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Tissue- and Cell-Specific Mitochondrial Defect in Parkin-Deficient Mice

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Abstract

Loss of Parkin, encoded by PARK2 gene, is a major cause of autosomal recessive Parkinson’s disease. In Drosophila and mammalian cell models Parkin has been shown to play a role in various processes essential to maintenance of mitochondrial quality, including mitochondrial dynamics, biogenesis and degradation. However, the relevance of altered mitochondrial quality control mechanisms to neuronal survival in vivo is still under debate. We addressed this issue in the brain of PARK2−/− mice using an integrated mitochondrial evaluation, including analysis of respiration by polarography or by fluorescence, respiratory complexes activity by spectrophotometric assays, mitochondrial membrane potential by rhodamine 123 fluorescence, mitochondrial DNA content by real time PCR, and oxidative stress by total glutathione measurement, proteasome activity, SOD2 expression and proteins oxidative damage. Respiration rates were lowered in PARK2−/− brain with high resolution but not standard respirometry. This defect was specific to the striatum, where it was prominent in neurons but less severe in astrocytes. It was present in primary embryonic cells and did not worsen in vivo from 9 to 24 months of age. It was not associated with any respiratory complex defect, including complex I. Mitochondrial inner membrane potential in PARK2−/− mice was similar to that of wild-type mice but showed increased sensitivity to uncoupling with ageing in striatum. The presence of oxidative stress was suggested in the striatum by increased mitochondrial glutathione content and oxidative adducts but normal proteasome activity showed efficient compensation. SOD2 expression was increased only in the striatum of PARK2−/− mice at 24 months of age. Altogether our results show a tissue-specific mitochondrial defect, present early in life of PARK2−/− mice, mildly affecting respiration, without prominent impact on mitochondrial membrane potential, whose underlying mechanisms remain to be elucidated, as complex I defect and prominent oxidative damage were ruled out.

Introduction

Mitochondrial dysfunction has long been thought to play a key role in Parkinson’s disease (PD) pathogenesis. Parkinsonian syndromes are induced in humans and animals by complex I inhibitors, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or rotenone, and complex I activity has been reported to be reduced in different tissues from patients, suggesting that this defect may contribute to neuronal degeneration in PD [1,2]. Mitochondrial dysfunction has also been suggested to contribute to oxidative damage and apoptosis in idiopathic PD brains [3]. Identification of genetic causes of familial PD reinforced a central role for mitochondria in PD pathogenesis [4,5]. Indeed most autosomal recessive forms were found to be caused by the alteration of a gene encoding a protein localized to mitochondria, either in particular contexts, as in the case of Parkin [6] and DJ-1 [7], or constitutively for PINK1 [8]. Furthermore, studies in various models and from many independent laboratories have provided evidence for interaction between the PARK2 and PINK1 genes and their protein products in a common pathway centered on maintenance of mitochondrial quality [9]. In cell models Parkin and PINK1 regulate the elimination of dysfunctional mitochondria through mitophagy [10–15]. Parkin and PINK1 have also been involved in mitochondrial fission and fusion [16–18], mitochondrial transport [19,20], and mitochondrial biogenesis [21,22], processes that are also relevant to mitochondrial quality. Consistent with these findings, PARK2 or PINK1 inactivation have been reproducibly linked with partial mitochondrial depolariza-
tion [23–25], reduced respiration rates [22,24,26,27] and/or reduction in the enzymatic activity of complex I of the mitochondrial electron transport chain [24–30].

However, the in vivo relevance of the PINK1/Parkin-dependent mitochondrial quality control mechanisms for neuronal cells has recently been questioned [31,32]. In addition, PINK1- or Parkin-deficient mouse models have less consistently shown mitochondrial defects than cellular models, possibly due to differences in the ages and tissues examined and in the methodologies used [26,33–37]. Several groups, including ours, have generated Parkin-deficient mice and shown that, in general, they do not present with overt signs of neuronal degeneration in the nigrostriatal pathway, even at high ages [38–42]. In some cases, including our model, moderate alterations in dopamine handling evocative of presymptomatic changes have been reported in the striatum, such as increased extracellular dopamine content, enhanced dopamine metabolism via monoamine oxidase and decreased motor activation following amphetamine-induced dopamine-release [38,39]. Therefore, despite the lack of manifest parkinsonian phenotype, these mice may be valuable for investigation of early disease-related modifications and compensatory mechanisms. To clarify discrepancies in the literature regarding the role of mitochondrial dysfunction in these models [35,37], we have investigated mitochondrial functions, including respiration, respiratory complexes activity and inner membrane potential, in mice carrying a germline homozygous deletion of PARK2 exon 3 [39].

To address potential technical biases underlying the literature discrepancies, we analyzed respiration in different mitochondrial and tissue preparations, including crude mitochondrial pellets from striatum and cortex, purified mitochondria from whole brain, and post-nuclear supernatants from midbrain and striatum; we also examined primary embryonic neurons and astrocytes from striatum or cortex. We provide evidence for tissue- and cell-type-specific reduction of mitochondrial respiratory capacity in PARK2−/− mice. These defects were observed in the absence of overt oxidative damage, decreased mitochondrial membrane potential or defective enzymatic activities of the respiratory chain complexes, thus leaving open their underlying mechanisms.

