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α -Synuclein and mitochondrial bioenergetics regulate tetrahydrobiopterin levels in a human dopaminergic model of Parkinson disease

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Abstract

Parkinson disease (PD) is a multifactorial disease resulting in preferential death of the dopaminergic neurons in the substantia nigra. Studies of PD-linked genes and toxin-induced models of PD have implicated mitochondrial dysfunction, oxidative stress, and the misfolding and aggregation of α -synuclein (α -syn) as key factors in disease initiation and progression. Many of these features of PD may be modeled in cells or animal models using the neurotoxin 1-methyl-4-phenylpyridinium (MPP⁺). Reducing oxidative stress and nitric oxide synthase (NOS) activity has been shown to be protective in cell or animal models of MPP⁺ toxicity. We have previously demonstrated that siRNA-mediated knockdown of α -syn lowers the activity of both dopamine transporter and NOS activity and protects dopaminergic neuron-like cells from MPP⁺ toxicity. Here, we demonstrate that α -syn knockdown and modulators of oxidative stress/NOS activation protect cells from MPP⁺-induced toxicity via postmitochondrial mechanisms rather than by a rescue of the decrease in mitochondrial oxidative phosphorylation caused by MPP⁺ exposure. We demonstrate that MPP⁺ significantly decreases the synthesis of the antioxidant and obligate cofactor of NOS and TH tetrahydrobiopterin (BH₄) through decreased cellular GTP/ATP levels. Furthermore, we demonstrate that RNAi knockdown of α -syn results in a nearly twofold increase in GTP cyclohydrolase I activity and a concomitant increase in basal BH₄ levels. Together, these results demonstrate that both mitochondrial activity and α -syn play roles in modulating cellular BH₄ levels.

Keywords

α -Synuclein; Tetrahydrobiopterin GTPCH; Parkinson disease Bioenergetics; MPP⁺; DAT; Free radicals

Parkinson disease (PD) is the second most common neurodegenerative disorder, resulting in the motor symptoms of bradykinesia, dyskinesia, and akinesia. The motor symptoms

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associated with the disease are a result of the loss of dopaminergic neurons in the substantia nigra pars compacta (SNPc). α -Synuclein (α -syn), a major component of Lewy bodies, has been implicated in idiopathic and familial PD etiology through both mutations and gene multiplications [1–4]. However, the initiating factor that ultimately leads to selective loss of dopaminergic cells remains elusive in idiopathic PD. However, evidence from a number of cellular and animal models expressing familial PD mutations, as well as the long-established link between PD and environmental toxins, suggests mitochondrial dysfunction as a candidate for one of the earliest perturbations in cell physiology [5,6]. Mitochondrial dysfunction leads to both decreased ATP production and increased generation of reactive oxygen species (ROS), resulting in numerous cellular alterations including an increase in cytosolic dopamine, posttranslation modification of a number of proteins modulating their function, and an increase in misfolding and aggregation of proteins such as α -syn [7,8].

Mitochondrial dysfunction in PD patients may be demonstrated by decreased complex I activity [9,10], decreased Ψ_m , and imbalances in mitochondrial fission and fusion as reviewed by Henchcliffe and Beal [11]. In idiopathic PD, mitochondrial dysfunction may be driven by the accumulation of somatic mitochondrial DNA mutations, which are increased in the aging brain and further still in PD patients and impaired mitophagy [12]. Mitochondrial dysfunction in PD may be modeled using the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or its active metabolite 1-methyl-4-phenylpyridinium (MPP^+) [13–15]. MPP^+ is known to be taken up into cells expressing the dopamine transporter (DAT) and to inhibit mitochondrial complex I, resulting in the production of superoxide and decreased ATP production [16,17]. Complex I inhibition by MPP^+ results in numerous downstream processes, including increased intracellular iron accumulation via IRE-mediated transferrin receptor expression [18], proteasome inhibition [19], and NOS activation [9], ultimately resulting in caspase-mediated apoptosis.

