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The Neurogenic Basic Helix-Loop-Helix Transcription Factor NeuroD6 Enhances Mitochondrial Biogenesis and Bioenergetics to Confer Tolerance of Neuronal PC12-NeuroD6 Cells to the Mitochondrial Stressor Rotenone

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Abstract

The fundamental question of how and which neuronal specific transcription factors tailor mitochondrial bioenergetics to the need of developing neuronal cells has remained largely unexplored. In this study, we report that the neurogenic basic helix-loop-helix transcription factor NeuroD6 possesses mitochondrial biogenic properties by amplifying the mitochondrial DNA content and TFAM expression levels, a key regulator for mitochondrial biogenesis. NeuroD6-mediated increase in mitochondrial biogenesis in the neuronal progenitor-like PC12-NEUROD6 cells is concomitant with enhanced mitochondrial bioenergetic functions, including increased expression levels of specific subunits of respiratory complexes of the electron transport chain, elevated mitochondrial membrane potential and ATP levels produced by oxidative phosphorylation. Thus, NeuroD6 augments the bioenergetic capacity of PC12-NEUROD6 cells to generate an energetic reserve, which confers tolerance to the mitochondrial stressor, rotenone. We found that NeuroD6 induces an adaptive bioenergetic response throughout rotenone treatment involving maintenance of the mitochondrial membrane potential and ATP levels in conjunction with preservation of the actin network. In conclusion, our results support the concept that NeuroD6 plays an integrative role in regulating and coordinating the onset of neuronal differentiation with acquisition of adequate mitochondrial mass and energetic capacity to ensure energy demanding events, such as cytoskeletal remodeling, plasmalemmal expansion, and growth cone formation.

Keywords

NeuroD family; neuronal differentiation; mitochondrial biogenesis; mitochondrial membrane potential; oxidative phosphorylation; mitochondrial stressors

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Introduction

Mitochondrial dysfunction critically affects the developing and mature central nervous system given its high-energy demand [1, 2]. Autism spectrum disorder (ASD) is the latest identified neurodevelopmental disorder with mitochondrial perturbations [3]. Developing neurons require high levels of ATP to fuel plasmalemmal biogenesis and cytoskeletal assembly associated with axonal and dendritic growth, processes that are estimated to consume approximately 50% of cellular ATP [4]. Since mitochondria produce the large majority of intracellular ATP through the oxidative phosphorylation system (OXPHOS), developing neurons are vitally dependent on robust mitochondrial biogenesis and bioenergetics. OXPHOS involves a series of redox reactions during which electrons are transferred through several multimeric respiratory enzyme complexes I, III, and IV, establishing a mitochondrial membrane potential ($\Delta\Psi_m$) due to proton gradient between the mitochondrial matrix and the inner mitochondrial membrane, with ATP being synthesized by complex V, also called ATP synthase upon return of protons to the mitochondrial matrix [5]. The OXPHOS capacity of mitochondria is under a coordinated bi-genomic regulation due to the limited coding capacity of mitochondrial DNA (mtDNA), which only encodes 13 out of 80 protein subunits composing the OXPHOS system, with the remainder being nuclear-encoded and imported into mitochondria [6]. In contrast, the protein machinery responsible for mitochondrial biogenesis, more precisely mtDNA replication, is solely nuclear-encoded [7]. The mitochondrial-specific transcription factor Tfam is an essential regulator for *in vivo* mitochondrial biogenesis, as reflected by the early embryonic lethality of *Tfam* null mice and a limiting determinant of mtDNA copy number [8, 9]. Moreover, decreased Tfam expression levels in neurons of mutant mice result in mitochondrial respiratory chain defects [10], while mtDNA depletion in humans results in severe mitochondrial diseases, such as mtDNA depletion syndrome [11].

Although major progress has been made toward elucidating the transcriptional network regulating mitochondrial biogenesis and bioenergetics via the ubiquitous transcriptional factors NRF-1-NRF-2 and the co-activator PGC-1 [12], little is known about the identity of neuronal-specific transcriptional factors tailoring mitochondrial functions to the onset of neuronal differentiation. Our recent studies have addressed this gap in our knowledge by demonstrating that a direct correlation between mitochondrial mass and expression of the neurogenic basic helix-loop-helix (bHLH) transcription factor NeuroD6 during the early stages of neuronal differentiation [13]. Furthermore, our gene set enrichment analysis of our genome-wide microarray studies has revealed a link between NeuroD6 and a cluster of mitochondrial bioenergetic-related genes [14]. Finally, NeuroD6 sustained the mitochondrial biomass and low levels of ROS during oxidative stress [15]. Thus, the goal of the present study was to determine whether NeuroD6 could coordinate mitochondrial biogenesis and bioenergetics with the onset of neuronal differentiation. This role would be in concordance with NeuroD6 embryonic expression being triggered at E11.5, a time when neuronal progenitor cells undergo cell cycle withdrawal and initiate neuronal differentiation [16, 17]. We found that NeuroD6 mediates mitochondrial biogenesis by concomitantly increasing mtDNA copy number and Tfam expression levels. Furthermore, NeuroD6 promotes mitochondrial bioenergetic functions by increasing the expression of key subunits of the respiratory complexes, the mitochondrial membrane potential and ATP levels, thereby generating an energetic reserve. Finally, NeuroD6 endows the PC12-NEUROD6 cells with tolerance to the mitochondrial stressor rotenone, an inhibitor of the respiratory complex I (NADH: nicotinamide adenine dinucleotide ubiquinone oxidoreductase) by utilizing this increased basal energetic capacity, thus preventing a deleterious mitochondrial bioenergetic deficit and subsequent cell death.

Materials and methods

Cell culture

Control PC12 and PC12-NEUROD6 cells (previously called PC12-Nex1) were generated as described [18] and grown in the presence of F12K medium (Invitrogen) containing 15% horse serum (Invitrogen), 2.5% fetal bovine serum (Invitrogen). Since the three generated PC12-NEUROD6 clones (PC12-Nex1-M A, B and C) displayed similar response upon NGF exposure and withdrawal of trophic factors [18–20], we used the PC12-NEUROD6 clone A to remain consistent with our previous studies pertaining to NeuroD6 impact on the mitochondrial biomass and bioenergetic-related genes in the absence or presence of oxidative stress [13–15]. For immunocytochemistry, cells were grown on poly-D-lysine coated coverslips. For live cell confocal microscopy, cells were seeded onto 35 mm² PDL-coated glass bottom plates (Warners Instruments).

