson's disease in Asia. *Parkinsonism Relat Disord.* 2007; 13:89–92. [PubMed: 17222580]
- Ferree A, et al. Regulation of physiologic actions of LRRK2: focus on autophagy. *Neurodegener Dis.* 2012; 10:238–41. [PubMed: 22204929]
- Friedman LG, et al. Disrupted autophagy leads to dopaminergic axon and dendrite degeneration and promotes presynaptic accumulation of alpha-synuclein and LRRK2 in the brain. *J Neurosci.* 2012; 32:7585–93. [PubMed: 22649237]
- Galter D, et al. MitoPark mice mirror the slow progression of key symptoms and L-DOPA response in Parkinson's disease. *Genes Brain Behav.* 2010; 9:173–81. [PubMed: 20002202]
- Galter D, et al. LRRK2 expression linked to dopamine-innervated areas. *Ann Neurol.* 2006; 59:714–9. [PubMed: 16532471]
- Giesert F, et al. Expression analysis of Lrrk1, Lrrk2 and Lrrk2 splice variants in mice. *PLoS One.* 2013; 8:e63778. [PubMed: 23675505]
- Gomez-Suaga P, et al. LRRK2 delays degradative receptor trafficking by impeding late endosomal budding through decreasing Rab7 activity. *Hum Mol Genet.* 2014; 23:6779–96. [PubMed: 25080504]
- Greggio E, et al. The Parkinson's disease kinase LRRK2 autophosphorylates its GTPase domain at multiple sites. *Biochem Biophys Res Commun.* 2009; 389:449–54. [PubMed: 19733152]
- Greggio E, et al. The Parkinson disease-associated leucine-rich repeat kinase 2 (LRRK2) is a dimer that undergoes intramolecular autophosphorylation. *J Biol Chem.* 2008; 283:16906–14. [PubMed: 18397888]
- Healy DG, et al. Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study. *Lancet Neurol.* 2008; 7:583–90. [PubMed: 18539534]
- Hernandez D, et al. Regulation of presynaptic neurotransmission by macroautophagy. *Neuron.* 2012; 74:277–84. [PubMed: 22542182]
- Herzig MC, et al. High LRRK2 levels fail to induce or exacerbate neuronal alpha-synucleinopathy in mouse brain. *PLoS One.* 2012; 7:e36581. [PubMed: 22615783]

