Loss of functional OPA1 unbalances redox state: implications in dominant optic atrophy pathogenesis

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Introduction
Dominant optic atrophy (DOA) is characterized by moderate to severe loss of visual acuity with insidious onset in early childhood. DOA penetrance may be as low as 40% and prevalence is 1:50,000 worldwide.¹ Up to now, there is no effective treatment for this complex pathology. The majority of DOA patients (~75%) harbor mutation in the OPA1 gene coding for a mitochondrial GTPase.² There are 280 different OPA1 mutations (http/mitodyn.org), the majority of which result in premature termination ensuing OPA1 haploinsufficiency.¹ There is marked inter- and intrafamilial variability in the rate of disease progression, and recent studies show a severe multisystemic disorder associated with some OPA1 mutations (DOA⁺ syndrome).³⁻⁶ These patients present additional
neurological complications, such as ataxia, sensorineural deafness, sensory motor neuropathy as well as chronic progressive external ophthalmoplegia and myopathy. Moreover, a recent case described two DOA families which also presented parkinsonism. Altogether these recent findings are of major pathophysiological importance, and highlight the widespread deleterious consequences of OPA1 mutations, not only for retinal ganglionic cells (RGCs) but also for other neuronal populations.

The OPA1 gene encodes a mitochondrial inner membrane protein facing the intermembrane space with various functions including inner membrane fusion, cristae structuration, mitochondrial DNA maintenance, mitochondrial energetic modulation, and protection from apoptosis. Patients’ skin fibroblasts, muscles, or lymphoblasts revealed impairments in mitochondrial morphology, respiration, and energetics, and also displayed a loss of mitochondrial DNA integrity and an increase in sensitivity to apoptosis. However, it is still not clear if DOA patients do or do not develop energetic defects (see for review refs. 10, 16). Mitochondria from DOA or DOA+ mice models present an increase in mitochondrial fragmentation, cristae disorganization, mitophagy, and cytochrome c oxidase deficiency. In Caenorhabditis elegans, mutations of the OPA1 gene ortholog, eat-3, cause mitochondrial fragmentation and hypersensitivity to oxidative stress, but do not lead to cell death. Heterozygous mutations of OPA1 in Drosophila melanogaster result in a shortened life span, increased reactive oxygen species (ROS) production, sensitivity to oxidative stress, and defects in the activity of respiratory chain complexes. These two DOA invertebrate models link the critical generation of ROS with OPA1 dysfunction. We sought to investigate if ROS production promotes DOA pathogenesis in mammals using various complementary models such as (1) a DOA mouse model, (2) a canonical ex vivo neuronal model of OPA1 haplosufficiency, and (3) fibroblasts from DOA and DOA+ patients.

Our results show that OPA1 haplosufficiency lead to a decrease in mitochondrial respiration, accompanied by an increase in mitochondrial ROS production. This associated increase is linked to a decrease in aconitate activity and an increase in antioxidant defenses, in both primary cultured neurons and in mice. ROS production is countered by the activation of the nuclear factor (erythroid-derived 2)-like 2 (NRF2) transcription factor and its downstream targets. Loss of functional OPA1 imbalanced the redox state, which sensitzes cells to exogenous pro-oxidative stresses. We believe this phenomenon is a molecular mechanism underlying DOA pathogenesis.

**Materials and Methods**

**Cell culture**

**Wistar rats**

All animals (n = 45 pregnant rats, 350 embryos) in this study were ethically maintained and used as ethical laws of CNRS (Centre National de la Recherche Scientifique) and FRBT (Fédération de Recherche en Biologie de Toulouse). The animal facility called Genotoul Anexpo is maintained in accordance to F.E.L.A.S.A organism (Federation of European Laboratory Animal Science Associations). Animals were housed in groups of six and kept in a specific pathogen-free and temperature-controlled facility on a 12-h light/dark cycle. Pregnant Wistar rats are delivered by Janvier company 48 h before being sacrificed and were handled in a calm room. Day 17 embryos were removed from pregnant Wistar rats (Janvier, France) under intraperitoneal pentobarbitol (Sigma-Aldrich, UK) anesthesia. The rats were then immediately sacrificed. Cortices were dissected, enzymatically dissociated with papain (10 U/mL, Sigma-Aldrich, UK), then exposed for 5 min in a solution that inactivated papain: DNase I (Invitrogen, Thermo Fisher Scientific, Waltham, USA) and B27 (Gibco, Thermo Fisher Scientific, Waltham, USA), diluted in Phosphate Buffer Saline (PBS) 1X with d-glucose (33 mmol/L, Sigma-Aldrich, UK). Cells were dissociated by trituration and filtered through a membrane (70 μm, BD Falcon). Cells were then purified through a BSA solution (8%, Sigma-Aldrich, UK) diluted in Neurobasal A-25 (Invitrogen, Thermo Fisher Scientific, Waltham, USA). Dishes, with or without glass coverslips, were coated with poly-d-lysine (0.1 mg/mL, Sigma-Aldrich, UK) 24 h prior to culturing. For each experiment, cortices from 8 to 12 embryos per rat are mixed. Experiments were reproduced three to eight times. Cultures were grown in Neurobasal® (Eurobio) supplemented with B27 (Invitrogen, Thermo Fisher Scientific, Waltham, USA), 2 mmol/L glutamine, 0.1% penicillin and streptomycin (Gibco, Thermo Fisher Scientific, Waltham, USA), 250 U/mL amphotericin (Invitrogen, Thermo Fisher Scientific, Waltham, USA), and 1 mmol/L lactic acid (Sigma-Aldrich, UK) at a density of 6 × 10^5 cells per cm².