Drug treatments

When measuring $\Delta\Psi_m$ with TMRM or JC-10, PC12 and PC12-NEUROD6 cells were treated with 5 μ M carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP, Sigma) during the incubation period with the $\Delta\Psi_m$ -dependent dye. FCCP (5 μ M) was also present in the medium during image acquisition. Oligomycin-treated (4 μ g/ml) cells were first labeled with Magnesium GreenTM (Mg-Gr; Invitrogen) and subsequently incubated into serum-containing phenol red-free F12K medium (Invitrogen) containing 4 μ g/ml oligomycin for 45 minutes prior to trypsinization. For rotenone treatment, cells were incubated with rotenone (1 μ M) for a period of 6, 24, or 48 hours. For antimycin A treatment, cells were incubated in antimycin A-containing medium (500 ng/ml) for a period of 24 hours followed by MitoSox and Hoechst labeling.

Mitochondrial DNA content by quantitative PCR

Total cellular DNA from PC12 and PC12-NEUROD6 cells was isolated by phenol-chloroform extraction using standard methods. MtDNA was quantified by normalizing the mitochondrial-encoded gene *mtco1* to the nuclear-encoded gene *Ndufv1* using qPCR and the $\Delta\Delta$ CT method. The primers were: 5'-ACCCCGCTATAACCCAATATCAGAC-3' (F, *mtco1*), 5'-TGGGTGTCCGAAGAATCAAAATAG-3' (R, *mtco1*), 5'-CGCCATGACTGGAGGTGAGGXAG-3' (F, *Ndufv1*), and 5'-GGCCCCGTAAACCCGATGTCTTC-3' (R, *Ndufv1*). SYB Green fluorescence detection for qPCR was performed using iCycler iQ Real-time detection System (Bio-Rad). A total of 10 ng total DNA was used in a 25 μ l reaction containing 1X SYBR Green iCycler iQ mixture and 0.2 μ M of forward and reverse gene-specific primers.

Mitochondrial fractionation

Mitochondrial enriched fractions were isolated from PC12 and PC12-NEUROD6 cells using a mitochondrial/cytosol fractionation kit (Sigma) according to the manufacturer's recommendations and as described [13]. Protein concentration was determined using the Bradford assay (Bio-Rad).

Immunoblot analysis

Proteins (40 μ g) were resolved on 10% NuPAGE Bis-Tris gels (Invitrogen) and transferred to a nitrocellulose membrane as described [15]. Primary monoclonal antibodies included: Complex I NDUSF3 (Mitosciences; 1:500), Complex I NDUSF4 (Mitosciences; 1:500), Complex III core2 protein (Mitosciences; 1:1000), Complex IV subunit I (Mitosciences; 1:500), Complex IV subunit III (Mitosciences; 1:500) ATP synthase subunit delta (Mitosciences; 1:250), ATP synthase subunit alpha (Mitosciences; 1:1000), Tfam (Santa-

Cruz; 1:500). The antigen-antibody complexes were detected using the Supersignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology) and corresponding secondary horseradish peroxidase-conjugated antibodies. To confirm equal loading, blots were stripped using RestoreTM Western blot stripping buffer (Pierce Biotechnology) and re-probed with the anti- α subunit of ATP synthase or GAPDH as indicated. Protein levels visualized by immunoblot analysis were quantified using the NIH (National Institute of Health) ImageJ software (<http://rsb.info.nih.gov/ij/>) and normalized against the loading control as described in [15].

Confocal microscopy

For fixed cells were acquired with an oil immersion 63x objective (NA 1.4) on a Zeiss LSM 710 confocal microscope. For live-cell confocal microscopy, cells were visualized with an oil immersion objective 63x (NA 1.4) on a Zeiss LSM 510 confocal microscope equipped with a TempModuleS and CO₂ Module (Zeiss) to maintain the temperature and CO₂ levels at 37°C and 5.0%.

Immunocytochemistry

PC12 and PC12-NEUROD6 cells were fixed and permeabilized, as described [13]. For the cytoskeletal immunocytochemistry experiments, cells were treated as described [21]. Briefly, cells were washed for 30 sec with PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, [pH 6.9]) and permeabilized with 0.2% Triton-X 100 in PHEM buffer containing 10 μ M taxol (Invitrogen) followed by fixation with 4.0% paraformaldehyde in PHEM buffer. Polymerized F-actin and tubulin were labeled with Alexa Fluor® 488-phalloidin (Invitrogen) and anti- β -III tubulin monoclonal antibody (Covance; 1:500), respectively, as described [13]. When examining expression of mtDNA and Tfam, the following primary antibodies were used: monoclonal anti-DNA (American Research Products; 1:100) and goat anti-Tfam (Santa-Cruz; 1:250). Samples were mounted using Prolong Gold antifade reagent with the nuclear counterstain DAPI (Invitrogen).

Measurement of ATP levels

To measure the levels of intracellular ATP by flow cytometry, cells were grown in the presence or absence of rotenone (1 μ M) followed by Mg-Gr labeling (10 μ M) for 30 min as described [15]. Data were collected from 20,000 cells for each sample and experiment (n=5) using a FACScalibur flow cytometer (BD Bioscience) before being analyzed using CellQuant software (BD Bioscience). To analyze Mg-Gr labeling by live-cell confocal microscopy, the laser power, gain, offset, and iris were kept constant during image acquisition, such that differences in fluorescence intensity could be directly compared among samples.

Measurements of $\Delta\Psi_m$ using TMRM

Cells were incubated with 25 nM tetramethylrhodamine methyl ester (TMRM; Invitrogen) at 37° C for 60 min. Imaging was performed in the presence of 5 nM TMRM diluted in serum containing phenol red-free F12K medium. Using NIH Image J software (<http://rsb.info.nih.gov/ij/>), the maximal intensity of TMRM labeling within the nuclear compartment delineated by DAPI staining as well as the standard deviation (S.D.) were calculated. All pixels with a value less than or equal to the maximal nuclear labeling were considered as cytoplasm, while all pixels with a value greater than the maximal nuclear labeling plus three S.D. were considered mitochondrial specific [22]. The average intensity of the cytoplasmic (F_c) and mitochondrial (F_m) regions was calculated using AxioVision v. 4.7.2 (Zeiss Imaging Software). A sigma filter of 32 was applied to reduce noise while preserving fine structures. Images were segmented to define the “mitochondria” and

“cytoplasmic” regions using threshold values. “Mitochondria” less than 5 pixels in size were excluded from the analysis. A watershed mode was used to automatically separate touching mitochondria such that they were measured as distinct mitochondria. The average F_m of a cell was then calculated by averaging the fluorescence intensity of all the individual mitochondria present (between 25–100) within the cell. Finally, the cytoplasmic regions used to determine the average F_c value were restricted to regions within a few micrometers of mitochondria as described [23]. For each sample, measurements were taken from a minimum of 60 cells per experiment ($n=3$). Data were expressed as the mean F_m/F_c ratio normalized against the mean F_m/F_c ratio of control PC12 cells $\pm$ S.D. For flow cytometry analysis, PC12 and PC12-NEUROD6 cells were labeled with 25 nM TMRM for 45 min as described above. Data were collected from 20,000 events for each sample per experiment ($n=4$) using a FACScalibur flow cytometer and analyzed using CellQuant software. Data were normalized against the TMRM-emitted fluorescence intensity of PC12 cells $\pm$ S.D.