- Herzig MC, et al. LRRK2 protein levels are determined by kinase function and are crucial for kidney and lung homeostasis in mice. *Hum Mol Genet.* 2011; 20:4209–23. [PubMed: 21828077]
- Higashi S, et al. Localization of Parkinson's disease-associated LRRK2 in normal and pathological human brain. *Brain Res.* 2007a; 1155:208–19. [PubMed: 17512502]
- Higashi S, et al. Expression and localization of Parkinson's disease-associated leucine-rich repeat kinase 2 in the mouse brain. *J Neurochem.* 2007b; 100:368–81. [PubMed: 17101029]
- Hindle S, et al. Dopaminergic expression of the Parkinsonian gene LRRK2-G2019S leads to non-autonomous visual neurodegeneration, accelerated by increased neural demands for energy. *Hum Mol Genet.* 2013; 22:2129–40. [PubMed: 23396536]
- Hinkle KM, et al. LRRK2 knockout mice have an intact dopaminergic system but display alterations in exploratory and motor co-ordination behaviors. *Mol Neurodegener.* 2012a; 7:25. [PubMed: 22647713]
- Hinkle KM, et al. LRRK2 knockout mice have an intact dopaminergic system but display alterations in exploratory and motor co-ordination behaviors. *Mol Neurodegener.* 2012b; 7:25. [PubMed: 22647713]
- Hulihan MM, et al. LRRK2 Gly2019Ser penetrance in Arab-Berber patients from Tunisia: a case-control genetic study. *Lancet Neurol.* 2008; 7:591–4. [PubMed: 18539535]
- Ishihara L, et al. Screening for Lrrk2 G2019S and clinical comparison of Tunisian and North American Caucasian Parkinson's disease families. *Mov Disord.* 2007; 22:55–61. [PubMed: 17115391]
- Ishihara L, et al. Clinical features of Parkinson disease patients with homozygous leucine-rich repeat kinase 2 G2019S mutations. *Arch Neurol.* 2006; 63:1250–4. [PubMed: 16966502]
- Kachergus J, et al. Identification of a novel LRRK2 mutation linked to autosomal dominant parkinsonism: evidence of a common founder across European populations. *Am J Hum Genet.* 2005; 76:672–80. [PubMed: 15726496]
- Kay DM, et al. Escaping Parkinson's disease: a neurologically healthy octogenarian with the LRRK2 G2019S mutation. *Mov Disord.* 2005; 20:1077–8. [PubMed: 16001413]
- Keating DJ. Mitochondrial dysfunction, oxidative stress, regulation of exocytosis and their relevance to neurodegenerative diseases. *J Neurochem.* 2008; 104:298–305. [PubMed: 17961149]
- Kontgen F, Stewart CL. Simple screening procedure to detect gene targeting events in embryonic stem cells. *Methods Enzymol.* 1993; 225:878–90. [PubMed: 8231892]
- Lee BD, et al. Inhibitors of leucine-rich repeat kinase-2 protect against models of Parkinson's disease. *Nat Med.* 2010; 16:998–1000. [PubMed: 20729864]
- Lee JW, et al. Behavioral, neurochemical, and pathologic alterations in bacterial artificial chromosome transgenic G2019S leucine-rich repeated kinase 2 rats. *Neurobiol Aging.* 2015; 36:505–18. [PubMed: 25174649]
- Lewis PA, Manzoni C. LRRK2 and human disease: a complicated question or a question of complexes? *Sci Signal.* 2012; 5:pe2. [PubMed: 22253261]
- Li X, et al. Enhanced striatal dopamine transmission and motor performance with LRRK2 overexpression in mice is eliminated by familial Parkinson's disease mutation G2019S. *J Neurosci.* 2010; 30:1788–97. [PubMed: 20130188]
- Li X, et al. Leucine-rich repeat kinase 2 (LRRK2)/PARK8 possesses GTPase activity that is altered in familial Parkinson's disease R1441C/G mutants. *J Neurochem.* 2007; 103:238–47. [PubMed: 17623048]
- Li Y, et al. Mutant LRRK2(R1441G) BAC transgenic mice recapitulate cardinal features of Parkinson's disease. *Nat Neurosci.* 2009; 12:826–8. [PubMed: 19503083]
- Liao J, et al. Parkinson disease-associated mutation R1441H in LRRK2 prolongs the “active state” of its GTPase domain. *Proc Natl Acad Sci U S A.* 2014; 111:4055–60. [PubMed: 24591621]
- Lin X, et al. Leucine-rich repeat kinase 2 regulates the progression of neuropathology induced by Parkinson's-disease-related mutant alpha-synuclein. *Neuron.* 2009; 64:807–27. [PubMed: 20064389]
- Liu HF, et al. LRRK2 R1441G mice are more liable to dopamine depletion and locomotor inactivity. *Ann Clin Transl Neurol.* 2014; 1:199–208. [PubMed: 25356398]