Cortical neurons (1 × 10⁶) were electroporated using the Rat Neuron Nucleofector Kit (Amaxa, Lonza) according to the manufacturers’ optimized protocol with 3 μg of control luciferase-targeting (siCtrl, D-001210-02, Dharmacon GE, UK) or OPA1-targeting (siOPA1, target sequence GAUUGUGCUGACUUUAUA, Dharmacon GE, UK) small interfering RNA (Dharmacon GE, UK).
Patients’ fibroblasts

Fibroblasts obtained by skin biopsy from consenting DOA patients and healthy volunteers were cultured in Dulbecco’s modified Eagle’s medium 4.5 g/L glucose (DMEM, Invitrogen, Thermo Fisher Scientific, Waltham, USA), supplemented with 10% Fetal Bovine Serum (FBS). penicillin (100 U/mL) and streptomycin (100 mg/mL), and maintained for up to 20 passages. The use of patient fibroblasts for research purpose in neurogenetics was approved by the “Comité de Protection des Personnes Ouest II – Angers” (No. CB 2014/02).

Opa1+/− mice

Mice were kept in a 12-h light/12-h dark cycle with food and water available ad libitum in full-barrier facilities free of specific pathogens. Mouse breeding and all experimental procedures were performed according to ethical laws of CNRS (Centre National de la Recherche Scientifique) and Fédération de Recherche en Biologie de Toulouse (FRBT). The animal facility called Genotoul Anexpo is maintained in accordance to F.E.L.A.S.A organism (Federation of European Laboratory Animal Science Associations). Two sanitary controls are effectuated per year following the set point (Armand, 2016). The B6;C3-tm1Lm1Jed mouse strain (abbreviated Opa1+/−) mouse has been previously described by Alavi and colleagues.13,20,23 Wild-type (Opa1+/+) littermates were systematically used as control. All examined mice were sacrificed either at 4 or 10 months old. After sacrifice, adult brains cortices were isolated and separated from hippocampus and cerebellum area. Each of those areas has been snap frozen in liquid nitrogen and stored at −80°C. Cortices were thawed on ice and 20 mg of tissue as been isolated and processed for immunoblot experiments.

Measurement of oxygen consumption and ATP/ADP levels

Oxygen consumption rates (OCR) were performed using the XF24 Extracellular Flux Analyser (Seahorse Bioscience, North Billerica, MA). Neurons (3 × 105) transfected with siCtrl or siOPA1 were plated on XF24 microplates 6 days before OCR measurements. Dual-analyte sensor cartridges were soaked in XF Calibrant Solution (Seahorse Bioscience, USA), supplemented with 10% Fetal Bovine Serum (FBS). penicillin (100 U/mL) and streptomycin (100 mg/mL), and maintained for up to 20 passages. The use of patient fibroblasts for research purpose in neurogenetics was approved by the “Comité de Protection des Personnes Ouest II – Angers” (No. CB 2014/02).

Immunoblot analysis

Neurons, human fibroblasts, and 20 mg of DOA mice models and control littermate mice cortices were lysed for 30 min in a buffer containing 50 mmol/L Tris-HCl pH 7.5, 250 mmol/L NaCl, 5 mmol/L Ethylene Diamine Tetraacetic acid (EDTA), 5 mmol/L Ethylene Glycol Tetraacetic Acid (EGTA), 1 mmol/L dithiothreitol, 0.1% Triton X-100, 0.1% SDS, 1% deoxycholate, 1% tergitol type NP-40, and protease inhibitors (Complete protease inhibitor mixture, Roche Applied Science, UK). After sonication, cell lysates were centrifuged at 20,000 g at 4°C for 10 min. Lysate from frozen mice cortices were obtained using the same protocol, but adding a step of dounce homogenization. Total protein concentration was determined in the supernatant using the Bradford protein assay (Bio-Rad). Proteins (100–200 μg) were separated by SDS-PAGE (8–15%) and transferred onto nitrocellulose membranes (Whatman, Protran, Sigma-Aldrich, UK). Free binding sites were blocked with 5% nonfat dry milk, 0.2% Tween 20 in Tris buffer saline, pH 7.6 (blocking buffer). The membranes were probed with various primary antibodies: anti-OPA1 (1/300, BD Bioscience, USA), anti-actin (1/25000, Chemicon, Merck Millipore, UK), anti-HSP60 (1/8000, Sigma-Aldrich, UK), anti-citrate synthase (1/3000, Abcam, Cambridge, USA), anti-VDAC (1/1000, Abcam, Cambridge, USA), anti-TOM20 (1/2000, Abcam, Cambridge, USA), anti-aconitase (1/500, Abcam, Cambridge, USA), anti-SOD1 and anti-SOD2 (1/2000, Epitomics, Abcam, Cambridge, USA), anti-catalase (1/3000, Abcam, Cambridge, USA), anti-NQO1 (1/3000, Abcam, Cambridge, USA), anti-mitofilin (1/1000 Abcam, Cambridge, USA), anti-MFN1 (1/1000, Abnova, Taien City, Taiwan), and anti-GSTP1 (1/8000, Oxford Biochemical Research, Euromedex, France), and incubated overnight at 4°C in blocking buffer. After chemiluminescent detection of horseradish peroxidase-conjugated secondary antibody (1/10000, Abcam, Cambridge, USA), scanned photographic films were quantitatively analyzed using Image J software.
**Immunocytochemistry**