Both oxidative stress and NOS activation have been shown to be required for MPP^+ -induced cell death in various animal and cell models. Both neuronal NOS (nNOS) and inducible NOS-knockout mice show reduced dopaminergic cell loss, and pharmacological inhibition of nNOS with 7-NI significantly attenuated astrocytic infiltration and neurotransmitter depletion, in response to MPTP [20–22]. The activity of the constitutive NOS isoforms (endothelial NOS, eNOS) is regulated primarily by calcium fluxes, mediated by calmodulin, and by the availability of 5,6,7,8-tetrahydrobiopterin (BH4). In the absence of BH4, NOS dimerization is unable to occur, resulting in incomplete electron transfer from the oxidase domain to the reductase domain of the homodimer. This “uncoupling” results in the donation of electrons to molecular oxygen, rather than arginine, and the production of superoxide instead of nitric oxide, resulting in the increased production of peroxynitrite [23]. The pathogenic effects of NOS uncoupling have been well documented, particularly in relation to atherosclerosis and endothelial dysfunction [24]. BH4 is synthesized from GTP in a process regulated by GTP cyclohydrolase I (GTPCH), forming 3,4-dihydroneopterin-3'-triphosphate and ultimately resulting in the conversion of sepiapterin to BH4 by sepiapterin reductase. BH4 is also required as a cofactor for the phenylalanine, tryptophan, and tyrosine hydroxylases, meaning decreases in BH4 levels can result in decreased neurotransmitter production [25]. Furthermore BH4 may also act as an antioxidant, being oxidized to 7,8-dihydrobiopterin (BH2) or further to biopterin (B), and the ratio of reduced to oxidized

biopterins (BH₄:(BH₂ + B)) may serve as a marker of cellular redox status. Oxidized BH₂ may be recycled to BH₄ by the action of dihydrofolate reductase and has been demonstrated to be important for the maintenance of cellular BH₄ levels and maintenance of NOS coupling under conditions of oxidative stress [26,27]. Levels of BH₄ have been shown to be decreased in PD patients and in the MPP⁺ model of PD, although the mechanisms have not been fully investigated [28,29].

We and others have previously shown that reduction of cellular α -syn levels protects cell and animal models from MPP⁺/MPTP toxicity [16,30,31]. Furthermore, we have previously shown that RNAi-mediated knockdown of α -syn is able to reduce the activity of DAT and also to reduce NOS activation in response to MPP⁺ [16,30]. In this study, we seek to understand the mechanisms by which α -syn knockdown confers its protective effects on MPP⁺-treated cells and the role(s) the NOS and tyrosine hydroxylase (TH) cofactor and antioxidant BH₄ plays in this process. We first confirm that RNAi-mediated α -syn knockdown protects BE(2)-M17 human dopaminergic model neurons from MPP⁺ toxicity. We then demonstrate that neither α -syn knockdown nor the presence of antioxidants significantly attenuates MPP⁺-induced mitochondrial impairment, indicating that both of these interventions protect cells from MPP⁺ toxicity by postmitochondrial mechanisms. We subsequently investigated how MPP⁺ and cellular α -syn levels affect the availability of BH₄ in the BE(2)-M17 cell line and demonstrate that, in this cellular model, MPP⁺ decreases BH₄ levels as a result of impaired BH₄ synthesis, as opposed to oxidation of BH₄, with this process driven by reduced mitochondrial ATP production. Reduction of cellular α -syn levels resulted in an increase in basal BH₄ levels driven by an increase in GTPCH activity. The implications of α -syn-mediated regulation of the cofactor and antioxidant, BH₄, in Parkinson disease are discussed.