Materials and Methods

Ethics statement

All experiments were approved by the “Comité d’Éthique pour l’expérimentation animale Charles Darwin” (Ce5/2009/052).

Generation of experimental groups

PARK2−/− mice were generated and brought into the C56Bl/6j genetic background as previously described [39,42]. Experimental groups of age-matched littermate PARK2−/− and wild type mice were generated by intercross of heterozygous PARK2−/− mice. Genotypes were determined by PCR amplification [42]. After weaning, male and female mice were separated and maintained at a maximum of five littermates per cage, with food and water ad libitum, and at constant temperature (20°C ± 2°C) with a 08:00–18:00 light cycle. The experiments were performed on female mice (n = 42 per genotype) as follows, with n corresponding to the number of animals per genotype, 24-month-old mice were used for respiration analyses on crude mitochondrial pellets (n = 8), purified mitochondrial pellets (n = 2) or post-nuclear supernatants (n = 5), and oxidative stress evaluation with glutathione and proteasome studies (n = 5), 9-month-old mice for respiration analyses on post-nuclear supernatants (n = 7), 12-month-old mice for respiratory chain complexes assays (n = 7), and oxidative stress evaluation with glutathione and proteasome studies (n = 5). For all applications, mice were killed by decapitation, without prior anesthesia.

Tissue preparation

After mouse decapitation, liver and brain were rapidly removed and the regions of interest dissected on ice. All samples were manually homogenized using a Dounce homogenizer with a Teflon pestle. The scheme depicting the different tissue preparations and their use is shown in Figure S1.

Crude mitochondrial pellets from the striatum or cortex were obtained by homogenization in 9 volumes of Buffer A (50 mM Tris HCl, 5 mM Glucose, 1 mM Pyruvate, 1 mM EGTA, 150 mM KCl, pH 7.5). Centrifugation for 5 min at 1000 g gave post-nuclear supernatant. The nuclei and tissue particles in the pellet was subjected to a second identical cycle of homogenization and centrifugation. The two post-nuclear supernatants were collected and centrifuged for 10 min at 10,000 g to obtain the crude mitochondrial pellet that was diluted in 120 and 240 μL of Buffer A for striatal and cortical mitochondria respectively. These suspensions were used for respiration and protein assay. Between 5 and 10 μg mitochondrial proteins were used per run of respiration.

Purified brain mitochondria were obtained by homogenization of a whole brain in 1 mL Mannitol-Sucrose (MS) isolation medium (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.4) containing 0.1 mg/mL fatty acid-free bovine serum albumin (FAB-BSA) [43]. The homogenate volume was brought to 1.5 mL. The supernatant of a first centrifugation during 6 min at 500 g was centrifuged during 10 min at 14 000 g to obtain the crude mitochondrial pellet that was diluted in 200 μL of 12% Percoll solution in MS buffer, layered on top of a discontinuous Percoll gradient (200 μL of 12% Percoll on top of 1 mL of 24% Percoll in MS buffer) and centrifuged during 18 min at 17 000 g. The resulting mitochondrial pellet was washed twice with MS buffer with centrifugation during 7 min at 17 000 g the first time and during 5 min at 14 000 g the second time. The last purified mitochondrial pellet was diluted in 100 μL MS buffer and used for respiration (5 μg of mitochondrial proteins per run) and protein assays.

Lever, ventral midbrain and striatal post-nuclear supernatants were obtained by homogenization in 9 volumes of 300 mM sucrose, 1 mM EGTA, 5 mM Tris HCl pH 7.4, followed by centrifugation during 10 minutes at 1000 g. The post-nuclear supernatants were directly used for respiration and protein assays, as well as for the analysis of inner mitochondrial membrane potential. Each run of respiration assay used 300 μg of protein of post-nuclear supernatant from midbrain or striatum and 1 mg from liver.

Primary striatal or cortical neurons were prepared at embryonic day 16 by a method adapted from Friedman et al [44]. In summary, following centrifugation through a BSA cushion, primary neurons were seeded onto poly-ornithine and laminin coated Seashore 24-well plates (175,000 cells/well for striatal neurons and 140,000 cells/well for cortical neurons) and cultured for 7 days at 37°C, 5% CO2 in Neurobasal medium supplemented with 2% B27, 1 mM sodium pyruvate and 1 mM glutamax.