Neurons were fixed for 15 min at 37°C with PBS containing 3.7% formaldehyde and permeabilized for 15 min in PBS, 1% bovine serum albumin, 0.3% TritonX-100, and for 10 min at −20°C in methanol. Nonspecific sites were blocked for 1 h in PBS containing 5% goat serum, 3% bovine serum albumin, and 0.5% Tween 20 (blocking solution). Polyclonal antibodies against NRF2 (1/50, Santa Cruz Biotechnology, Dallas, TX, USA) were incubated overnight at 4°C in blocking solution. Neurons were then incubated with...
Figure 1. Redox state is imbalanced in a dominant optic atrophy (DOA) mouse model. (A) Histograms representative of OPA1 protein quantities, assessed by immunoblot, in cortices of 4 and 10 months old control mice (gray bars) and Opa1+/− mice (striped bars). OPA1 quantities are significantly decreased in both Opa1+/− (0.70 ± 0.10 arbitrary units [AU]) and 10 (0.50 ± 0.05 AU) months old mice when compared to 4 (1.09 ± 0.10 AU) and 10 (1.16 ± 0.09 AU) months old Opa1+/+ mice. Results are expressed as mean ± standard error of the mean (SEM) for 4 and 10 months old mice (n = 6–9). Statistical significance was determined by a two-way analysis of variance (ANOVA), *P < 0.05, **P < 0.01. (B) Histogram representative of aconitase activities in cortices of 4 and 10 months old Opa1+/− (striped bars) and Opa1+/+ control (gray bars) cortices. Aconitase activity is significantly lower in 4 (1.82 ± 0.04 AU) and 10 (2.30 ± 0.36 AU) months old Opa1+/− mice when compared to 4 (2.67 ± 0.03 AU) and 10 (2.81 ± 0.22 AU) months old control Opa1+/+ mice, respectively. Aconitase proteins quantities are unchanged in DOA mouse model’s cortices. Results are expressed as mean ± SEM (n = 9 for 4 months old mice and n = 6 for 10 months old mice). Statistical significance was determined by a two-way ANOVA, *P < 0.05. (C) Histogram representative of SOD1, SOD2, and catalase proteins quantities relative to actin, assessed by immunoblot, in 4 and 10 months old Opa1+/− (striped bars) and Opa1+/+ (gray bars) mice cortices. SOD1 proteins quantities are significantly increased in 10 months old (1.48 ± 0.17 AU) Opa1+/− mice compared to 4 months old (0.76 ± 0.08 AU) Opa1+/+ mice. Likewise, SOD2 proteins quantities are significantly increased in 10 months old (1.55 ± 0.11 AU) Opa1+/− mice compared to 4 months old (0.98 ± 0.09 AU) Opa1+/+ mice. Catalase quantity is unchanged in Opa1+/− and Opa1+/+ 4 and 10 months old mice. Results are expressed as mean ± SEM (n = 9 for 4 months old mice and n = 6 for 10 months old mice). Statistical significance was determined using a two-way ANOVA, *P < 0.05, **P < 0.01.

Statistical analysis

Most of the experiments were statistically treated with Student’s paired t-test because of the systematic comparison between siCtrl and siOPA1 neurons. OCRs between siCtrl and siOPA1 neurons were investigated using a two-way analysis of variance (ANOVA) with a Sidak’s multiple comparison test. Nucleus NRF2 raw integrated densities in siCtrl and siOPA1 neurons were carried out using a nonparametric test (Mann–Whitney test). The results presented in Figures 1 and 3 were assessed by a two-way ANOVA with a Sidak’s multiple comparison test. *P values: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 were considered statistically significant.