Materials and methods

All reagents were purchased from Sigma–Aldrich (UK) or Fisher Scientific (UK), unless otherwise stated. All cell culture reagents, including siRNA, were purchased from Invitrogen (UK), unless otherwise stated. All reagents for HPLC analysis were of electrochemical grade. Antibodies were obtained as follows: mouse monoclonal anti- α -syn antibody (clone 4B12), 1:1000 (Covance); polyclonal rabbit anti-DAT antibody, 1:1000, and polyclonal rabbit anti-dopamine receptor 3 antibody, 1:500 (Alpha-Diagnostics); polyclonal rabbit anti-vesicular monoamine transporter 2 (VMAT2) antibody, 1:2000 (Abcam); monoclonal mouse anti-dopamine receptor 2 antibody (clone B-10), 1:250 (Santa Cruz Biotechnology); polyclonal rabbit anti-GIRK2, 1:200 (Alomone Laboratories); and monoclonal mouse anti-GTPCH (clone 4A12), 1:1000 (AbD Serotec).

Cell culture

Human BE(2)-M17 neuroblastoma cells (ECACC No. 95011816) and human SH-SY5Y neuroblastoma cells (ECACC No. 94030304) were obtained from the European Collection of Cell Cultures and used within 20 passages of the original vial. Cells were cultured in Opti-MEM supplemented with 10% fetal bovine serum (FBS; v/v) and 100 U/ml penicillin, 0.1 mg/ml streptomycin, or Dulbecco's modified Eagle's medium (DMEM) supplemented

with 10% (v/v) FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine at 37 °C and in 5% CO₂. Cells were transfected as described [16]; briefly, BE(2)-M17 or SH-SY5Y cells were plated at a density of 3×10^5 cells/well in a six-well plate (plates were coated with 0.1% (w/v) poly-L-lysine; Sigma) and allowed to adhere overnight. For each well to be transfected, 3 µl of Lipofec-tamine 2000 was resuspended in 300 µl of Opti-MEM (OM) and incubated for 5 min at room temperature and then added to siRNAs, SNCA1 or mutated control oligo (MUT12), resuspended in OM (50 nM) [16]. After 5 min, the siRNA and LF2000 mixtures were combined and complexes were allowed to form for 20 min before addition to the cells. After 6 h, 900 µl of OM with 30% (v/v) FBS was added to the wells and the medium replaced after 24 h. MPP⁺ (Sigma) was dissolved in medium supplemented with 5% (v/v) FBS and passed through a 0.22-µm filter. Cells were treated with MPP⁺ 36 h after plating or transfection by aspiration of the medium and replacement with medium containing 5% (v/v) FBS and 10–100 µM MPP⁺. For GTPCH activity, cells were incubated in the presence/absence of 10 mM guanosine for 20 h before harvest [32].

Immunofluorescence

Cells were cultured in 24-well plates containing glass coverslips and allowed to adhere overnight. Cells were fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature and permeabilized with immunofluorescence (IF) block solution for 30 min at room temperature. Primary antibodies were diluted in IF block solution and incubated overnight at 4 °C with gentle shaking. Cells were washed four times in IF wash solution and appropriate AlexaFluor 488 IgG secondary antibodies (Invitrogen) were applied for 45 min at room temperature with gentle rocking and protected from light. Cells were incubated with DAPI diluted 1:2000 in IF wash solution for 10 min and then washed three times. The cells were mounted using Vectashield mounting medium (Vector Laboratories) and imaged using fluorescence microscopy (Nikon). For cell survival assays, cells cultured in 12-well plates were transfected with siRNA and treated with MPP⁺ for 36 h. Cells were washed twice in phosphate-buffered saline (PBS) and either imaged using bright-field microscopy or incubated with propidium iodide (PI) for 30 min and then imaged by fluorescence microscopy. The numbers of adherent cells remaining attached to the plate and the percentage of PI-positive (necrotic) cells were quantified.