Astrocytes were isolated from striatum or cortex of newborn mice as previously described [45] and cultured in poly-L-lysine coated tissue culture flask (75 cm2) for 7 days. Astrocytes were then trypsinized, plated in Seashore 24-well plates (40,000 cells/well) and cultured for another 4 days at 37°C, 5% CO2 in Dulbecco’s modified Eagle’s medium F12 supplemented with 10% fetal bovine serum and 1% penicillin streptomycin.
Respiration assays

For mitochondrial preparations or post-nuclear supernatants, the rate of oxygen consumption was measured in a Clark-type electrode. Mitochondrial pellets were analyzed by classical respirometry using Hansatech electrode (Cambridge, UK). The respiration medium was 100 mM KMES, 5 mM KH₂PO₄, 1 mM EGTA, 3 mM EDTA, 0.4% w/v FAF-BSA, pH 7.4 for crude mitochondrial pellets. It was 125 mM KCl, 1 mM MgCl₂, 2 mM KH₂PO₄, 20 mM HEPES, 200 μM EGTA, 0.2% w/v FAF-BSA, pH 7.4 for purified mitochondria. Post-nuclear supernatants were analyzed by high resolution respirometry with an Oroboros apparatus (Innsbruck, Austria) using 100 mM KCl, 40 mM sucrose, 10 mM TES, 5 mM MgCl₂, 1 mM EGTA, 0.4% w/v FAF-BSA, pH 7.2 as respiration medium.

Common recorded parameters were the respiration rate in the presence of 10 mM glutamate, 5 mM malate and 1 mM ADP, in the presence of 1 μg/mL oligomycin, a complex V inhibitor, and in the presence of 1 mM KCN (= non-respiratory oxygen consumption). These parameters allowed the calculation of state 3 respiration = respiration in the presence of substrates+ADP - non-respiratory oxygen consumption, state 4 respiration = respiration in the presence of oligomycin - non-respiratory oxygen consumption, and respiratory control ratio = state 3/state 4, evaluating the efficiency of the oxidative phosphorylation coupling. Two additional parameters were recorded with Oroboros electrode: basal respiration (in the presence of substrates before ADP addition) and maximal respiration rate (maximal rate obtained by progressive additions of 1.25 μM carbonyl cyanide m-chlorophenyl hydrazone (cccp), a protonophore dissipating the inner membrane potential, performed after the step with addition of oligomycin). These additional parameters were used to calculate the respiratory reserve = maximal respiration - basal respiration.

For primary neurons and astrocytes, respiration was analyzed in a XF24 extracellular flux analyzer (Seahorse Bioscience) in the presence of 125 mM KCl, 1 mM MgCl₂, 20 mM HEPES, 200 μM EGTA, 0.2% w/v FAF-BSA, pH 7.4 for purified mitochondria. Post-nuclear supernatants were analyzed by high resolution respirometry with an Oroboros apparatus (Innsbruck, Austria) using 100 mM KCl, 40 mM sucrose, 10 mM TES, 5 mM MgCl₂, 1 mM EGTA, 0.4% w/v FAF-BSA, pH 7.2 as respiration medium.

Mitochondrial activities

The maximal velocity of respiratory complexes and citrate synthase activities was evaluated using standardized spectrophotometric assays used in the diagnosis of human mitochondrial diseases [47] while complex V activity was assayed using the ATP hydrolysis assay [48].

Determination of the mitochondrial transmembrane potential

The mitochondrial membrane potential was evaluated in post-nuclear supernatants using the fluorescent probe rhodamine 123, which accumulates in the mitochondrial compartment according to the Nerst law [49]. Incubation was performed at room temperature with 2 to 5 μg protein of post-nuclear supernatants/mL of respiration medium with 250 nM rhodamine 123, and in diverse bioenergetic conditions: condition A = substrates (10 mM glutamate+5 mM malate), condition B = A+1 mM ADP, condition C = B+1 μM oligomycin, condition D = C+1 μM cccp, condition E = C+2 μM cccp, condition F = C+4 μM cccp, condition G = C+8 μM cccp, condition H = C+10 μM cccp and 1 mM KCN. Reading was performed with an Accuri flow cytometer. Results were expressed in mV as calculated by Nerst equation after normalization of the fluorescence to the size of the objects as measured by their forward scatter, taking into account the proportion of mitochondrial objects (% of energizable objects upon maximal energization), evaluating the mitochondrial volume in the optic chamber of Accuri flow cytometer (estimated for brain at 3% of the volume analyzed), and using condition H for evaluation of the zero fluorescence.

Quantification of mitochondrial DNA

Quantification of mitochondrial DNA was performed in striatum by quantitative real time PCR using as standard a plasmid with the murine mitochondrial DNA-encoded MT-CO2 gene as an insert [50]. Results were expressed as copy number of mitochondrial DNA/10 pg of total DNA, which roughly represent a normal diploid genome.

Determination of cytosolic and mitochondrial glutathione levels

Total glutathione levels were determined using 20 μg proteins of crude mitochondrial pellet or cytosol from striatum and midbrain with a modified version of the recycling enzymatic assay based on the reduction of each GSSG molecule to give two GSH molecules [51]. Standard curves were obtained with various concentrations of GSSG and the values were thus expressed as GSSG equivalent. Total glutathione content therefore equals 0.5 GSH+GSSG and is expressed in nmol of glutathione/mg of protein.