- Longo F, et al. Genetic and pharmacological evidence that G2019S LRRK2 confers a hyperkinetic phenotype, resistant to motor decline associated with aging. *Neurobiol Dis.* 2014; 71:62–73. [PubMed: 25107341]
- MacLeod D, et al. The familial Parkinsonism gene LRRK2 regulates neurite process morphology. *Neuron.* 2006; 52:587–93. [PubMed: 17114044]
- Mandemakers W, et al. LRRK2 expression is enriched in the striosomal compartment of mouse striatum. *Neurobiol Dis.* 2012; 48:582–93. [PubMed: 22850484]
- Manzoni C, et al. Inhibition of LRRK2 kinase activity stimulates macroautophagy. *Biochim Biophys Acta.* 2013; 1833:2900–10. [PubMed: 23916833]
- Matta S, et al. LRRK2 Controls an EndoA Phosphorylation Cycle in Synaptic Endocytosis. *Neuron.* 2012; 75:1008–21. [PubMed: 22998870]
- Melrose H, et al. Anatomical localization of leucine-rich repeat kinase 2 in mouse brain. *Neuroscience.* 2006; 139:791–4. [PubMed: 16504409]
- Melrose HL, et al. Impaired dopaminergic neurotransmission and microtubule-associated protein tau alterations in human LRRK2 transgenic mice. *Neurobiol Dis.* 2010; 40:503–17. [PubMed: 20659558]
- Melrose HL, et al. A comparative analysis of leucine-rich repeat kinase 2 (Lrrk2) expression in mouse brain and Lewy body disease. *Neuroscience.* 2007; 147:1047–58. [PubMed: 17611037]
- Migheli R, et al. LRRK2 affects vesicle trafficking, neurotransmitter extracellular level and membrane receptor localization. *PLoS One.* 2013; 8:e77198. [PubMed: 24167564]
- Miklavc P, et al. Surfactant secretion in LRRK2 knock-out rats: changes in lamellar body morphology and rate of exocytosis. *PLoS One.* 2014; 9:e84926. [PubMed: 24465451]
- Miyajima T, et al. The mouse/human cross-species heterodimer of leucine-rich repeat kinase 2: Possible significance in the transgenic model mouse of Parkinson's disease. *Neurosci Lett.* 2015
- Mortiboys H, et al. Mitochondrial impairment in patients with Parkinson disease with the G2019S mutation in LRRK2. *Neurology.* 2010; 75:2017–20. [PubMed: 21115957]
- Ness D, et al. Leucine-rich repeat kinase 2 (LRRK2)-deficient rats exhibit renal tubule injury and perturbations in metabolic and immunological homeostasis. *PLoS One.* 2013; 8:e66164. [PubMed: 23799078]
- Ng CH, et al. Parkin protects against LRRK2 G2019S mutant-induced dopaminergic neurodegeneration in *Drosophila*. *J Neurosci.* 2009; 29:11257–62. [PubMed: 19741132]
- Nichols RJ, et al. Substrate specificity and inhibitors of LRRK2, a protein kinase mutated in Parkinson's disease. *Biochem J.* 2009; 424:47–60. [PubMed: 19740074]
- Niu J, et al. Leucine-rich repeat kinase 2 disturbs mitochondrial dynamics via Dynamin-like protein. *J Neurochem.* 2012; 122:650–8. [PubMed: 22639965]
- Ohta E, et al. Dominant-negative effects of LRRK2 heterodimers: a possible mechanism of neurodegeneration in Parkinson's disease caused by LRRK2 I2020T mutation. *Biochem Biophys Res Commun.* 2013; 430:560–6. [PubMed: 23220480]
- Orenstein SJ, et al. Interplay of LRRK2 with chaperone-mediated autophagy. *Nat Neurosci.* 2013; 16:394–406. [PubMed: 23455607]
- Ozelius LJ, et al. LRRK2 G2019S as a cause of Parkinson's disease in Ashkenazi Jews. *N Engl J Med.* 2006; 354:424–5. [PubMed: 16436782]
- Papkovskaia TD, et al. G2019S leucine-rich repeat kinase 2 causes uncoupling protein-mediated mitochondrial depolarization. *Hum Mol Genet.* 2012; 21:4201–13. [PubMed: 22736029]
- Parisiadou L, et al. LRRK2 regulates synaptogenesis and dopamine receptor activation through modulation of PKA activity. *Nat Neurosci.* 2014; 17:367–76. [PubMed: 24464040]
- Paxinos, G.; Franklin, K. *The Mouse Brain in Stereotaxic Coordinates.* Academic Press; San Diego: 2001.
- Piccoli G, et al. LRRK2 controls synaptic vesicle storage and mobilization within the recycling pool. *J Neurosci.* 2011; 31:2225–37. [PubMed: 21307259]
- Ramonet D, et al. Dopaminergic neuronal loss, reduced neurite complexity and autophagic abnormalities in transgenic mice expressing G2019S mutant LRRK2. *PLoS One.* 2011; 6:e18568. [PubMed: 21494637]