Measurements of $\Delta\Psi_m$ using the ratiometric dye JC-10

Cells were incubated with 4 μ M JC-10 (Enzo Life Sciences) at 37 °C for 30 min, washed twice, and imaged in serum-containing phenol red-free F12K medium. During imaging, the duration of light exposure was minimized and kept constant for all samples and emissions at 520 nm and 590 nm were detected simultaneously. The laser power, gain, offset, and iris were kept constant to allow direct comparison between all samples. To calculate the F_r/F_g ratio, individual mitochondria ($n=20$) from each cell were hand-traced and the average fluorescence intensity of each signal within the region of interest was recorded using NIH Image J software. For each sample, measurements were taken from 115–200 cells and three independent experiments. Heat map images were constructed using Volocity software (Mountain View). When applicable, data were expressed as the number of mitochondria with an F_r/F_g ratio corresponding to the indicated 0.1 increment or as percent of the F_r/F_g ratio relative to PC12 ($t=0$) or PC12-NEUROD6 ($t=0$) $\pm$ S.D.

Cell survival assay

PC12 and PC12-NEUROD6 cells were seeded at 60% confluency (5×10^5 cells/ml) in the absence or presence of rotenone (1 μ M) for specific periods of time, as indicated in the figure legends. Cells were incubated with 2 μ M CellTracker GreenTM (Invitrogen) for 45 min, washed with PBS, trypsinized, collected by centrifugation (1100 g) and resuspended in 1.0 ml of PBS containing 5.0×10^5 red (580/605) fluorescent microspheres (Invitrogen). Data were collected from 20,000 events for each sample per experiment ($n=3$) using a FACScalibur flow cytometer and analyzed using CellQuant software. Survival rate following rotenone exposure was determined by calculating the density of cells relative to the number of beads as described [24]. To determine the density of cells, the number of cells was divided by the total number of events, beads, and cells. The percentage of survival rate was calculated by dividing the cell density of rotenone-treated cells with the cell density of untreated cells.

Quantification of polymerized microtubules and F-actin

The levels of polymerized microtubules and F-actin in PC12 and PC12-NEUROD6 cells grown in the absence or presence of rotenone (1 μ M) was determined after tubulin extraction based on the fluorescence intensity of β III-tubulin and phalloidin labeling, respectively, as described [21]. Each cell was hand-traced and the average fluorescence intensity of each signal was measured using NIH Image J software for 50–70 cells per experiment in three independent experiments. Data are expressed as the mean fluorescence intensity normalized against the fluorescence intensity of untreated PC12 cells $\pm$ S.D.

Measurements of ROS levels

When indicated, PC12 and PC12-NEUROD6 cells were first treated with rotenone (1 μ M) or antimycin A (500 ng/ml) for the indicated period of time followed by labeling with 0.5 μ g/ml Hoechst 33342 for 30 min and 2.5 μ M MitoSOX Red (Invitrogen) for 10 min at 37°C as described [15]. The mean fluorescence intensity of MitoSOX labeling was calculated for each cell using NIH Image J software. For each sample, measurements were taken from 150 cells in three independent experiments. Data were expressed as the mean fluorescence intensity normalized against the fluorescence intensity of the untreated PC12 cells $\pm$ S.D.

Statistical analysis

Statistical analyses were performed using the unpaired student t-test, with p-value of less than 0.05 considered statistically significant. Data are expressed as means $\pm$ S.D.

Results

NeuroD6 expression is associated with enhanced mitochondrial biogenesis

On the basis of the correlation between NeuroD6 expression and mitochondrial mass established in our previous studies [13], we investigated whether NeuroD6 could modulate the mitochondrial biomass by enhancing mitochondrial biogenesis. To address this question, we took advantage of our neuronal paradigm, the PC12-NeuroD6 cell line, which constitutively expresses NeuroD6 and spontaneously recapitulates the first three stages of neuronal differentiation with 44% of PC12-NeuroD6 cells being at stage I defined as the lamellipodia stage, 28% at stage II associated with neurite outgrowth, and 25% at stage III corresponding to axonal outgrowth [13, 18], as defined by the Banker's group [25]. The PC12-NeuroD6 cells behave as neuronal progenitor cells poised to execute terminal differentiation with cell cycle withdrawal upon neurotrophic cues [18, 26], thereby mimicking the restrictive proliferation potential of NeuroD6⁺ committed neural progenitors during neurogenesis [27]. We initially measured mtDNA content as an indicator of mitochondrial biogenesis by real-time quantitative PCR using total DNA isolated from control PC12 and PC12-NEUROD6 cells. We found a two-fold increase in mtDNA copy number in the presence of NeuroD6 expression (Fig. 1A), which is in concordance with our previously published study indicating a two-fold increase in mitochondrial mass determined by flow cytometry and quantitative confocal fluorescence microscopy [13]. We confirmed by immunocytochemistry using an anti-DNA antibody increased mtDNA content in the presence of NeuroD6 expression (Fig. 1B). We next examined whether increased mtDNA copy number was accompanied by increased expression levels of the nuclear-encoded mitochondrial specific transcription factor, Tfam, since mtDNA copy number is directly correlated to TFAM protein levels [8, 9]. The TFAM protein is synthesized as a precursor form (nuclear form) with a molecular weight of 28 kDa, which contains a mitochondrial targeting peptide removed upon processing after import to mitochondria, giving rise to the mitochondrial form with a molecular weight of 25 kDa [28]. By immunoblot analysis, we detected more than two-fold increase of the nuclear and mitochondrial forms of TFAM protein in PC12-NEUROD6 cells (Fig. 1C). Finally, confocal microscopy confirmed increase of TFAM expression in the presence of NeuroD6 expression (Fig. 1D). Notably, we found enrichment of the TFAM protein at the tip of the growing neurites in stage II PC12-NEUROD6 cells, a neuronal stage characterized by outgrowth of neuritic processes (Fig. 1D). TFAM protein remained enriched in the growth cones of stage III PC12-NEUROD6 cells, a stage during which axonal outgrowth occurs (Fig. 1D). Collectively, our results support the notion that NeuroD6 expression mediates an increase in mitochondrial mass by enhancing mitochondrial biogenesis during the early stages of neuronal differentiation.