Quantification of protein expression

Protein samples were prepared as follows: 5 µg of protein was heated to 95 °C in 1 × Laemmli buffer for 10 min. Proteins were then separated by SDS–polyacrylamide gel electrophoresis at 200 V. Depending on protein size, 10, 12, or 15% resolving gels were used. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) at 100 V for 70 min. Protein molecular weight was estimated using Spectra multicolor broad-range protein ladder (Fermentas). Membranes were blocked in 5% (w/v) nonfat milk diluted in Tris-buffered saline/Tween 20 (TBST) for 1 h at room temperature or overnight at 4 °C. For primary antibody incubation, antibodies were diluted in 1% (w/v) nonfat milk diluted in TBST and membranes incubated for 1 h at room temperature or overnight at 4 °C with gentle shaking. Blots were washed three times with TBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Bio-Rad), diluted 1:5000 in 5% (w/v) nonfat milk, for 1 h at room temperature with gentle shaking. After

being washed, the membranes were imaged using ECL+ (GE Healthcare) on a ChemiDoc XRS system (Bio-Rad) and the optical density of each band was quantified using ImageJ software [33]. For comparative expression assessment, expression was normalized to the intensity of the β -actin band.

^3H -labeled dopamine (^3H]DA) uptake assay

^3H]DA uptake measurements were performed on cells in six-well plates as described previously [30]. BE(2)-M17 cells were washed three times in D-PBS + Ca^{2+} + Mg^{2+} (PBS⁺⁺; Invitrogen). Cells in six-well plates were incubated with 1 ml/well ^3H]DA (GE Healthcare) diluted in PBS⁺⁺ (10 nM) with or without 10 μM mazindol for up to 15 min. After incubation, ^3H]DA solution was aspirated and cells were washed twice in ice-cold PBS⁺⁺ and then lysed in 1 ml of NaOH (1 M). Cell lysate (500 μl) was added to 5 ml of OptiPhase SuperMix (PerkinElmer) and scintillation quantified using a scintillation counter (Beckman Coulter). Results were normalized to protein content measured in a sample of the cell lysate using the BCA assay.

Quantification of intracellular catecholamine content by HPLC

Cells (5×10^5) were harvested into 500 μl HClO_4 (100 mM) containing ascorbate (50 μM) and stored at -80°C until analysis. On the day of analysis, the samples were thawed and homogenized and centrifuged through a 0.22- μm filter (VWR) at 13,000g for 3 min at 4°C . Samples (50 μl) were injected onto an isocratic HPLC system and quantified using an LC-4B electrochemical detector and a carbon working electrode held at +0.7 V vs a Ag/AgCl reference electrode (Decade SDC, Antec). HPLC separation was performed using a 250-mm Microsorb C18 reverse-phase column (Agilent) and a mobile phase comprising methanol (13% v/v), NaH_2PO_4 (120 mM), EDTA (0.8 mM), and sodium octane sulfonate (3.2 mM), pH 3.27, and the flow rate was fixed at 1 ml/min. Catecholamine content was determined relative to authentic, freshly prepared standards and normalized to sample protein content, as determined by BCA assay.

Measurement of mitochondrial oxygen consumption

BE(2)-M17 cells in culture or 48 h post-siRNA transfection were plated at a density of 3×10^4 or 1.5×10^4 for cells/well (for analysis using the Seahorse XF 24 or XF 96 (Seahorse Bioscience), respectively) 24 h before metabolic flux analysis. One hour before the start of the assay, the cells were washed and the medium was replaced with XF assay medium (modified DMEM, pH 7.4; Seahorse Biosciences) supplemented with glucose (25 mM) and sodium pyruvate (2 mM) and incubated at 37°C at atmospheric CO_2 levels. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in cultured BE(2)-M17 cells before the injection of 100 μM MPP⁺ or medium alone (control) using the XF24 or the XF96 analyzer (Seahorse Biosciences). Maximal and minimal OCR was measured in BE(2)-M17 cells treated with 300 nM FCCP or 2 μM oligomycin (injection A) and then with 300 nM FCCP or 3 μM rotenone and antimycin A (injection B). The change in the oxygen consumption rate (OCR %) was monitored for 270vmin post-injection of MPP⁺ in a minimum of six wells ($n=3$). The effects of compounds affecting MPP⁺ uptake and storage or modulators of oxidative stress/NOS were investigated by pretreating cells with dopamine (30 nM), reserpine (10 μM), mazindol (100 μM), L-NAME (1 mM), BH4 (10