20S proteasome peptidase activity

The chymotrypsin-like activity of the proteasome was assayed in cytosol from striatum and midbrain with the fluorogenic peptide succinyl-Leu-Leu-Val-Tyr-amidomethylcoumarin (LLVY-AMC) as substrate. 20 μg cytosolic proteins were incubated with 12.5 μM substrate in 25 mM Tris, pH 7.5 in a final volume of 200 μl [52]. Enzymatic kinetics was monitored in a temperature-controlled microplate fluorimetric reader (Fluostar Galaxy; BMG, Stuttgart, Germany). Excitation/emission wavelengths were 350 nm/440 nm.
Western blot analyses

30 μg of proteins of crude mitochondrial pellets from striatum or midbrain were solubilized overnight at room temperature in sample buffer at a SDS/protein ratio = 30. Proteins were separated by electrophoresis in 4–20% pre-cast gradient gels (Biorad, Hertfordshire, UK) and transferred on nitrocellulose membrane (Protran Whatman, Dassel, Germany). Antibodies were rabbit polyclonal anti-SOD2 from Sigma and anti-nitrotyrosine from Molecular Probes, mouse monoclonal anti-VDAC from Mitoscience and rabbit anti-4-hydroxynonenal (HNE) produced in the team [52]; they were visualized using peroxidase-conjugated secondary antibodies from Jackson ImmunoResearch (Newmarket, UK).

Statistical methods

Statistics were performed with SigmaPlot 12.5 software. Non-parametric tests were used unless the data were shown to have a normal distribution allowing the use of parametric tests. The numbers given indicate independent measurements. Animals were not pooled but always individually evaluated. Mean comparisons were performed with Mann and Whitney test or unpaired Student t test while paired comparisons were done by the Wilcoxon signed-rank test or paired Student t test. Multiple comparison with the Holm-Sidak test was used after verification of equal variance to address the relative influence of age, genotype and their interaction.

Results

High resolution respirometry and cell-type specific analysis detect a respiration defect in PARK2+/− mice

Reductions in uncoupled respiratory rates have previously been observed in the striatum or the substantia nigra of PARK2+/− mice by two independent groups, with however partial inconsistency with respect to the associated state 3 and state 4 respiration rates [35,37]. Both reports relied on the analysis with standard oxygraphy of relatively crude mitochondrial pellets obtained by two successive centrifugation steps. Under these conditions, the observed respiratory control ratios (state 3/state 4 respiration) were low (below 3.5), reflecting relatively poor coupling of the electron transport chain with ATP synthesis [35,37].

We performed similar experiments in aged mice considering that they were more likely to express a defect because of the lack of neurodegenerative phenotype in PARK2+/− mice [35,37,41] and the relationship of PD with ageing. Crude mitochondrial pellets from striatum and cortex were prepared from the brain of 24-month-old mice (Figure S1). State 3 and state 4 respiration as well as the respiratory control ratio were similar in PARK2+/− and wild type mice (Figure 1A, B, C). As in the previous studies, the respiratory control ratios were relatively low (Figure S2).

Considering that mild respiration defects might not be detectable in crude mitochondrial pellets we next prepared whole brain purified mitochondria from 24-month-old mice and found similar state 3 and state 4 respiration as well as respiratory control in PARK2+/− and wild-type mice (Figure 1D). Although mitochondrial enrichment of the preparation was shown by the higher specific respiratory rates as compared to those of crude mitochondrial pellets, the respiratory control ratio of these purified mitochondria was only slightly increased (Figure S2). Considering the need to use whole brain instead of specific brain region, the improvement of respiratory control ratio did not appear sufficient to pursue that procedure in a higher number of animals.

Furthermore we reasoned that altered mitochondria were likely to be eliminated during mitochondrial purification. That procedure might thus be inappropriate when addressing mitochondrial quality control as is the case in the absence of Parkin. Very low respiratory rates such as those observed without mitochondrial enrichment can only be analyzed with high resolution respirometry using Oroboros technology. We therefore optimized this technique for the analysis of post-nuclear supernatants. The relative rapidity of this tissue preparation allowed the parallel study of striatum, midbrain and liver from each mouse. With this method the respiratory control ratios were above 10 in midbrain and striatum, indicating excellent mitochondrial preservation. These ratios were similar between PARK2+/− and wild type mice (Figure S2). An example of curve obtained with 300 g striatum post-nuclear supernatant is shown in Figure 2A. 24-month-old PARK2+/− mice showed significant decreased respiratory reserve in striatum when compared to wild type mice (p = 0.028 using Mann and Whitney test). The concomitant decrease of state 3 respiration in striatum just failed to reach significance (p = 0.06.

**Figure 1.** Respiration was similar in brain mitochondrial preparations from 24-month-old PARK2+/− and wild type mice examined with standard oxygraphy. (A) Representative experiment with a crude mitochondrial pellet from striatum. Blue curve = oxygen (O2) concentration expressed as nmol/mL; red curve = rate of oxygen consumption expressed as nmol O2/mL and min; ADP = addition of 400 μM ADP; OM = addition of 1 μg/mL oligomycin, a complex V inhibitor; KCN = addition of 1 mM KCN (non-respiratory oxygen consumption); (B, C, D) Bars showing state 3 respiration (respiration in the presence of substrates +ADP – non-respiratory oxygen consumption) and state 4 respiration (respiration in the presence of oligomycin – non-respiratory oxygen consumption) on complex I substrates (glutamate+malate); black bars = PARK2+/−, white bars = wild type; data are expressed as nmol O2/mL and mg proteins and are shown as means ± SD; two animals, one per genotype, were analyzed the same day; the numbers between brackets indicate the number of individual animals of each genotype; (B) results from crude mitochondrial pellets from striatum (n = 6), (C) crude mitochondrial pellets from cortex (n = 7) and (D) purified mitochondria from whole brain (n = 2).