NeuroD6 increases expression levels of key subunits of respiratory complexes and ATP levels

Based on our gene set enrichment analysis highlighting a correlation between NeuroD6 and mitochondrial energy-transducing genes [14], we next examined whether NeuroD6 constitutive expression altered the expression status of mitochondrial respiratory complexes that comprise the electron transport chain (ETC). Using mitochondrial-enriched fractions for immunoblot analyses, we observed more than a two-fold increase of the nuclear-encoded subunits NDUFS3 and NDUFS4 of complex I (Fig. 2A, B), which are both critical for the overall activity of complex I, a respiratory complex that plays a pivotal role in cellular energy homeostasis being the point of entry for electrons to the respiratory chain [29, 30]. We next examined the expression levels of the nuclear-encoded core 2 protein of complex III, which maintains the integrity of the respiratory complex III, and found its expression levels unaltered in the presence of NeuroD6 expression (Fig. 2A, B). In contrast, the expression levels of the nuclear-encoded subunit IV and the mitochondrial-encoded subunits mtco1 and mtco3 of complex IV substantially increased upon NeuroD6 overexpression (Fig. 2A, B). Finally, we found that expression levels of the 3 subunit of ATP synthase (complex V), which is part of the stalk connecting the F1 and F0 moieties thereby playing an important role in energy coupling of complex V [31], augmented by a two-fold factor in the presence of NeuroD6 expression, while expression levels of the α subunit of complex V remained unaltered (Fig. 2A, B).

We next investigated whether increased expression levels of subunits of the respiratory complexes in PC12-NeuroD6 cells led to increased ATP levels produced by OXPHOS. To assess ATP levels in live PC12 and PC12-NEUROD6 cells, we used the magnesium green (Mg-Gr) dye, which has been successfully employed to monitor changes in ATP levels in live cells such as cultured chick ciliary sensory neurons, spinal neurons, hair cells and live patient fibroblasts [4, 32–34]. Fluorescence emission from the Mg-Gr dye is directly proportional to the concentration of intracellular free Mg^{2+} concentration. Since the affinity of Mg^{2+} for ATP is approximately 10 times higher than its affinity for ADP or AMP [35], the fluorescence intensity is inversely correlated with the intracellular levels of ATP [4]. By live-cell confocal microscopy, we observed a substantial decrease in fluorescence intensity in PC12-NeuroD6 cells, indicative of increased intracellular ATP levels (Fig. 2C). To determine whether ATP levels were the result of OXPHOS, Mg-Gr labeled PC12 and PC12-NEUROD6 cells were treated with oligomycin (4 μ g/ml), an inhibitor specific to ATP synthase, and analyzed by live-cell confocal microscopy while keeping the settings of labeling and imaging constant between untreated and oligomycin-treated cells. Oligomycin elevated the intensity of overall Mg-Gr fluorescence in both PC12 and PC12-NEUROD6 cells, indicative of decreased ATP levels produced by oxidative phosphorylation (Fig. 2C). Our flow cytometry analysis revealed significantly elevated ATP levels in the presence of NeuroD6 expression (Fig. 2D; Supplemental Fig. 1). Collectively our results are congruent with the notion that NeuroD6 expression increases the bioenergetic capacity of PC12-NEUROD6 cells.

NeuroD6 expression increases the mitochondrial membrane potential of PC12-NEUROD6 cells

We extended our studies by investigating whether NeuroD6 expression could alter the mitochondrial bioenergetic status by modulating the mitochondrial membrane potential $\Delta\Psi_m$, the paramount element of the proton-motive force that links electron transport to ATP synthesis [36]. We used two distinct cationic fluorescent lipophilic dyes, TMRM and JC-10, an enhanced derivative of JC-1, both of which accumulate in the mitochondrial matrix in a $\Delta\Psi_m$ -dependent manner.

We initiated our studies with the TMRM dye using two complementary quantification methods, live-cell confocal microscopy and flow cytometry. Using live-cell confocal microscopy, fluctuation of $\Delta\Psi_m$ was assessed by calculating the mitochondrial-to-cytoplasmic dye fluorescence ratio (Fm/Fc) [23], as described in the *Materials and methods* section. We found that NeuroD6 augmented $\Delta\Psi_m$ of PC12-NEUROD6 cells by 33%, when compared to that of PC12 cells (Fig. 3A). To confirm that TMRM concentration and time of exposure were appropriate to measure reliable and meaningful variations of $\Delta\Psi_m$, PC12 and PC12-NEUROD6 cells were exposed to FCCP (5 μ M), a proton ionophore known to induce mitochondrial depolarization, resulting in a reduced $\Delta\Psi_m$. FCCP treatment resulted in decreased TMRM-emitted fluorescence signal in both PC12 and PC12-NEUROD6 cells by 23% and 29%, respectively (Fig. 3A), which is in keeping with the reported $\Delta\Psi_m$ decline in sensory neurons exposed to similar FCCP concentration [23]. To perform a more powerful semi-quantitative analysis of $\Delta\Psi_m$, we took a flow cytometry approach, which encompassed 20,000 events per experiment. The flow cytometry analysis corroborated the NeuroD6-mediated increase of $\Delta\Psi_m$ revealed by live-cell confocal microscopy (Fig. 3B).

We supplemented our $\Delta\Psi_m$ analysis by using the ratiometric dye JC-10, which selectively enters mitochondria in a $\Delta\Psi_m$ -dependent fashion to exist as monomers or aggregates at low or high concentrations, respectively. Since JC-10 aggregation is accompanied by an emission shift from 535 nm (green) to 590 nm (red), the ratio of red to green emission (F_r/F_g) is reflective of $\Delta\Psi_m$ fluctuations [37]. Ratiometric analysis by live-cell confocal microscopy revealed an increase in red emission from JC-10 aggregates in PC12-NEUROD6 cells, indicating that the mitochondrial membrane became more polarized upon NeuroD6 expression (Fig. 4A). Exposure to FCCP (5 μ M) produced a $\Delta\Psi_m$ loss in both JC-10 labeled PC12 and PC12-NEUROD6 cells (Supplemental Fig. 2). We assessed the fluctuation of $\Delta\Psi_m$ by quantifying the F_r/F_g and found that PC12-NEUROD6 cells displayed a 20% increase of $\Delta\Psi_m$ (Fig. 4B), in concordance with the TMRM-derived mitochondrial membrane potential analysis (Fig. 3A). After generating a heat map of $\Delta\Psi_m$ variations using pseudo-colors representing increments of 0.1 unit of the F_r/F_g ratio, we analyzed the distribution of the F_r/F_g ratio among the mitochondrial population in each cell type. We found that the PC12 cells harbored the largest fraction of mitochondria with a F_r/F_g ratio between 0.40 and 0.49 corresponding to a green-blue coloring, while the PC12-NEUROD6 cells displayed the largest fraction of mitochondria with a F_r/F_g ratio in the range of 0.90 – 0.99, illustrated by a yellow-red coloring (Fig. 4C). Collectively, the TMRM and JC-10 analyses provide the first demonstration that the neurogenic bHLH transcription factor NeuroD6 modulates the mitochondrial bioenergetic status by increasing polarization of the mitochondrial membrane.