Results

Opa1+/− transgenic mice cortices show imbalanced redox state

To address the question of the implication of ROS as a determinant of DOA pathogenesis in mammals, we investigated whether a redox imbalance could be evidenced in vivo in a mouse model of DOA.20

As expected, a decrease in OPA1 levels was evidenced in Opa1+/− transgenic mice cortices compared to litter mate Opa1+/+ mice (Fig. 1A). Cortices from 4 and 10 months old DOA mice were analyzed for aconitase activity. Aconitase was shown to be highly sensitive to oxidation due to damaged FeS core and inhibition of its activity is routinely used as a signature of increased mitochondrial ROS production.27–29 Downregulation of OPA1 correlated with a 47% and 57% inhibition of aconitase activity in 4 and 10 months old Opa1+/− transgenic mice compared to litter mate Opa1+/+ mice, respectively (Fig. 1B). Moreover, aconitase activity was slightly decreased (38%) from 4 to 10 months in Opa1+/− transgenic mice (Fig. 1B). This drop could not be attributed

Alexa fluor 488-conjugated secondary antibodies (1/300, Molecular Probes), labeled with 0.25 µg/mL Hoechst in PBS over 5 min and mounted in Mowiol. Immunolabeling was visualized under a fluorescence microscope (Nikon Eclipse 80i or Zeiss 710 Big) and images were acquired using NIS-Element (Nikon Digital Sight DUS2 camera) or ZEN 2011 software. Nucleus raw integrated densities (sum of pixel values) of NRF2 by μm² in neurons were measured using Image J software and confocal images.

Aconitase and catalase activities and GSH/GSSG measurements

Aconitase activities measurements were performed using a protocol described previously.25 The photochrome was measured at 340 nm using the UVIKON Spectrophotometer 922. Catalase activity was determined by measuring decomposition of H₂O₂ at 240 nm as described previously.26 Reduced (GSH) and oxidized (GSSG) measurements were performed by reverse-phase high-performance liquid chromatography as described previously.26 For both aconitase and catalase activities measurement, 20 mg of DOA mice and control mice cortices were used per replicate of experiment and 1 × 10⁶ cortical neurons ex vivo transfected with either siCtrl or siOPA1 has been harvested for the assay.

Rotenone treatment and cellular viability

Acute oxidative stress was induced in DIV6 (6 days in vitro) cultured neurons by 1 h incubation in neurobasal medium containing 500 nmol/L rotenone (Sigma-Aldrich, UK). Then, rotenone was removed and cells were incubated for 3 h in neurobasal medium.

Neuronal viability was estimated by trypan blue exclusion and counting picnotic nuclei after DAPI staining as described in Bertholet et al.21

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OPA1 Influences Redox State

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A. siCtrl siOPA1

B. siCtrl siOPA1

C. siCtrl siOPA1

D. siCtrl siOPA1

E. Ea

Eb

F. siCtrl siOPA1
Figure 2. OPA1 downregulation decreases mitochondrial respiration, induces the nuclear translocation of NRF2, and increases both catalase quantity and activity in cortical neurons ex vivo. (A) Representative immunoblots and histograms showing protein levels of OPA1 (inner membrane), citrate synthase (matrix), HSP60 (matrix), VDAC (outer membrane), and TOM20 (outer membrane) relative to actin in siOPA1- (gray bars) and siCtrl-transfected (white bars) neurons. Only OPA1 protein quantity is drastically decreased (0.23 ± 0.06 AU) in siOPA1 neurons when compared to controls (1.08 ± 0.16 AU). Results are expressed as mean ± SEM (n = 5–8). Statistical significance was determined by Student’s paired t-test, ***P < 0.001. (B) Oxygen consumption rates were measured at 6 days in vitro (DIV6) in neurons transfected with control small interfering RNA (siCtrl, black line) or small interfering RNA against OPA1 (siOPA1, dotted line). Spontaneous mitochondrial respiration is significantly lower in siOPA1-transfected neurons (0.48 ± 0.07 pmol/min per µg) than in controls (0.92 ± 0.11 pmol/min per µg). After 0.6 µmol/L oligomycin injection, cell respiration is also significantly lower in siOPA1 neurons (0.20 ± 0.03 pmol/min per µg) than in controls (0.40 ± 0.06 pmol/min per µg). After 6 µmol/L FCCP injection, maximal respiration is significantly lower siOPA1 neurons (0.44 ± 0.09 pmol/min per µg) than in controls (0.59 ± 0.16 pmol/min per µg). Finally, 50 mMol/L rotenone and 0.18 µmol/L antmycin A injections inhibit mitochondrial respiration. Results are expressed as mean ± SEM (n = 3). Statistical significance was determined by a two-way analysis of variance (ANOVA), ***P < 0.01 and ****P < 0.001. (C) ATP over ADP ratio is unchanged in siOPA1 neurons (gray bar) when compared to siCtrl-treated (white bar) cells. Results are expressed as mean ± SEM (n = 3). (D) Aconitase activity is lower in siOPA1-treated neurons (2.2 ± 0.4 µU/mg) (gray bars) when compared to control cells (3.0 ± 0.5 µU/mg) (white bars). Results are expressed as mean ± SEM (n = 5). Statistical analysis was determined by Student’s paired t-test, *P < 0.05. (E) a: Representative immunoblots and histogram showing that OPA1 downregulation in neurons has no effect on the aconitate quantity relative to actin. Results are expressed as mean ± SEM (n = 6). Statistical significance was determined by Student’s paired t-test, *P < 0.05. (E) b: Histogram represents fluorescence intensity (pixels sum/µm²) of nuclear NRF2 detected by Image J software, which is higher in siOPA1 neurons (471.4 ± 85 pixels sum/µm²) (gray bars) than in controls (412.8 ± 62 pixels sum/µm²) (white bars). Results are expressed as mean ± SEM (n=800 cells per condition, n = 5). Statistical significance was determined by a two-way ANOVA, ***P < 0.001. Scale bar: 5 µm. (F) Catalase activity is increased in siOPA1 neurons (5.56 ± 0.66 µmol/min per mg) when compared to controls (3.44 ± 0.69 µmol/min per mg). Results are expressed as mean ± SEM (n = 5). Representative immunoblots and protein quantities of catalase in siOPA1 (gray bars) and siCtrl (white bars) neurons relative to actin. Catalase quantity is increased in siOPA1 (1.6 ± 0.33 AU) when compared to controls (0.85 ± 0.14 AU). Results are expressed as mean ± SEM (n = 7). Statistical significance was determined by Student’s paired t-test, *P < 0.05, **P < 0.01.