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with Mann and Whitney test) (Figure 2B). State 4 respiration was not affected. The decreased state 3 respiration and respiratory reserve in midbrain did not reach significance (Figure 2C) and liver respiration was similar in both genotypes (Figure 2D).

Using the same approach we then studied younger mice at 9 months of age to address the presence of the respiratory defect earlier in life. PARK2\(^{-/-}\) mice presented with a decrease in state 3 respiration rate and respiratory reserve in midbrain and striatum that was similar to the pattern observed in aged mice (Figure 2B). As previously observed in aged mice, the decrease was only significant in the striatum (p = 0.05 for state 3 respiration with Mann and Whitney test).

Liver showed a significant age-associated decrease in respiratory reserve capacity in both genotypes (Figure 2D). In contrast the respiratory capacity in striatum and midbrain post-nuclear supernatants was not modified by age.

Even precisely limited brain regions contain distinct cell populations, which essentially are astrocytes and neurons. These cells present with strikingly different energetic metabolism and thus may develop different responses to the absence of Parkin [53]. Using Seahorse technology to evaluate the respiration of cultured cells in multi-wells plates, we addressed this question by comparing PARK2\(^{-/-}\) and wild type pure primary embryonic neurons or neonatal astrocytes from striatum or cortex (Figure 3). In striatal neurons, the maximal respiration rate and spare respiratory capacity were significantly reduced in the absence of Parkin. In contrast they were similar in PARK2\(^{-/-}\) and wild type cortical neurons. Astrocytes strikingly differed from neurons with respect to their uncoupled maximal respiration, which was equal or often lower than their initial respiration. As a result, their spare respiratory capacity appeared null or negative. This observation contrasted with previously reported maximal respiration rates in astrocytes that were at least two-fold higher than basal respiration using Seahorse protocols without the step of oligomycin inhibition [54,55]. We thus considered that our protocol was not suitable for reliable evaluation of maximal respiration and only analyzed initial respiration of astrocytes, which appeared mildly reduced in PARK2\(^{-/-}\) striatum and normal in cortex.

At the end of these diverse analyses of respiration it could be concluded that the absence of parkin was associated with a mild but significant striatal respiratory defect that did not appear to worsen with age and was even detectable in embryonic neurons.

Figure 2. High resolution respirometry reveals a respiration defect in PARK2\(^{-/-}\) mice. (A) Representative experiment with striatal post-nuclear supernatant. Blue curve = oxygen concentration \(O_2\) expressed as nmol/mL; red curve = rate of oxygen consumption expressed as nmoles \(O_2/\)mL and min; successive additions: ADP = 1 mM ADP in the medium with 10 mM glutamate+5 mM malate; OM = 1 \(\mu\)g/mL oligomycin, cccp = successive additions of 1.25 \(\mu\)M (up to the maximal respiration rate independent from ATP synthase capacity); KCN = 1 mM KCN (non-respiratory oxygen consumption); (B, C, D) Bars showing state 3 respiration, state 4 respiration and respiratory reserve on complex I substrates (glutamate+malate) in post-nuclear supernatants from (B) striatum, (C) midbrain and (D) liver of 24- and 9-month-old PARK2\(^{-/-}\) (black bars) and wild type mice (white bars); all data are expressed as nmoles \(O_2/\)mL and mg proteins and shown as means ± SD; two animals, one per genotype, were analyzed the same day; the data were obtained from 6 to 9 individual animals of each genotype. * p<0.05 using Mann and Whitney test. In liver, state 3 respiration rate and respiratory reserve significantly decreased with age in both wild type mice (p = 0.011 and <0.001 when comparing state 3 respiration and respiratory reserve respectively between 9 and 24 months of age using Mann and Whitney test) and in PARK2\(^{-/-}\) mice (p = 0.019 and <0.001 when comparing state 3 respiration and respiratory reserve respectively between 9 and 24 months of age using Mann and Whitney test). doi:10.1371/journal.pone.0099898.g002
Mitochondrial inner membrane potential of PARK2−/− mice was normal but showed greater sensitivity to uncoupling with ageing in the striatum.

Low inner membrane potential (ΔΨm) is one of the expected consequences of a respiration defect. Furthermore it has been proposed to be an important signal for Parkin translocation to mitochondria and mitophagy [56]. To determine whether the absence of Parkin translates in vivo into the presence of depolarized mitochondria, we analyzed the ΔΨm in midbrain, striatum and liver of PARK2−/− and wild type mice, at 9 and 24 months of age (Figure 4). The reliability of the method was demonstrated in the three tissues by the significant decrease in ΔΨm upon ADP stimulation (condition B versus A, p<0.001 with Mann and Whitney test), its increase upon inhibition of the ATP synthase activity by oligomycin (condition C versus B, p<0.001), and decrease with addition of uncoupler above 1 μM (condition C versus E, F or G, p<0.001). The mean ΔΨm did not significantly differ between PARK2−/− and wild type mice in any of the tissues.