NeuroD6 confers tolerance to the mitochondrial stressor rotenone

We next investigated whether NeuroD6 could endow the PC12-NEUROD6 cells with greater respiratory plasticity and intrinsic ability to cope with mitochondrial stressors. We exposed PC12 and PC12-NEUROD6 cells to the specific inhibitor of complex I, rotenone, as it is the major entry point of electrons into the ETC, thereby contributing to the establishment of proton gradient required for ATP synthesis [30]. Moreover, it has been established that 50% of PC12 cells undergo apoptosis 24 hours after treatment with rotenone (1 μ M) [38, 39]. Thus, we determined the viability of PC12 and PC12-NEUROD6 cells upon exposure to rotenone for 6, 24, and 48 hours by flow cytometry using fluorescence beads, as apoptotic PC12 cells loose adherence, making inappropriate to quantify cell death using a LIVE/DEAD fluorescence-based assay. After six hours of rotenone exposure, the viability of PC12 cells was already compromised with 35% of cell death, while PC12-NEUROD6 cells displayed negligible cell loss (Fig. 5). After 48 hours of rotenone, PC12 cells underwent massive cell death (83%), while PC12-NEUROD6 cells displayed a robust

survival rate, with only 17% of cell death (Fig. 5). Thus, NeuroD6 endows the PC12-NEUROD6 cells with increased survival capacity upon exposure to rotenone, which is keeping with our previous findings of NeuroD6 neuroprotective properties upon oxidative stress and growth factor withdrawal [15, 19].

NeuroD6 prevents rotenone-induced loss of $\Delta\Psi_m$ and ATP depletion

On the basis that NeuroD6 promotes increased polarization of the mitochondrial membrane, we hypothesized that NeuroD6-mediated resistance to rotenone may be in part attributable to sustained $\Delta\Psi_m$ levels. Thus, we performed a time course analysis of $\Delta\Psi_m$ fluctuation in PC12 and PC12-NEUROD6 cells upon exposure to rotenone (1 μ M) using the ratiometric dye JC-10 to remain consistent with published studies using rotenone-treated PC12 cells [39]. We quantified the F_m/F_c ratios by generating a heat map of $\Delta\Psi_m$ variations throughout rotenone treatment using pseudo-colors. After six hours of rotenone exposure, PC12 cells displayed a 17% decrease in $\Delta\Psi_m$, while PC12-NEUROD6 cells exhibited a negligible and not statistically significant decline in $\Delta\Psi_m$ (Fig. 6A, B). PC12 cells showed a further accentuated loss of $\Delta\Psi_m$ reaching 36% and 40% after 24 and 48 hours of rotenone exposure, respectively (Fig. 6A), in keeping with previous studies [39]. In contrast, PC12-NEUROD6 cells displayed a different pattern of $\Delta\Psi_m$ fluctuation and kinetics, with a transient decrease of $\Delta\Psi_m$ (26%) after 24 hours of rotenone, before returning to near basal levels (83%) after 48 hours of treatment (Fig. 6B). The fact that PC12-NEUROD6 cells possess a greater $\Delta\Psi_m$ capacity than PC12 cells prior to rotenone exposure allows PC12-NEUROD6 cells to absorb this transient decline without compromising their survival.

We next investigated whether NeuroD6 could prevent rotenone-induced ATP deficit. We quantified intracellular ATP levels during rotenone treatment of live PC12 and PC12-NEUROD6 cells by flow cytometry using Mg-Gr labeling. After 6 hours of rotenone exposure, PC12 cells already displayed mild loss of ATP levels before losing more than half of their original ATP levels after 24 hours of rotenone exposure (Fig. 6C; Supplemental Fig. 3). ATP levels could not be accurately quantified in rotenone-treated PC12 cells for 48 hours, as their survival rate was severely compromised with more than 83% cell death (Fig. 5). In contrast, PC12-NEUROD6 cells exhibited a negligible ATP loss throughout rotenone exposure (Fig. 6C). Thus, NeuroD6 confers tolerance of the PC12-NEUROD6 cells to the mitochondrial stressor by sustaining ATP levels and limiting $\Delta\Psi_m$ fluctuations.

NeuroD6 expression prevents disruption of the actin network upon rotenone exposure

Given that rotenone affects the cytoskeletal integrity by depolymerizing microtubules upon binding to tubulin [40] or inhibiting actin polymerization by interfering with the activity of Rho GTPases [41] in conjunction with NeuroD6 ability to remodel the actin and cytoskeletal networks [13, 14], we explored whether NeuroD6-mediated protection to rotenone exposure could also occur by preserving the cytoskeletal architecture.

We examined the effect of rotenone on microtubule polymerization by performing a microtubule extraction to remove the unpolymerized tubulin monomers prior to labeling polymerized microtubules and F-actin network. This technique effectively and selectively removes soluble tubulin without affecting the cytoskeletal fraction containing the polymerized microtubules and therefore the fluorescence intensity of tubulin labeling remains proportional to the amount of polymerized microtubules [21, 42]. Upon phalloidin-labeling, our quantification analysis revealed a 60% decrease in levels of polymerized F-actin in PC12 cells after 6 hours of rotenone exposure, while levels of polymerized F-actin remained unaffected in rotenone-treated PC12-NEUROD6 cells, even after 48 hours (Fig. 7). In contrast, levels of polymerized microtubules in PC12 remained unaltered throughout rotenone treatment in PC12 and PC12-NEUROD6 cells (Fig. 7). Thus, NeuroD6 may in part

confer tolerance to rotenone by preserving the interplay between F-actin network and its cargo, the mitochondria, known to play a critical role in developing neurons [13, 43].