- Ross OA, et al. Association of LRRK2 exonic variants with susceptibility to Parkinson's disease: a case-control study. *Lancet Neurol.* 2011; 10:898–908. [PubMed: 21885347]
- Ross OA, et al. Analysis of Lrrk2 R1628P as a risk factor for Parkinson's disease. *Ann Neurol.* 2008; 64:88–92. [PubMed: 18412265]
- Saez-Atienzar S, et al. The LRRK2 inhibitor GSK2578215A induces protective autophagy in SH-SY5Y cells: involvement of Drp-1-mediated mitochondrial fission and mitochondrial-derived ROS signaling. *Cell Death Dis.* 2014; 5:e1368. [PubMed: 25118928]
- Saha S, et al. LRRK2 modulates vulnerability to mitochondrial dysfunction in *Caenorhabditis elegans*. *J Neurosci.* 2009; 29:9210–8. [PubMed: 19625511]
- Sanders LH, et al. LRRK2 mutations cause mitochondrial DNA damage in iPSC-derived neural cells from Parkinson's disease patients: reversal by gene correction. *Neurobiol Dis.* 2014; 62:381–6. [PubMed: 24148854]
- Schapansky J, et al. Membrane recruitment of endogenous LRRK2 precedes its potent regulation of autophagy. *Hum Mol Genet.* 2014; 23:4201–14. [PubMed: 24682598]
- Sen S, et al. Dependence of leucine-rich repeat kinase 2 (LRRK2) kinase activity on dimerization. *J Biol Chem.* 2009; 284:36346–56. [PubMed: 19826009]
- Sharp T, et al. An in vivo study of dopamine release and metabolism in rat brain regions using intracerebral dialysis. *J Neurochem.* 1986; 47:113–22. [PubMed: 3711893]
- Skibinski G, et al. Mutant LRRK2 toxicity in neurons depends on LRRK2 levels and synuclein but not kinase activity or inclusion bodies. *J Neurosci.* 2014; 34:418–33. [PubMed: 24403142]
- Sossi V, et al. Increase in dopamine turnover occurs early in Parkinson's disease: evidence from a new modeling approach to PET 18 F-fluorodopa data. *J Cereb Blood Flow Metab.* 2002; 22:232–9. [PubMed: 11823721]
- Sossi V, et al. Changes of dopamine turnover in the progression of Parkinson's disease as measured by positron emission tomography: their relation to disease-compensatory mechanisms. *J Cereb Blood Flow Metab.* 2004; 24:869–76. [PubMed: 15362717]
- Sossi V, et al. Dopamine turnover increases in asymptomatic LRRK2 mutations carriers. *Mov Disord.* 2010; 25:2717–23. [PubMed: 20939082]
- Stafa K, et al. Functional interaction of Parkinson's disease-associated LRRK2 with members of the dynamin GTPase superfamily. *Hum Mol Genet.* 2014; 23:2055–77. [PubMed: 24282027]
- Sterky FH, et al. Altered dopamine metabolism and increased vulnerability to MPTP in mice with partial deficiency of mitochondrial complex I in dopamine neurons. *Hum Mol Genet.* 2012; 21:1078–89. [PubMed: 22090423]
- Su YC, Qi X. Inhibition of excessive mitochondrial fission reduced aberrant autophagy and neuronal damage caused by LRRK2 G2019S mutation. *Hum Mol Genet.* 2013
- Sulzer D, et al. Mechanisms of neurotransmitter release by amphetamines: a review. *Prog Neurobiol.* 2005; 75:406–33. [PubMed: 15955613]
- Tan EK. Identification of a common genetic risk variant (LRRK2 Gly2385Arg) in Parkinson's disease. *Ann Acad Med Singapore.* 2006; 35:840–2. [PubMed: 17160203]
- Taymans JM, et al. Distribution of PINK1 and LRRK2 in rat and mouse brain. *J Neurochem.* 2006; 98:951–61. [PubMed: 16771836]
- Tong Y, et al. Loss of leucine-rich repeat kinase 2 causes age-dependent bi-phasic alterations of the autophagy pathway. *Mol Neurodegener.* 2012; 7:2. [PubMed: 22230652]
- Tong Y, et al. R1441C mutation in LRRK2 impairs dopaminergic neurotransmission in mice. *Proc Natl Acad Sci U S A.* 2009; 106:14622–7. [PubMed: 19667187]
- Tong Y, et al. Loss of leucine-rich repeat kinase 2 causes impairment of protein degradation pathways, accumulation of alpha-synuclein, and apoptotic cell death in aged mice. *Proc Natl Acad Sci U S A.* 2010; 107:9879–84. [PubMed: 20457918]
- Trushina E, et al. Defects in mitochondrial dynamics and metabolomic signatures of evolving energetic stress in mouse models of familial Alzheimer's disease. *PLoS One.* 2012; 7:e32737. [PubMed: 22393443]