Opa1+/- mice. However, to reinforce our data we monitored antioxidant defenses induction as a consequence of increased ROS levels. The protein levels of superoxide dismutases 1 and 2 (SOD1 and SOD2), that catalyze the dismutation of superoxide anion into hydrogen peroxide, and the levels of catalase, detoxifying hydrogen peroxide into water and oxygen, were estimated by immunoblotting in 4 and 10 months old Opa1+/- mice cortices and control (Fig. 1C). The data reveal an increase in SOD1 and SOD2, while catalase is unchanged in 10 months Opa1+/- mice cortices when compared to 4 months Opa1+/- mice. No difference was observed in control mice (Fig. 1C).

Altogether these findings suggest that during the 6 months interval (between 4 and 10 months) there is an excess of mitochondrial ROS in Opa1+/- mice that may induce an antioxidant response.

**OPA1 downregulation imbalances neuronal redox state in vitro and activates NRF2 pathway**

To decipher the molecular mechanisms underlying the excess of ROS in DOA mice, we used a previously characterized in vitro neuronal model of OPA1 haploinsufficiency.21

Primary cortical neurons transfected with siRNA directed against OPA1 mRNA (siOPA1) showed 70% decrease in OPA1 protein levels at day 6 post-transfection when compared to neurons treated with control siRNA (siCtrl) (Fig. 2A). Interestingly, citrate synthase, HSP60, VDAC, and TOM20 levels were unchanged (Fig. 2A), showing that mitochondria quantity was not affected by OPA1 downregulation.

While OPA1 downregulation is known to negatively impact respiration in various cell lines,30–32 we evaluated here for the first time the effect of OPA1 downregulation on respiration in neurons using the Seahorse XF24 analyzer (Seahorse Bioscience) (Fig. 2B). In siOPA1- and siCtrl-transfected neurons, rotenone and antimycin treatment considerably inhibited the OCR, showing that more than 95% of respiration was due to mitochondria. In siCtrl-transfected neurons, oligomycin inhibited ATP-linked respiration, while addition of the protonophore FCCP, which uncouples oxidation and phosphorylation, resulted in maximal OCR. In siOPA1-transfected neurons, spontaneous respiration was reduced by 32%, when compared to siCtrl-treated neurons. Furthermore, the ATP-linked respiration and the maximal OCR were reduced by 34% and 46%, respectively. Contrary to siCtrl-transfected cells, the maximal OCR in siOPA1-treated cells was not significantly different than spontaneous OCR.

Therefore, depletion of OPA1 in neurons induced a decrease in spontaneous, ATP-linked respiration and maximal mitochondrial respiration without affecting the mitochondrial biomass. A decrease in mitochondrial respiration could lead to energetic failure. However, we showed that neither ATP nor ADP levels, and thus the...
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**OPA1 Influences Redox State**

**Figure A**
- **Non-treated cells** vs. Rataenane 500nM (1h)
- Percentage of viable cells

**Figure B**
- **Non-treated cells** vs. Rataenane 500nM (1h)
- Necrotic nuclei (%)

**Figure C**
- **Non-treated cells** vs. Rataenane 500nM (1h)
- Nuclear fluorescence intensity (pixels sum/μm²)

**Figure D**
- **SOD1** and **SOD2**
- Relative quantities (AU)
- **Catalase**
- Relative quantities (AU)
ratio of ATP over ADP, changed upon OPA1 downregulation in cortical neurons (Fig. 2C). The ratio of ATP/ADP (and the amounts of these molecules) in the steady state is the result of synthesis and consumption. So if it remains stable while cellular respiration in phosphorylation conditions decreases, it could be the consequence of a consumption decrease to the same extent as synthesis. In the context of neurons it can be reflected by a decrease in the functionality of these cells. This was shown in Berto-hole et al.21 by the reduction of synaptogenesis and dendritogenesis in OPA1 downregulated neurons.