Figure 3. Maximal respiration is reduced in striatal neurons from PARK2−/− mice. Representative experiments with neurons or astrocytes from striatum or cortex: the traces represent the evolution of the respiration rate (in pmol O2/minute) in Seahorse plates seeded with cells from PARK2−/− (black circles) and wild type (white circles) mice. Sequential additions are: oligomycin (OM) (0.25 μg/ml for neurons and 0.5 μg/ml for astrocytes), fccp (FP) (3 μM for cortical neurons and 1 μM for other cells), and rotenone+antimycin (RA) (50 nM rotenone+150 μg/ml antimycin A). Bars below the traces show the means and SD of basal respiration, maximal respiration and spare respiratory capacity (SRC = maximal – basal respiration) of 3 independent tests, each performed in 6 to 12 independent wells. * p<0.05 using Mann and Whitney test.

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conditions and tissues examined, excluding excessive accumulation of partially depolarized mitochondria in the absence of Parkin.

In both PARK2<sup>−/−</sup> and wild type brain ageing had a clear impact on the ΔΨm response to increasing doses of the uncoupler cccp with a significant drop of the mean ΔΨm under 8 μM cccp treatment from 9 to 24 months of age in the striatum and midbrain (p = 0.022 and 0.035 in midbrain and p = 0.001 and < 0.001 in striatum of PARK2<sup>−/−</sup> and wild type mice respectively and p = 0.181 and 0.138 in midbrain). The drop of ΔΨm between condition C (highest value under oligomycin) and condition F or G (uncoupled by either 4 or 8 μM cccp) was then calculated for each mouse. In striatum and under 8 μM cccp only, that drop was significantly higher in PARK2<sup>−/−</sup> than in wild type mice (p = 0.041 with Mann and Whitney test).

In liver, despite a similar trend, the changes with age in the mean response of ΔΨm to uncoupling did not reach significance in wild type or PARK2<sup>−/−</sup> mice.

In conclusion the absence of Parkin did not significantly alter the mean ΔΨm in midbrain, striatum and liver of PARK2<sup>−/−</sup> mice.

Figure 4. The inner mitochondrial membrane potential is normal in midbrain and striatum of PARK2<sup>−/−</sup> mice. Dark circles = PARK2<sup>−/−</sup>, white circles = wild type; ΔΨ<sub>m</sub> = inner mitochondrial membrane potential expressed as mV after transformation of the fluorescent rhodamine 123 signal using the Nernst equation, as explained in the methods section; A = ΔΨ<sub>m</sub> in the presence of glutamate+malate; B = A+1 mM ADP; C = B+1 μM oligomycin; D = C+1 μM cccp; E = C+2 μM cccp; F = C+4 μM cccp; G = C+8 μM cccp. The data were obtained in parallel to the analysis of respiration; two animals, one per genotype, were analyzed the same day; the data obtained from seven 9-month-old mice and six 24-month-old ones of each genotype, they are expressed as mean and SD. doi:10.1371/journal.pone.0099898.g004
but it was associated in striatum with a greater impact of ageing on the $\Delta\psi$ m response to uncoupling.

The respiratory defect in PARK2$^{--}$ mice was not due to defective complex I activity, altered mitochondrial content or overt oxidative stress

Complex I activity has previously been reported to be lowered in cells from PD patients with PARK2 mutations [25,29,30] and in a Parkin-depleted zebrafish model [57]. Such a defect may underlie the reduction in striatal tissue respiration and the increased sensitivity of $\Delta\psi$ m to uncoupling in PARK2$^{--}$ mice. To address this possibility, we used spectrophotometric methods recently standardized for the diagnosis of human mitochondrial diseases [47] to measure the respiratory chain activities in striatal post-nuclear supernatants and crude mitochondrial pellets of cortex and midbrain from one-year-old PARK2$^{--}$ and wild type mice. All the respiratory chain activities examined were similar in PARK2$^{--}$ and wild type mice (Table 1).

Post-translational modifications regulating the activity of mitochondrial proteins involved in the import, catabolism or oxidative phosphorylation of respiratory substrates could also underlie the respiratory defect observed in PARK2$^{--}$ mice. Previous studies have reported alterations in proteins involved in the defense against oxidative stress in these mice [35,36]. We therefore searched for evidence of oxidative stress in midbrain and striatum of PARK2$^{--}$ mice. Ageing however being known to have significant influence on oxidative stress, we analyzed the combined influence of age and genotype on the parameters of oxidative stress using multiple comparison by the Holm-Sidak test. Proteasome activity significantly increased with age in striatum ($p<0.001$) but not midbrain, without influence of the genotype (Table 2). Similarly the midbrain mitochondrial glutathione content increased with age ($p<0.001$ when comparing 12-month-old to 24-month-old mice) without influence of the genotype. In contrast, significant interaction between age and genotype was observed for the striatal mitochondrial glutathione content ($p=0.015$). Ageing led to a significant increase in striatal mitochondrial glutathione in wild type mice ($p<0.001$ when comparing 12-month-old to 24-month-old wild type mice) but had no effect in PARK2$^{--}$ mice. At 12 months of age the striatal mitochondrial glutathione levels were significantly higher in PARK2$^{--}$ mice than in wild type mice ($p<0.011$ when comparing 12-month-old wild type to 12-month-old PARK2$^{--}$ mice with Mann and Whitney test). Cytosolic glutathione levels did not change with age in midbrain. In striatum cytosolic glutathione levels significantly decreased with age ($p<0.001$ when comparing 12-month-old to 24-month-old mice without influence of the genotype, Figure 5); interaction between age and genotype just failed to reach significance ($p=0.056$).