- Tsika E, et al. Conditional expression of Parkinson's disease-related R1441C LRRK2 in midbrain dopaminergic neurons of mice causes nuclear abnormalities without neurodegeneration. *Neurobiol Dis.* 2014; 71:345–58. [PubMed: 25174890]
- Walker MD, et al. Behavioral deficits and striatal DA signaling in LRRK2 p.G2019S transgenic rats: a multimodal investigation including PET neuroimaging. *J Parkinsons Dis.* 2014; 4:483–98. [PubMed: 25000966]
- Wang X, et al. DLP1-dependent mitochondrial fragmentation mediates 1-methyl-4-phenylpyridinium toxicity in neurons: implications for Parkinson's disease. *Aging Cell.* 2011; 10:807–23. [PubMed: 21615675]
- Wang X, et al. LRRK2 regulates mitochondrial dynamics and function through direct interaction with DLP1. *Hum Mol Genet.* 2012; 21:1931–44. [PubMed: 22228096]
- Webber PJ, et al. Autophosphorylation in the leucine-rich repeat kinase 2 (LRRK2) GTPase domain modifies kinase and GTP-binding activities. *J Mol Biol.* 2011; 412:94–110. [PubMed: 21806997]
- West AB, et al. Differential LRRK2 expression in the cortex, striatum, and substantia nigra in transgenic and nontransgenic rodents. *J Comp Neurol.* 2014; 522:2465–80. [PubMed: 24633735]
- Westerink BH. Sequence and significance of dopamine metabolism in the rat brain. *Neurochem Int.* 1985; 7:221–7. [PubMed: 20492917]
- Westerlund M, et al. Developmental regulation of leucine-rich repeat kinase 1 and 2 expression in the brain and other rodent and human organs: Implications for Parkinson's disease. *Neuroscience.* 2008a; 152:429–36. [PubMed: 18272292]
- Westerlund M, et al. Lrrk2 and alpha-synuclein are co-regulated in rodent striatum. *Mol Cell Neurosci.* 2008b; 39:586–91. [PubMed: 18790059]
- Yakhine-Diop SM, et al. G2019S LRRK2 mutant fibroblasts from Parkinson's disease patients show increased sensitivity to neurotoxin 1-methyl-4-phenylpyridinium dependent of autophagy. *Toxicology.* 2014; 324:1–9. [PubMed: 25017139]
- Zetterstrom T, et al. In vivo measurement of extracellular dopamine and DOPAC in rat striatum after various dopamine-releasing drugs; implications for the origin of extracellular DOPAC. *Eur J Pharmacol.* 1988; 148:327–34. [PubMed: 3384000]
- Zetterstrom T, et al. Effect of dopamine D-1 and D-2 receptor selective drugs on dopamine release and metabolism in rat striatum in vivo. *Naunyn Schmiedebergs Arch Pharmacol.* 1986; 334:117–24. [PubMed: 2946964]
- Zhou H, et al. Temporal expression of mutant LRRK2 in adult rats impairs dopamine reuptake. *Int J Biol Sci.* 2011; 7:753–61. [PubMed: 21698001]
- Zigmond MJ, et al. Increased dopamine turnover after partial loss of dopaminergic neurons: compensation or toxicity? *Parkinsonism Relat Disord.* 2002; 8:389–93. [PubMed: 12217625]
- Zimprich A, et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron.* 2004; 44:601–7. [PubMed: 15541309]

Highlights

- New LRRK2 gene targeted (G2019S knock in) mouse model
- G2019S mice have significantly elevated brain LRRK2 kinase activity
- One G2019S mutant allele is sufficient to impair dopamine transmission
- Middle-aged G2019S mice display a reduction in striatal dopamine release
- Aged G2019S mice have altered mitochondrial morphology and dynamics

Fig. 1. Generation and expression analysis in G2019S KI mice

(a) Schematic of targeting design, showing the G2019S mutation in exon 41. The PKG-Neo-pA cassette was removed by Cre-mediated deletion. (b) Confirmation, via cDNA sequencing, of the presence of the two base mutagenesis which was required to change the codon that encodes amino acid 2019 from GGG to AGC in exon 41. (c) Expression analysis of striatal cDNA via quantitative RT-PCR for genes LRRK1, LRRK2, MAPT and SNCA did not reveal any differences between genotypes. Data expressed as mean $\pm$ SEM. (d) Protein expression in the hemi-brain (detected by LRRK2 antibody MJFF2) was similar at 3

months between genotypes. (e) Protein levels of LRRK2 and TH in the striatum at 3, 12 and 20 months. GAPDH is the loading control.