Levels of superoxide anions do not contribute to rotenone-induced cell death

Finally, we investigated whether NeuroD6 could also mediate tolerance to rotenone by controlling levels of superoxide anions ($O_2^{\cdot-}$) since NeuroD6 acts as regulator of ROS homeostasis upon oxidative stress mediated by trophic factor removal [15] and ROS production contributes to rotenone-induced apoptosis in some neuronal paradigms [37, 44, 45]. We evaluated ROS levels in PC12 and PC12-NEUROD6 cells upon rotenone exposure by live-cell imaging confocal microscopy using the redox-sensitive dye MitoSOXTM. As a control, cells were treated with the complex III inhibitor, antimycin A (500 ng/ml), known to increase $O_2^{\cdot-}$ levels in PC12 cells [46, 47]. As expected, antimycin A-treated PC12 cells showed significantly increased $O_2^{\cdot-}$ levels, while treated-PC12-NEUROD6 cells displayed negligible $O_2^{\cdot-}$ levels (Fig. 8), in keeping with NeuroD6 conferring tolerance to oxidative stress through a comprehensive antioxidant response [15]. In contrast, rotenone exposure did not provoke an increase in $O_2^{\cdot-}$ levels in PC12 cells even after 24 hours (Fig. 8), which resulted in more than 60% of cell death (Fig. 5). Similarly, PC12-NEUROD6 cells displayed negligible $O_2^{\cdot-}$ levels after 24 hours of rotenone exposure (Fig. 8). Collectively, these results make it unlikely that rotenone compromised cell survival through elevated levels of superoxide anions.

Discussion

This study documents for the first time a neurogenic modulation of mitochondrial biogenesis and bioenergetics by the bHLH differentiation factor NeuroD6, which induces neuronal differentiation and cell cycle exit of neuronal progenitor cells during brain development [16, 17]. We report three novel roles for NeuroD6: first, it promotes mitochondrial biogenesis during the early stages of neuronal differentiation. Second, NeuroD6 enhances the bioenergetic capacity of the neuronal-like PC12-NEUROD6 cells by augmenting the mitochondrial membrane potential and ATP levels produced by OXPHOS, thereby generating an energetic reserve. And third, NeuroD6 confers tolerance to the mitochondrial stressor, rotenone, by inducing an adaptive bioenergetic response, which consists of maintaining the mitochondrial membrane potential and ATP levels, while preserving the integrity of the actin network.

At present, the question of how and which neuronal-specific transcription factors tailor mitochondrial biogenesis and bioenergetics to the need of developing neurons has remained largely unexplored, as most transcriptional studies have been conducted in the context of adipocyte and muscle cell differentiation [48]. These studies revealed that the ubiquitously expressed and nuclear-encoded transcriptional coactivator peroxisome proliferator-activated receptor- γ coactivator 1 (PGC-1) and the transcriptional regulators NRF-1, NRF-2 (GABP) manipulate the nucleo-mitochondrial interactions to regulate mitochondria biogenesis and bioenergetics [49]. Recent studies have revealed NRF-1-mediated regulation of the expression of all ten nuclear-encoded subunits of the respiratory complex IV in mature neurons [50–52].

However, a gap in our knowledge remains about the transcriptional regulator(s) coupling of mitochondrial biogenesis and bioenergetic to the outset of neuronal differentiation. In this study, we took advantage of our neuronal-like PC12-NEUROD6 cell line, in which NeuroD6 expression spontaneously recapitulates the first four stages of neuronal differentiation and coordinates increase of the mitochondrial biomass with cytoskeletal rearrangement and a gradient of microtubule-binding proteins compatible with mitochondrial trafficking during axonal outgrowth [13]. Although the PC12-NEUROD6

cellular paradigm has inherent limitations, such as the lack of lineage diversity, it presents the clear advantage of providing a homogeneous experimental platform to study NeuroD6 contribution for linking mitochondrial homeostasis to neuronal differentiation while circumventing the well-established functional redundancy with the other members of the NeuroD family, NeuroD, NeuroD2, and NeuroD4 [16, 17].

Although our previous study has highlighted NeuroD6 ability to influence mitochondrial mass at the outset of neuronal differentiation prior to neurite outgrowth [13], the question of whether NeuroD6 directly affects mitochondrial biogenesis was not addressed. In this study, we report that NeuroD6 concomitantly increases the mtDNA content and the expression levels of the TFAM regulator, known to be essential for *in vivo* mitochondrial biogenesis and maintenance of the mtDNA copy number [8, 9]. The significance of our finding is two-fold: 1) neuronal mitochondrial biogenesis has not been the subject of extensive studies and therefore its molecular mechanism remains unknown; and 2) manipulation of mitochondrial biogenesis may prove clinically relevant to alleviate mitochondrial defects associated with specific neurodevelopmental disorders and neurodegenerative diseases [53]. Considering that developing neurons are critically dependent on mitochondria to provide optimal ATP levels [4], the integrative role of NeuroD6 for concurrently regulating neuronal differentiation and mitochondrial biogenesis is advantageous to tailor at the transcriptional level the bioenergetic requirement to specific developmental stages during neurogenesis.

The present findings that NeuroD6 modulates the mitochondrial membrane potential and ATP levels produced by OXPHOS are congruent with our published results of increased expression of bioenergetic-related genes upon NeuroD6 expression during the early stages of neuronal differentiation [14] and the preponderance of mitochondria with an elongated morphology in PC12-NEUROD6 cells [13, 15], characteristic of energized mitochondria [54, 55]. Synchronization between enhanced mitochondrial membrane potential and neurite outgrowth has also been demonstrated in immortalized hippocampal neuroblasts [56] and upon local NGF signaling in axons of cultured embryonic chick sensory neurons [23]. Finally, our collective findings of neuronal-specific transcriptional regulation of mitochondrial bioenergetics and biogenesis are particularly pertinent in light of the recent evidence that differential mRNA levels of OXPHOS components are most likely the results of a coordinated tissue-specific transcriptional regulation [57] and that half of the mammalian mitochondrial proteome is tissue-specific [58].

NeuroD6-mediated tolerance to the mitochondrial stressor rotenone may be due to the increased baseline of the mitochondrial membrane potential and ATP levels in PC12-NEUROD6 cells prior to exposure to the stressor. It may also result from the fact that NeuroD6 prevented a mitochondrial bioenergetic collapse by sustaining ATP levels accompanied by a transient and mild decline of the mitochondrial membrane potential. Similar transient bioenergetic decline has been reported in response to changes in energy metabolites without resulting in cell death [59]. NeuroD6-mediated tolerance to mitochondrial stressor is in keeping with our previous studies showing NeuroD6 maintaining ATP levels and mitochondrial mass and integrity via the anti-apoptotic Bcl2 response after growth factor removal to promote cellular survival [15, 19]. Finally, the observation that rotenone-induced apoptosis of PC12 cells under the conditions used in this study is the result of ATP deficiency instead of oxidative stress is congruent with the recently formulated ATP limitation hypothesis for the rotenone model in the context of high ATP demand [60] and chronic activation of NMDA glutamate receptors in cultured rat cerebellar granule neurons [61]. It is also consistent with the emerging view that mitochondrial impairment may be a pivotal contributor to neurodegenerative diseases and aging, independently to oxidative stress [62]. Finally, the lack of superoxide anions in rotenone-treated PC12 cells may be a consequence of high levels of ROS detoxifying

enzymes, such as SOD1, SOD2 and Gpx1, along with recently identified powerful suppressors of ROS, such as PGC-13 and PINK1 [15, 19, 63–66]. It is also plausible that in PC12 cells electrons may predominantly enter at the level of complex III instead of complex I, which combined with their intrinsic anti-oxidant response may in part result in the lack of detected rotenone-mediated ROS, in contrast with high levels of generated ROS in the presence of antimycin A, a specific inhibitor for complex III.