In parallel nitrotyrosine (NT) and 4-hydroxynonenal (HNE), two different protein oxidative adducts, were searched for in crude mitochondrial pellets using western blot analysis. At 12 months of age there was a significant increase in both adducts in the striatum of PARK2$^{--}$ compared to wild type mice ($p=0.001$ and $p=0.016$ for NT and HNE respectively with Mann and Whitney) (Figure 6). At 24 months of age, PARK2$^{--}$ striatum showed increase in NT only ($p=0.028$ compared with wild type mice) whereas wild-type mice showed an increase of HNE in midbrain ($p=0.003$).

The steady-state of the mitochondrial superoxide dismutase 2 (SOD2), normally increased in the presence of mitochondrial oxidative stress [59], was normal at 12 months of age and slightly increased at 24 months of age in PARK2$^{--}$ mice compared to wild type mice ($p=0.016$ using Mann and Whitney test) (Figure 7).

Altogether, our results show an increase in oxidative stress in the striatum mitochondria of PARK2$^{--}$ mice (increased glutathione levels, increased oxidative adducts and, with age, increased SOD2 expression). That increase is however moderate (normal SOD2 expression at 12 months of age, normal proteasome activity).

### Discussion

Recent work, based on the analysis of cell models overproducing Parkin and subjected to pharmacological treatments leading to immediate and global depolarization of the mitochondrial network, attributed to Parkin a central role in the clearance of dysfunctional mitochondria [12]. These studies predict that loss of function of the PINK1/Parkin pathway should lead to progressive mitochondrial impairment, possibly representing a primary

### Table 1. Activities of mitochondrial respiratory chain complexes in 12-month-old PARK2$^{--}$ and wild type mice.

| Complex   | Striatum | Midbrain | Cortex |
|-----------|----------|----------|--------|
| Complex I | PARK2$^{--}$ | Wild type | PARK2$^{--}$ | Wild type | PARK2$^{--}$ | Wild type |
| Complex II| 91±26 | 93±20 | 133±31 | 138±38 | 121±27 | 111±20 |
| Complex II | 76±24 | 86±21 | 248±28 | 231±64 | 190±45 | 183±38 |
| Combined I+III | 118±42 | 97±30 | 304±81 | 326±86 | 232±82 | 283±73 |
| Complex III | 78±47 | 60±19 | 372±121 | 376±71 | 696±411 | 583±154 |
| Complex IV | 398±78 | 386±84 | 1585±598 | 1875±794 | 959±221 | 1112±267 |
| Complex V | NA | NA | 194±139 | 171±59 | 170±94 | 185±87 |
| Citrate synthase | 455±33 | 458±45 | 194±146 | 171±162 | 170±96 | 185±93 |

Striatum = post-nuclear supernatants from striatum; Midbrain = crude mitochondrial pellet from ventral midbrain; Cortex = crude mitochondrial pellet from occipital cortex; complex I = rotenone sensitive NADH ubiquinone oxidoreductase; complex II = succinate ubiquinone oxidoreductase; combined I+III = succinate cytochrome c oxidoreductase; complex III = antimycin sensitive ubiquinol cytochrome c oxidoreductase; complex IV = cytochrome c oxidase; complex V = F$_{1}$F$_{0}$ ATP synthase; complex V assay requires mitochondrial preparation and was therefore not performed on striatal post-nuclear supernatants; data are expressed as nanomoles/minute and mg proteins and as means ± SD; each series of values represents 7 independent samples from each genotype.

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mechanism of neurodegeneration in autosomal recessive PD. However, whether Parkin-dependent mitochondrial quality control plays indeed a role in vivo under physiological conditions and during aging, remains debated.

We addressed this issue by revisiting mitochondrial functions in PARK2<sup>−/−</sup> mice using recently developed, robust and sensitive technologies. The simultaneous analysis of minimal amounts of post-nuclear supernatants from different tissues with high resolution respirometry revealed a reliable respiratory defect specifically in the striatum of PARK2<sup>−/−</sup> mice, which was not exacerbated by ageing. The presence of this defect was confirmed in primary embryonic striatal neurons and was not found in cortical neurons from PARK2<sup>−/−</sup> mice using the Seahorse technology, supporting its region-specificity and early appearance in life.

Contrary to previous observations in cell and zebrafish models [25,29,57], the constitutive deletion of PARK2 in mice did not result in significant changes in mitochondrial membrane potential, overt oxidative stress markers or defective activity of mitochondrial complex I or other respiratory chain complexes despite the presence of significant respiratory defect. With ageing however the mitochondrial potential in the striatum showed increased sensitivity to uncoupling, with a greater impact in PARK2<sup>−/−</sup> than in wild type mice.