Impaired MRC functioning could lead to an imbalance in the redox state because of increased electron leaks. In agreement, we showed that aconitase activity was reduced by 33% in siOPA1-treated neurons (Fig. 2D), with no effect in aconitase protein quantity, suggesting that OPA1 downregulation would induce an increase in mitochondrial ROS levels in cultured neurons.21

Again, to reinforce these data we analyzed the NRF2 transcription factor pathway which accounts in large parts for the oxidative stress responses, and monitored some of its antioxidant target genes by analyzing SOD1, SOD2, and catalase protein levels.33 Since a hallmark of NRF2 activation is its nuclear translocation, we studied its intracellular localization. In neurons ex vivo, the decreased quantity of OPA1 induced a 1.3-fold increase in nuclear NRF2 fluorescence compared to siCtrl cells (Fig. 2E). In siOPA1-transfected neurons, no statistical differences in SOD1 and 2 (data not shown) were detected, while we uncovered an increase in both catalase quantity (88%) and activity (61%) (Fig. 2F).

We thus concluded that downregulation of OPA1 induced NRF2 activation leading to upregulation of, at least, one of its target genes, catalase.

**Supplemental oxidative stress challenges viability of OPA1-downregulated neurons**

Since OPA1 downregulation leads to increased mitochondrial ROS levels that induce antioxidant response, we addressed the impact of an additional acute oxidative stress in OPA1 downregulated cells. A supplemental stress would therefore be deleterious for cell viability. Neurons were thus incubated with rotenone, a potent inhibitor of respiratory complex I that induces a major oxidative stress.34,35 We checked this hypothesis by estimating the neuronal viability by trypan blue exclusion assay (Fig. 3A) as well as the number of picnotic nuclei by DAPI staining (Fig. 3B) of siCtrl- and siOPA1-transfected neurons upon acute treatment of rotenone (1h, and 500 nmol/L). siOPA1-transfected cells showed 8% decreased viability when compared to siCtrl-treated neurons (Fig. 3A). Accordingly, the number of picnotic nuclei is increased by 34% in siOPA1 versus siCtrl rotenone-treated neurons (Fig. 3B). These results suggest that antioxidant defenses may be overwhelmed in OPA1 downregulated conditions. We checked this hypothesis by analyzing the NRF2 nuclear translocation (Fig. 3C), as well as SOD1, SOD2, and catalase protein quantities (Fig. 3D). Upon acute rotenone treatment, NRF2 translocation was increased by 1.2-fold in siCtrl neurons, while it was unchanged in siOPA1 neurons (Fig. 3C). Accordingly, a 1.5-fold increase in SOD1 protein quantity was observed in control cells, while no change occurred in siOPA1 neurons (Fig. 3D). No difference was observed for SOD2 protein quantities in siOPA1 and siCtrl neurons treated or not with rotenone (Fig. 3D). Moreover, catalase protein quantity, which is increased by 32% in basal condition in siOPA1 neurons when compared to control neurons, did not increase and even diminished by 32%, suggesting that downregulation of OPA1 results in maximal catalase induc-
tion that cannot be further increased upon rotenone-induced oxidative stress.

**Cellular antioxidant defenses are impaired in certain fibroblasts from DOA patients**

We next addressed the question of antioxidant defenses in DOA patients’ fibroblasts (Table S1). SOD1, SOD2, and catalase were studied by immunoblot analysis (Fig. 4A). Fibroblasts from five healthy volunteers (C1–C5) and eight DOA patients (P1–P8) were analyzed. Even though heterogeneity exists between healthy volunteers and DOA patients in antioxidant defenses, some patients showed altered expression of antioxidant genes at the protein level (Fig. 4A and Fig. S2). DOA patients P1, P3, P5, and P7 expressed low levels of SOD1 and SOD2 proteins, while catalase was not different (Fig. 4A). To note, the levels of these antioxidant proteins are not correlated with the age of patients (Table S1 and Fig. S3).