Finally, NeuroD6 conferred protection to rotenone exposure by preserving the actin network in PC12-NEUROD6 cells, consistent with the role of NeuroD6 in favoring polymerization of the actin network via increased expression of several actin-remodeling proteins [13, 14]. This is relevant in the context of NeuroD6 differentiation properties [13, 16–18, 20] as the impact of rotenone on the cytoskeletal network is further influenced by the degree of neuronal differentiation. Rotenone disrupts actin dynamics in differentiating cultured E18 hippocampal neurons, thereby preventing axonal outgrowth [41], whereas it induces depolymerization of the microtubule network in mature dopaminergic and serotonergic neurons [67–69]. NeuroD6-mediated protection against rotenone-induced mitochondrial bioenergetic deficit may prove relevant to autism in light of recent studies highlighting the contribution of the environmental neurotoxin rotenone to autism pathogenesis, as expression levels of complex I are diminished in the frontal cortices of autistic children in keeping with the increased frequency of complex I deficiency among autistic patients with defective oxidative phosphorylation, thereby exacerbating vulnerability of cortical neurons to rotenone [70, 71].

Conclusions

In summary, our findings demonstrate two novel roles for NeuroD6 in a neuronal-like context: 1) it stimulates mitochondrial biogenesis by concomitantly increasing mtDNA content and the key mitochondrial-specific regulator TFAM; and 2) it enhances mitochondrial bioenergetic functions by increasing expression levels of specific subunits of the respiratory complexes of the electron transfer chain, resulting in augmented mitochondrial membrane potential and levels of ATP produced by oxidative phosphorylation. This study combined with our previous studies [13–15] has strengthened the concept of NeuroD6 behaving as a pivotal coordinator to tailor mitochondrial biogenesis and bioenergetics with onset of neuronal differentiation to meet the energy demands. Thus, it is highly plausible that any defective coordination during neurogenesis may cause abnormal neuronal differentiation and cell migration and therefore abnormal neuronal circuitry resulting in neurodevelopmental disorders. Elucidating the *in vivo* interplay between NeuroD6 and mitochondrial biogenesis during embryonic neurogenesis will be an important direction in the future, which will ultimately provide novel insights on the molecular mechanisms responsible for many neurodevelopmental disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

bHLH	basic helix-loop-helix
ETC	electron transfer chain
NADH	nicotinamide adenine dinucleotide ubiquinone oxidoreductase
OXPHOS	oxidative phosphorylation
PGC-1	peroxisome proliferator-activated receptor- γ coactivator 1
ROS	reactive oxygen species

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Highlights

1. NeuroD6 induces mitochondrial biogenesis in neuroprogenitor-like cells
2. NeuroD6 augments the bioenergetic reserve of the neuronal PC12-NeuroD6 cells
3. NeuroD6 increases the mitochondrial membrane potential and ATP levels
4. NeuroD6 confers tolerance to rotenone via an adaptive mitochondrial response

Fig. 1. NeuroD6 expression modulates mitochondrial biogenesis

(A) NeuroD6 increases mtDNA content. MtDNA copy number was measured by qPCR from PC12 and PC12-NEUROD6 (referred to as NEUROD6) cells and normalized by $\Delta\Delta C(t)$. The data are expressed as mean $\pm$ S.D. ($n=3$; $*p<0.001$). (B) Confocal micrographs of PC12 and NEUROD6 cells stained for DNA (red) and the nuclear counterstain DAPI (blue). Images were acquired to visualize mtDNA instead of nuclear DNA. Scale bar: 10 μ m. (C) Immunoblot analysis showing increased expression of TFAM protein in PC12-NEUROD6 cells. The top panel shows a representative western blot with GAPDH as a loading control, while the bottom panel illustrates the quantification analysis of the nuclear and mitochondrial forms of the TFAM protein. Results are expressed as $\pm$ S.D. ($n=3$; $*p<0.05$). (D) Confocal micrographs of PC12 and NEUROD6 cells at distinct stages of neuronal differentiation stained for TFAM (green). Images were acquired to visualize mitochondrial TFAM instead of nuclear TFAM. Scale bar = 10 μ m. The arrow indicates TFAM enrichment at the tip of growing neurites.

Fig. 2. Increased expression levels of specific subunits of respiratory complexes and ATP levels produced by oxidative phosphorylation in PC12-NEUROD6 cells

(A) Western blots were performed using mitochondrial-enriched fractions isolated from PC12 and PC12-NEUROD6 cells with the α subunit of ATP synthase (complex V) as a loading control. (B) Quantification analysis of the immunoblot analyses. Results are expressed as $\pm$ S.D. ($n=3$; $*p<0.05$). (C) The relative ATP content in PC12 and PC12-NEUROD6 (NEUROD6) cells was examined by a fluorescent probe, magnesium green (Mg Green), whose fluorescence intensity is inversely correlated to the intracellular ATP level. Representative live cell confocal images from cells untreated or treated with oligomycin (4 μ g/ml). Scale bar = 10 μ m. (D) Flow cytometry analysis of intracellular ATP levels in Mg-Green labeled PC12 and NEUROD6 cells. Results are expressed as $\pm$ S.D. ($n=5$; $*p<0.05$).

Fig. 3. NeuroD6 expression increases the mitochondrial membrane potential ($\Delta\Psi_m$) of PC12-NEUROD6 cells

(A) Quantitative analysis of $\Delta\Psi_m$ by live cell confocal fluorescence microscopy. PC12 and PC12-NEUROD6 cells were labeled with the TMRM dye in the absence or presence of the uncoupler FCCP (5 μ M). The left-hand panel shows representative confocal images of TMRM-labeled PC12 and PC12-NEUROD6 (NEUROD6) cells. Merge panels show the overlay between TMRM labeling (red) and the corresponding DIC image. Scale bar = 10 μ m. The right-hand panel illustrates the quantification analysis of the ratio of F_m/F_c for untreated or FCCP-treated PC12 and NEUROD6 cells. Results are expressed as normalized mean of the ratio F_m/F_c to the untreated control PC12 cells $\pm$ S.D. ($n=3$; at least 60 cells counted for each cell type and experimental condition; * $p = 0.0473$ as compared to untreated PC12 cells). (B) Quantitative flow cytometry analysis of $\Delta\Psi_m$ using TMRM-labeled PC12 and PC12-NEUROD6 cells. The left-hand panel shows a representative histogram of TMRM-labeled PC12 and NEUROD6 cells. The right-hand panel shows results of the quantification analysis of PC12 (white) and NEUROD6 (black) cells. Results are expressed as the mean fluorescence intensity relative to PC12 cells $\pm$ S.D ($n=4$; * $p = 0.0005$).