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**Figure 5. Increase of the mitochondrial glutathione content in striatum of 12-month-old PARK2<sup>−/−</sup> mice.** Total glutathione (GSH+GS-SG) levels were determined in cytosols and isolated mitochondria from ventral midbrain and striatum of 12- and 24-month-old PARK2<sup>−/−</sup> (black bars) and wild-type mice (white bars), data are expressed as mean and SD of five independent measurements for each age and each genotype. * = p<0.05 using multiple comparison with Holm-Sidak test. Significant differences with age, without influence of the genotype, were observed for midbrain mitochondrial and striatal cytosolic glutathione content. Significant interaction between age and genotype was present for striatal mitochondrial glutathione content, which significantly increased with age only in wild type mice and was at 12 months of age significantly higher in PARK2<sup>−/−</sup> than in wild type mice.

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**Table 2. Proteasome activities were similar in PARK2<sup>−/−</sup> and wild type mice.**

| Tissue | Age     | Midbrain | Striatum |
|--------|---------|----------|----------|
|        | 12 months | 24 months | 12 months | 24 months |
| PARK2<sup>−/−</sup> | 116±89 | 84±12 | 43±8 | 77±110 |
| wild type | 94±37 | 80±13 | 43±4 | 63±7 |

Chymotrypsin-like activity of the proteasome. Results are expressed as nmoles of substrate cleaved/minute and milligram of protein and as mean±standard deviation of n=5 measurements.

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with this possibility, mitochondrial stress caused by the conditional accumulation of oxidative stress during ageing [63]. Consistent nervous system under stress conditions, for example following PINK1/Parkin pathway may become essential in the central cardiac tissue, the mitochondrial quality control function of the ultrastructure in myocytes during aging [62]. Therefore, as in infarction [61] and accumulation of mitochondria with abnormal defective clearing of damaged mitochondria following myocardiac infarction [61] and accumulation of mitochondria with abnormal ultrastructure in myocytes during aging [62]. Therefore, as in cardiac tissue, the mitochondrial quality control function of the PINK1/Parkin pathway may become essential in the central nervous system under stress conditions, for example following accumulation of oxidative stress during ageing [63]. Consistent with this possibility, mitochondrial stress caused by the conditional expression of mitochondrial unfolded ornithine transcarbamylase has been recently shown to lead to dopaminergic neurodegeneration and a parkinsonian phenotype in PINK1−/− mice [64].

Contrary to humans, the short life-time of rodents may not allow full unmasking of a phenotype following disruption of the PINK1/Parkin pathway, explaining the moderate mitochondrial defect and lack of overt neurodegeneration in PARK2−/− mice. Signs of oxidative damage were mild in aged PARK2−/− mice. Compensatory mechanisms such as the increase in glutathione levels, specifically in the mitochondrial fraction, may enhance the cellular antioxidant capacity and counteract oxidative stress in these mice. The relative content of reduced and oxidized glutathione should be determined in this fraction to properly evaluate the efficiency of this mechanism. Of note, we previously reported a selective increase in reduced glutathione content in total extracts from striatum and primary mesencephalic neurons of PARK2−/− mice [39]. The increase of SOD2, often used as a marker of oxidative stress, is also a protective mechanism efficiently neutralizing superoxide anions.

In conclusion, our work revealed an early respiration defect in PARK2−/− mice that may reflect a disease-relevant preclinical modification in autosomal recessive PD. Future studies are required to identify the molecular mechanisms underlying this modification and determine their relation to the mitochondrial quality control activity of the PINK1/Parkin pathway. To fully appreciate the role of PINK1/Parkin-dependent mitochondrial quality control in the physiopathology of autosomal recessive PD, it will also be essential to determine whether stress conditions
exacerbate mitochondrial dysfunction and unmask a neurodegenerative phenotype in the brains of PARK2+/− mice.

Supporting Information
Figure S1 Scheme of the different brain fractionation methods used to assess mitochondrial functions. (TIF)

Figure S2 Respiratory control ratios (RCR) in 24-month-old mice. Respiratory control ratios were calculated as state 3/state 4 respiration rate on glutamate+maltate as substrates; results are shown as mean and SD; numbers between brackets indicate the number of independent measurements in individual animals of each genotype (A) crude mitochondrial pellets from cortex (n = 8); crude mitochondrial pellets from striatum (n = 6) and purified mitochondrial pellets from whole brain (n = 2); (B) post-nuclear supernatants from striatum (n = 7), midbrain (n = 8) and liver (n = 8). (TIF)

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Author Contributions
Conceived and designed the experiments: MD CAG ALB CG RP FA OC AL. Performed the experiments: MD CAG ALB RFM CG MGP FA CSL AL. Analyzed the data: CAG ALB CG RP FA FB OC AL. Contributed reagents/materials/analysis tools: ALB CG RP FA AB OC AL. Wrote the paper: MD CAG ALB CG RP FB OC AL.

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