These results show actual heterogeneity of SODs content in patient fibroblasts. To determine if this heterogeneity could be linked to the status of OPA1 protein level in fibroblasts from DOA patients, we investigated the levels of both total OPA1 protein and of the ratio of the long (l-OPA1) relative to the short (s-OPA1) OPA1 isoforms (Fig. 4B). P1 and P3 presented a lower quantity

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**Figure 4.** Antioxidant defenses and OPA1 proteins levels in fibroblasts from dominant optic atrophy (DOA) patients. (A) Representative histograms of SOD1, SOD2, and catalase proteins levels relative to actin in five healthy volunteer’s skin fibroblasts (CTRL, C1–C5) and eight DOA patient’s skin fibroblasts (DOA, P1–P8) ex vivo. SOD1 CTRL: 1.15 ± 0.25 AU; SOD1 DOA: 0.62 ± 0.16 AU; SOD2 CTRL: 1.21 ± 0.19 AU; SOD2 DOA: 0.59 ± 0.16 AU; catalase CTRL: 0.82 ± 0.10 AU; catalase DOA: 0.96 ± 0.10 AU. Results are expressed as mean ± SEM (n = 3–6 per DOA and CTRL fibroblasts). (B) Representative histograms of OPA1 protein levels relative to actin in five healthy volunteer’s skin fibroblasts (CTRL, C1–C5) and eight DOA patient’s skin fibroblasts (DOA, P1–P8) ex vivo. Histogram representative of the ratio of the long (l-OPA1) to the short (s-OPA1) OPA1 isoforms. Results are expressed as mean ± SEM (n = 3–6 per DOA and CTRL fibroblasts). Statistical significance for the ratio of the long to the short OPA1 isoforms was determined by a one-way analysis of variance (ANOVA), **P < 0.01.
of OPA1. P5 and P7 presented differences of the ratio l-OPA1 over s-OPA1.

Discussion

In the present work, we clearly demonstrate that OPA1 dysfunction induces a decline in endogenous mitochondrial respiration in primary cultured neurons with a maximal mitochondrial respiration that does not exceed basal mitochondrial respiration. Defects in mitochondrial respiration have also been observed in several cell lines upon OPA1 knockdown. In agreement with our results, OPA1-depleted MEFs also showed a severe reduction in endogenous respiration, no effect after uncoupling, and a decrease in oxygen consumption mediated by complexes I, II, and IV.32 Studies using fibroblasts from DOA patients support the observation of a coupling defect of oxidative phosphorylation due to a reduction in complex IV at the protein level and heightened complex V activity, with no change in ATP production in either case.12 Defective oxidative phosphorylation with lowered ATP production has been found in DOA patient tissue analyzed by MR spectroscopy.36 However, the existence and the nature of the mitochondrial oxidative phosphorylation dysfunction are still controversial since other studies did not find respiratory defects in lymphoblastoid lines31 or in muscle biopsies of patients with DOA.8 Along these lines, cardiomyocytes derived from Opa1−/− mice also do not display any respiratory defects although mitochondrial morphology is altered.37

Mitochondrial respiration impairment due to OPA1 loss could originate from either downregulation/deregulation of MRC, or disorganization of the mitochondrial inner membrane structure,38 or both. Indeed, we have previously shown that the protein levels of certain subunits of MRC were decreased in siOPA1-transfected neurons.21 Besides, NDUFB8 and NDUFA9 from complex I, Core 1 and Core 2 from complex III, and Cox I and Cox VIc from complex IV decreased by 30–50%. Moreover, Zanna and colleagues have suggested a direct interaction between OPA1 and the respiratory chain complexes I, II, and III.14 On the other hand, a decrease in respiration in siOPA1-treated neurons could also damage the spatial organization of the MRC.39 The organization of respiratory chain complexes into supercomplexes (RCS) is essential for the assembly and stability of each complex41–43 and may influence the efficiency of electron transfer.44–46 Therefore, it is likely, as recently suggested, that the respiratory deficiency that we observed upon OPA1 downregulation in neurons results from disruption of RCS.47

Our studies using primary cortical neurons did not reveal any significant changes in ATP or ADP levels. This may indicate that either OPA1 knockdown is not sufficient to induce an energetic failure or any decrease in mitochondrial ATP synthesis is offset by a decrease in global ATP consumption. In addition, we found an increase in mitochondrial ROS production revealed by a decrease in antioxidant activity in OPA1-depleted neurons, consistent with prior findings in OPA1-mutant flies.48 We demonstrated that this increase in ROS production activates the NRF2 pathway, one of the primary mechanisms of cell detoxification.59 Indeed, downregulation of OPA1 in primary neurons induced relocation of the NRF2 transcription factor into the nucleus, where it upregulated target genes bearing antioxidant responsive promoter elements, such as catalase. Moreover, upregulation of antioxidant defenses also confirms that OPA1 downregulation promotes higher ROS production. Additional exogenous oxidative stress caused by rotenone reduced neuronal viability. These results are consistent with previous experiments showing hypersensitivity to oxidative stress in C. elegans and in D. melanogaster.17,18 Our data suggest that the viability impairment of siOPA1-treated neurons upon acute oxidative stress is due to inefficient antioxidant defenses. SOD1, SOD2, and catalase levels did not change upon acute oxidative stress in siOPA1 rotenone-treated cells, while SOD1 levels increased in rotenone-treated control cells. Our experiments performed in Opa1−/− mutant mice also found decreased aconitate activity and increased antioxidant defenses in Opa1−/− mouse cortices compared to controls, again suggesting excess mitochondrial ROS production.