Fig. 4. Ratiometric analysis shows NeuroD6-mediated increase of the mitochondrial membrane potential ($\Delta\Psi_m$) in PC12-NEUROD6 cells

(A) Representative confocal images of PC12 and PC12-NEUROD6 (NEUROD6) cells labeled with JC-10 (4 μ M). Emissions at 535 nm (monomers, green, depolarized) and 590 nm (JC-10 aggregates, red, polarized) were simultaneously recorded. Merge panels show the overlay between JC-10 monomers and aggregates. Scale bar = 10 μ m. (B) Ratiometric quantification of the F_r/F_g ratios representing $\Delta\Psi_m$ fluctuations in PC12 and NEUROD6 cells. (C) The left-hand panel depicts a representative heat map of the F_r/F_g ratio using pseudo-colors from JC-10 labeled PC12 and NEUROD6 cells. Mitochondria with a low F_r/F_g ratio (between 0.1 and 0.8 arbitrary units) are depicted in blue-green, while mitochondria with a high F_r/F_g ratio (above 1.0 arbitrary units) are illustrated in yellow-red, as indicated by the key for pseudo-colors. Scale bar=10 μ M. The right-hand panel illustrates the distribution of F_r/F_g ratios in JC-10 labeled PC12 and PC12-NEUROD6 cells. The F_r/F_g ratio of the mitochondria was measured in PC12 (dotted line) and PC12-NEUROD6 (solid line) cells from three independent experiments with $n = 1300-1500$ mitochondria for each cell type. The heat-map shown below indicates increments of arbitrary units for F_r/F_g ratio with corresponding pseudo-colors.

Fig. 5. NeuroD6 confers tolerance to the mitochondrial stressor rotenone

Cell survival was assessed by flow cytometry using fluorescent microspheres and cells labeled with CellTracker™ Green (2 μ M). PC12 (dotted line, triangles) and PC12-NEUROD6 (NEUROD6; solid line, squares) cells were grown in the presence of rotenone (1 μ M) for a period of 6, 24, or 48 hours prior to incubation with 5.0×10^5 red fluorescent microspheres prior to flow cytometry analysis. Results are expressed as percent of control (t=0) for PC12 and PC12-NEUROD6 cells $\pm$ S.D. (n=3).

Fig. 6. NeuroD6 expression prevents rotenone-induced $\Delta\Psi_m$ loss while sustaining ATP levels (A) Quantification analyses of the F_r/F_g ratio in rotenone-treated PC12 cells. The left-hand panel shows representative heat map-formatted confocal images of the F_r/F_g ratio in PC12 cells after rotenone exposure for the indicated periods of time. Scale bar = 10 μm . Decrease in the F_r/F_g ratio is illustrated by a shift from yellow-green-colored mitochondria to blue-colored mitochondria with the corresponding heat map scale of F_r/F_g ratios shown below. The right-hand panel represents the corresponding quantification analysis of the F_r/F_g ratio for rotenone-treated PC12 cells. Results are expressed as the percent control $\pm$ S.D. from three independent experiments ($n = 123 - 200$ cells for each cell type and experimental condition; $*p < 0.05$ and $**p < 0.001$ as compared to PC12 cells $t=0$). (B) Quantification analyses of the F_r/F_g ratio in rotenone-treated PC12-NEUROD6 cells. The left-hand panel shows representative heat map-formatted confocal images of the F_r/F_g ratio in PC12-NEUROD6 (NEUROD6) cells after rotenone exposure for the indicated periods of time, with the corresponding heat map scale of F_r/F_g ratios below. The right-hand panel represents the corresponding quantification analysis of the F_r/F_g ratio in rotenone-treated PC12-NEUROD6 cells after 6, 24, and 48 hr of treatment. Results are expressed as the percent control $\pm$ S.D. from three independent experiments ($n = 123 - 200$ cells for each cell type and experimental condition; $*p < 0.05$ as compared to control NEUROD6 cells). (C) Quantitative flow cytometry analysis of relative ATP levels in rotenone-treated PC12 and PC12-NEUROD6 cells. Results are expressed as the mean fluorescence intensity normalized against untreated PC12 $\pm$ S.D. ($n = 5$; $**p < 0.001$ as compared to untreated PC12 cells; $\ddagger p <$

0.05 as compared to untreated NEUROD6 cells). ATP levels were only assessed in NEUROD6 cells after 48 hours of rotenone treatment due to the massive PC12 cell death (Fig. 5).

Fig. 7. NeuroD6 expression prevents disruption of the actin network upon rotenone exposure
 (A) Time course analysis of the polymerized actin and microtubule networks in rotenone-treated PC12 and PC12-NEUROD6 cells. Cells were labeled with phalloidin-488 (green; left column), anti- β III tubulin (red; middle column), and DAPI (blue) following tubule extraction to exclusively detect polymerized microtubules. The right columns show the overlay between phalloidin, β III-tubulin and DAPI labeling. (B) Quantitative analysis of the microtubule and F-actin network before and after six hours of rotenone exposure. Results are expressed as the mean of fluorescence intensity normalized to untreated cells $\pm$ S.D. from 50–70 cells and three independent experiments (** $p < 0.001$ as compared to untreated PC12 cells).

Fig. 8. Levels of superoxide anions do not increase upon rotenone exposure in PC12 and PC12-NEUROD6 cells

(A) Live-cell confocal fluorescence microscopy on PC12 (left-hand panels) and PC12-NEUROD6 (NEUROD6; right-hand panels) cells in the absence ($t=0$; control) or presence of the mitochondrial stressors, rotenone (1 μ M) and antimycin A (500 ng/ml) for indicated periods of time. Untreated cells (top rows), rotenone-treated cells (2nd and 3rd rows), and antimycin A-treated cells for 24 hr (4th rows) were labeled with MitoSOX (red) and the nuclear counterstain Hoechst 33342 (blue). Scale bar = 10 μ m. (B) Quantification analysis of superoxide anions levels upon rotenone or antimycin A exposure. PC12 (white) and PC12-NEUROD6 (black) cells were untreated (control) or treated with rotenone or antimycin A for indicated periods of time before MitoSOX labeling. Results are expressed as the mean fluorescence intensity normalized against untreated PC12 cells $\pm$ S.D. from 150 cells for each cell type, duration of treatment and three independent experiments (* $p<0.05$ as compared to untreated PC12 cells).