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Fig. 2. Normal DA neuronal counts and levels but altered DA metabolism in aged G2019S mice (a) Stereological counts of DA neurons (TH positive) in 18–20 month old WT (N=5) and HOMO KI mice (N=3) are similar. (b) Examples of TH immunostained sections from WT and HOMO KI mice at the level of the nigra and striatum revealed similar neuron and process density. Striatal axonal neurochemistry was measured by HPLC measurements at 6 and 18 months (n=4 to 9 per group) (c) DA (d) DOPAC (e) HVA (f) metabolism ratio DOPAC/DA (g) metabolism ratio HVA/DA. Data expressed as mean $\pm$ SEM and analyzed by Student's T-test (a) or 2-way RM ANOVA (age and genotype as factors) followed by Tukey's Post hoc comparisons (c–g). *p<0.05, ** p<0.01, *** p<0.001

Fig. 3. Extracellular release of basal and amphetamine-stimulated DA is normal at 6 months, but impaired by 12 months in G2019S KI mice

Microdialysis collections taken from the striatum of freely moving WT, HET and HOMO mice (N=8–11 per genotype, per age) and analyzed by HPLC (a) Average extracellular baseline levels of DA at 6 months (b) time course of average baseline and amphetamine stimulated DA levels at 6 months (c) time course of % response to amphetamine (normalized to individual baselines) at 6 months (d) extracellular baseline levels of DA at 12 months (e) time course of baseline and amphetamine stimulated DA levels e at 12 months (f) time course of % response to amphetamine (normalized to individual baselines) at 12 months. N=8 mice per group. Data expressed as mean $\pm$ SEM, analyzed by 2-way RM ANOVA (time and genotype as factors) followed by Bonferroni's multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 4. Extracellular release of DOPAC and HVA metabolites remains intact in G2019S, resulting in significantly different extracellular metabolism ratios

(a) Average extracellular baseline levels of DOPAC at 12 months (b) Average extracellular baseline levels of HVA at 12 months. Individual sample extracellular metabolism ratios were computed and plotted (c) average of the individual sample metabolism ratio DOPAC/DA (d) the average of the individual sample metabolism ratio HVA/DA. N=8–11 mice per group, data expressed as mean $\pm$ SEM data and analyzed by ANOVA followed by Tukey's Post hoc comparisons. *p<0.05, ** p<0.01, *** p<0.001.

Fig. 5. HOMO G2019S mice travel longer distances in the open field at 6 months
 OFA parameters tested at 6 months (a–f). The distance traveled is significantly increased in HOMO mice. N=9–13 mice per group, data expressed as mean $\pm$ SEM, analyzed by ANOVA followed by Fisher's LSD comparisons * $p < 0.05$, ** $p < 0.01$.

Fig 6. HOMO G2019S mice have subtle OFA exploratory differences in the initial testing segment at 12 months

OFA parameters tested at 12 months (a–f). In the first testing segment HOMO mice displayed increased time in the center zone but more time freezing. N=9–13 mice per group, data expressed as mean $\pm$ SEM, analyzed by ANOVA followed by Fisher's LSD comparisons *p<0.05, ** p<0.01.

Fig. 7. Motor balance and coordination is not impaired in G2019S mice knock in mice
 Mice were placed on a rotating at 4–40rpm acceleration, increasing 1rpm every 5 seconds. The time to fall was recorded. N=9–13 mice per group, data expressed as mean $\pm$ SEM analyzed by ANOVA followed by Tukey's comparisons. ** $p < 0.01$ (HOMO vs HET) ## $p < 0.01$ (HOMO vs WT).

Fig. 8. LRRK2 kinase activity is significantly elevated in G2019S KI mice but LRRK2 levels are similar in WT, HET and HOMO mice

(a) LRRK2 kinase activity was measured using a radioactive assay with Nictide (Nichols et al., 2009) as a substrate. Activity was measured in hemi brains taken from WT, HET and HOMO at ages 4, 10–12 and 16–18 months (N=3 or 4 per group). Data expressed as mean $\pm$ SEM and analyzed by 2 way ANOVA (age and genotype as factors) followed by Tukey's Post hoc comparisons. ** $p < 0.01$, *** $p < 0.001$

(b–g) LRRK2 Levels were measured in lysates prepared WT, HET and HOMO hemi brains from mice aged 3 and 12 months in phosphate buffer solubilized LRRK2 (i.e. soluble fraction) or laemmli buffer solubilized LRRK2 (i.e. insoluble fraction). Immunoblots were probed with using MJFF2 (c41-2) antibody (b) Representative blots from mice aged 3 and 12 months of age. Signal density of the blot was quantified and normalized to loading control GAPDH or VDAC (c, d, f, g) or expressed as a ratio as soluble/insoluble (e, h). N=4–6 per group, data expressed as mean $\pm$ SEM and analyzed by ANOVA followed by Tukey's Post hoc comparisons.