Our results thus underscore the critical role of NRF2 signaling in DOA pathogenesis. To note, even if we observed a difference in the NRF2-induced target in our different DOA models, this is largely described in the literature (for review see ref. 49) as well as the different answers observed for the antioxidant defenses among the patient’s fibroblasts for example for Friedreich Ataxia.50 Because many studies focused on fibroblasts to understand cellular attempts common to different cells and particularly neurodegenerative diseases,7,14,50 we decided in this study to compare the effect of OPA1 dysfunction in DOA patients fibroblasts and neuronal models. In all our models, the NRF2 pathway is associated with activation of SOD1 or SOD2 or catalase, but not GSTP1 and NQO1. Indeed, these last two enzymes remain constant for all the patients as well as in neurons treated by siOPA1 or siCtrl, which supports distinguishing SOD1 or SOD2 or catalase as reliable markers.

Our results also show that DOA pathogenesis may be related to both the quantity of OPA1 protein and the relative levels of the five isoforms of OPA1. P1 and P3 present a decrease in OPA1 protein levels, suggesting that, in these two cases, haploinsufficiency may contribute to DOA pathogenesis. P5 and P7 present lower ratios of l-OPA1 to s-OPA1, which might also contribute to the
severity of this disease. Changes in the ratio of l-OPA1 to s-OPA1 were shown to impact OPA1 functions, and in a recent work, the level of l-OPA1 was presented as a possible therapeutic target for ischemia–reperfusion injury-related diseases at least for the retina because it could restore mitochondrial morphology. These results suggest that the protein profile of OPA1, beyond merely its genetic status, should be considered in future research.

As we showed here for DOA, dysfunctions in ROS metabolism may also underlie Leber’s hereditary optic neuropathy, glaucoma, and other neurodegenerative pathologies such as Alzheimer’s and Parkinson’s diseases. In our model, the redox state imbalance induced by OPA1 haploinsufficiency could escalate into an oxidative stress state upon exposure to factors such as UV light, blue light, smoke, aging, or diet. Interestingly, some studies described an increase in sensitivity of RGCs to blue light stimulation and to hypoxia. Additionally, within the retina, mitochondrial DNA lesions reach remarkable levels after exposure to endogenous ROS generated by the mitochondria themselves or from photosensitizers (for review see ref. 59). In this framework, it is noteworthy that numerous studies suggest that increasing mitochondrial antioxidant defenses may rescue optic nerve defects. Cellular systems that protect against oxidants include antioxidant defenses enzymes and oxidant scavengers. In addition to direct antioxidants, a number of antioxidant compounds are weak pro-oxidants with indirect antioxidant properties. Acting as weak pro-oxidants, these molecules have limited ability to cause oxidative damage, yet they are potent enough to activate the NRF2-mediated adaptive response, thereby protecting against existing or future high-level stresses. Given that our findings that OPA1 downregulation activates NRF2 pathway, the pro-oxidant compounds could be insufficient or dangerous therapeutic approaches for some DOA patients. Instead, direct testing of antioxidant compounds could offer a complementary pharmacological treatment for DOA. Accordingly, a recent study focused on idebenone, a benzoquinone that transfers electrons to complex III bypassing complex I, as a potential curative compound for DOA patients. Thus, it is of primary importance to identify patients able to induce an efficient NRF2 response. Our work could thus have a direct impact on both treatment and care of DOA patients. Indeed, the low expression of some antioxidant enzymes in several DOA patients could support their role as modifier factors for DOA.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Protein levels of MFN1 and mitofilin in OPA1 downregulated neurons. Representative immunoblots and histograms showing protein levels of mitofilin and mitofusin 1 (MFN1) relative to actin in siOPA1- (gray bars) and siCtrl-transfected (white bars) neurons. Results are expressed as mean ± SEM (n = 5). Statistical significance was determined by Student’s paired t-test.

Figure S2. DOA patient’s fibroblasts and control’s fibroblasts antioxidant proteins levels. (A) Representative immunoblots showing protein levels of SOD1, SOD2, catalase, GSTP1, NQO1, and actin, and histograms representing GSTP1 and NQO1 proteins quantities, in five healthy volunteer’s (CTRL) fibroblasts (C1–C5) and in eight DOA patient’s fibroblasts (P1–P8). (B) Representative immunoblots showing protein levels of OPA1 and actin.

Figure S3. DOA patients’ and controls’ fibroblasts antioxidant proteins levels relative to their age at the moment of the biopsies. Antioxidant proteins levels (SOD1, SOD2, catalase, GSTP1, NQO1, and actin, and histograms representing GSTP1 and NQO1 proteins quantities, in five healthy volunteer’s (CTRL) fibroblasts (C1–C5) and in eight DOA patient’s fibroblasts (P1–P8). (B) Representative immunoblots showing protein levels of OPA1 and actin.

Table S1. Clinical symptoms and mutations of DOA patients.