Fig. 9. Increases in the tau pSer 202/205 epitope immunoreactivity in HET and HOMO mice compared to WT mice at the striatal level

Sections taken at the level of the striatum from 2 different mice of each genotype with the corpus callosum area blown up to the right. Increased intensity of staining is noted in HET and HOMO G2019S mice. Str – striatum, cc-corpus callosum. Scale Bar 250µm.

Fig. 10. Increases in the tau pSer 202/205 epitope immunoreactivity in HET and HOMO mice compared to WT mice

Sections are at the level of the midbrain and the boxed area around the lateral hypothalamus is blown up to the right. Neuropil and white matter tract immunostaining is increased in HET and HOMOmice. cc- corpus callosum, pf parafascicular thalamic nuclei, psth – para-subthalamic nuclei, lh- lateral hypothalamus, pmn – pre-mammillary nuclei, sn-substantia nigra, reticular part, pmco – posteromedial cortical amygdaloid nucleus. Scale Bar = 150µm.

Fig. 11. Quantitative immunoblotting with tau antibodies reveals tau pSer 202/205 ~55kDa species is modestly increased in hemi-brain lysates of HOMO mice compared to WT
 (a) Representative immunoblots for epitopes CP-13 (pSer202/205), PHF-1 (pSer396/404)tau, total tau (tau-5) and axonal tau (tau-1) (b) Quantitative densitometry was performed from 2 separate blots for a total of N=5–6 per genotype. Data shown as min-versus-max boxes. Analyzed by ANOVA followed by Tukey's Post-hoc comparisons *p<0.05

Fig. 12. Progressive morphological changes in striatal mitochondria in G2019S KI mice
 (a) Normal mitochondria in WT mice 15 months. (b) Mitochondria in HET LRRK2 KI mice 15 months of age have altered distribution, clustering in the neuropils (#), which could indicate abnormal trafficking of the organelles. Asterisk denotes mitochondria with altered shape and cristae morphology (*). (c) Mitochondria in the striatum of HOMO LRRK2 KI mice acquire abnormal shape that resembles “beads-on-a-string” (#), and indicates altered mitochondrial fission which may be associated with reduced expression of Drp1 and Fis1 (see Figure 13). Note also the increased number of swollen mitochondria with altered cristae organization (*). (d) Higher power image of HOMO mouse at 15 months showing swollen/reorganized cristae (*). (e) WT control mouse at 23 months of age (f) HOMO mouse at 23 months, the mitochondria vary in size and shape as denoted by arrows, including abnormal “beads-on-a-string” (#) that have enlarged bulbous parts of organelle connected by thin double membranes devoid of matrix. N – nucleus; arrows – mitochondria. ^ - fibrils. (g, h) Higher power images of HOMO mouse at 23 months shows disorganized swollen cristae (*) and condensed mitochondria (#).

Fig. 13. Modest alterations in mitochondria are observed in the cortex and striatum of G2019S HOMO mice at 23 months

(a, b) cortex (c, d) hippocampus from WT and G2019S HOMO mouse 23 months of age. Note lipid droplets (white arrow), lipofuscin accumulation (*), mito with disorganized cristae (black arrows), cortical mitochondria appear larger and longer (#).

Fig. 14. Immunofluorescence confocal images with TOMM20 antibody reveal abnormal bead like structures

Staining is shown in coronal sections in the striatum from three different mice of each genotype aged 22–24 months. Red arrows point to abnormal threads connecting mitochondria. Scale bar = 10 μ m or 2 μ m for cropped images.

Figure 15a

Figure 15b

Figure 15c

Fig. 15. Immunoblots of mitochondrial proteins reveals changes in fission protein levels in G2019S KI mice

(a) Lysates were prepared from half brains and blotted for different mitochondrial proteins and autophagy markers. Left panel blots were normalized to GAPDH and right panel blots to vinculin. Each blot was normalized to its own loading control, two representative loading controls are shown. L=long form and S=short form of Opa-1.

(b) Immunoblots were quantified by densitometry. Data expressed as mean $\pm$ SEM and was analyzed by ANOVA followed by Tukey's Post-hoc comparisons * $p < 0.05$, ** $p < 0.01$.

a. TOTAL LYSATES (hippocampus, 20 months) **b. FRACTIONATED LYSATES (Striatum, 14 months)** **Fig. 16. Changes in phosphorylated Drp-1 616 in the hippocampus and striatum**

(a) Total hippocampal lysates from aged 20 months old mice shows a significant decrease in pDrp-1 616. pDrp-1 616 levels were normalized to Drp-1 levels. Data is mean $\pm$ SEM, analyzed by ANOVA and Tukeys post-hoc comparisons.

(b) Fractionated striatal lysates (cytosolic and mitochondrial enriched) were prepared from younger mice (14 months), HOMO mice have less Drp-1 and pDrp-1 616 in the mitochondrial fraction. VDAC is used as a marker to show mitochondrial fraction.

Table 1

Summary of digigait recorded indices in WT, HET and HOMO G2019S KI mice at 6 and 12 month.

Parameters (6 months)	WT	HET	HOMO
Stride length (cm)*	6.50 ± 0.33	6.87 ± 0.34 #	6.56 ± 0.43
Stride length variability	1.05 ± 0.21	1.02 ± 0.19	0.99 ± 0.22
Stride frequency (steps/sec)*	3.77 ± 0.21	3.56 ± 0.20	3.76 ± 0.26
Stride duration (sec)*	0.271 ± 0.014	0.287 ± 0.014 #	0.273 ± 0.018
% Stance duration	57.6% ± 3.4%	56.9% ± 2.2%	57.2% ± 3.7%
% Swing duration	42.4%	43.1%	42.8%
Step angle (deg)	67.2 ± 6.2	70.1 ± 4.8	69.3 ± 5.8
Fore paw angle (deg)	11.9 ± 2.1	11.0 ± 2.1	9.7 ± 3.8
Hind paw angle (deg)	15.8 ± 3.6	14.8 ± 3.0	15.4 ± 2.7
Hind paw drag (sq. mm)	2.5 ± 0.4	2.6 ± 0.5	2.7 ± 0.4
Total steps (no./paw)	18 ± 2	17 ± 1	18 ± 1
Gait symmetry (ratio)	1.00 ± 0.03	1.00 ± 0.01	0.99 ± 0.01

Parameters (12 months)	WT	HET	HOMO
Stride length (cm)	6.45 ± 0.25	6.58 ± 0.42	6.20 ± 0.54
Stride length variability	1.21 ± 0.38	1.14 ± 0.39	1.12 ± 0.41
Stride frequency (steps/sec)	3.81 ± 0.14	3.73 ± 0.24	3.98 ± 0.36
Stride duration (sec)	0.269 ± 0.011	0.275 ± 0.018	0.259 ± 0.023
% Stance duration	59.1% ± 2.7%	58.6% ± 2.3%	58.5% ± 4.0%
% Swing duration	40.9%	41.4%	41.5%
Step angle (deg)	61.9 ± 6.9	65.7 ± 7.9	62.0 ± 8.1
Fore paw angle (deg)	11.4 ± 1.4	11.5 ± 2.8	9.7 ± 1.9
Hind paw angle (deg)	18.4 ± 2.2	18.3 ± 3.1	19.3 ± 7.0
Hind paw drag (sq. mm)	2.63 ± 0.49	2.66 ± 0.31	2.62 ± 0.29
Total steps (no./paw)	19 ± 1	18 ± 2	17 ± 4
Gait symmetry (ratio)	0.98 ± 0.02	1.00 ± 0.02	1.00 ± 0.03

Mice were treadmill walking at 24cm/sec. Data analyzed by ANOVA, followed by Tukey's Post Hoc tests.

* ANOVA p<0.05,

Tukey's p<0.05 (compared to WT).

Table 2
Quantitative analysis of striatal mitochondria in aged G2019S HOMO and WT mice

Genotype	# of neurofils	Neurofil length, μm	# of Mito	Mito per neurofil length	Mito Length, μm	# Abnormal Mito
WT	84	296.6	213	0.76 ± 0.04	1.07 ± 0.05	3
HOMO	50	259.2	122	0.5 ± 0.05 ***	1.22 ± 0.07**	13

10–12 random electron micrograph images were analyzed per mouse. The number of mitochondria per neurofil length was analyzed by Student's T-test. Mitochondrial length was analyzed by the Kolmogorov-Smirnov test to allow comparison of the cumulative distributions between the two datasets.