μM), or ascorbic acid (100 μM) for 2 h before injection of MPP^+ . OCR was monitored for up to 270 min post- MPP^+ injection in a minimum of four wells (XF24) or six wells (XF96) ($n=3$). All OCR data were normalized as a percentage of baseline OCR at the time point before MPP^+ injection. Curves were fitted using a nonlinear regression analysis using GraphPad Prism 6 (GraphPad Software).

Biopterin quantification by HPLC with electrochemical detection

BH4, BH2, and B levels in cell lysates were determined by HPLC followed by electrochemical and fluorescence detection, as previously described [34]. Briefly, after treatment, cells were harvested, washed three times in PBS, and stored as pellets at -80°C . Cell pellets were resuspended in phosphate-buffered saline (50 mM), pH 7.4, containing dithioerythritol (1 mM) and EDTA (100 μM) and subjected to three freeze-thaw cycles. After centrifugation (5 min at 13,000 rpm and 4°C), the samples were transferred to new, cooled microtubes and precipitated with cold phosphoric acid (1 M), trichloroacetic acid (2 M), and dithioerythritol (1 mM). The samples were vigorously mixed and then centrifuged for 5 min at 13,000 rpm and 4°C . Samples were injected onto an isocratic HPLC system and quantified using sequential electrochemical (Coulchem III, ESA, Inc.) and fluorescence (Jasco) detection. HPLC separation was performed using a 250-mm ACE C18 column (Hichrom) and a mobile phase comprising sodium acetate (50 mM), citric acid (5 mM), EDTA (48 μM), and dithioerythritol (160 μM), pH 5.2 (all ultrapure, electrochemical HPLC grade), at a flow rate of 1.3 ml/min. Background currents of + 500 and $-50\ \mu\text{A}$ were used for the detection of BH4 on electrochemical cells E1 and E2, respectively. BH2 and biopterin were measured using a FP2020 fluorescence detector (Jasco). BH4, BH2, and B levels in samples were determined by comparison with authentic external standards (Shirks, Switzerland) and normalized to sample protein content, as determined by BCA assay.

GTPCH activity assay

GTPCH activity was assessed by the quantification of the conversion of GTP to 7,8-dihydroneopterin, as described in [35]. Cell pellets were resuspended in 300 μl Tris-HCl (100 mM), KCl (300 mM), EDTA (2.5 mM), phenylmethanesulfonyl fluoride (100 μM), and glycerol (10% v/v), pH 7.8, and freeze-thawed three times. After centrifugation (5 min at 13,000 rpm and 4°C) the supernatant was incubated with 10 mM GTP (final volume 300 μl) for 1 h at 37°C . Samples, or authentic neopterin standards, were subsequently incubated with 10 μl HCl (1 M) and 10 μl I/KI solution (1% w/v I in 2% KI) and incubated for 1 h. Samples were centrifuged (2 min at 13,000 rpm) and 10 μl ascorbate (100 mM), 10 μl NaOH (1 M), and calf intestinal alkaline phosphatase (0.32 U) were added to the supernatant (final volume 340 μl). Samples were centrifuged and then assessed using the biopterin electrochemical/fluorescence HPLC system detailed above.

Quantification of intracellular phosphorylated nucleosides

Intracellular GTP, GDP, GMP, and ATP levels were quantified by HPLC with absorbance detection. Nucleosides were extracted from cultured cells (3×10^6 cells/treatment) 24 h post- MPP^+ treatment. Cells were washed three times in ice-cold PBS and deproteinized with 100 μl trichloroacetic acid (1 M). Samples were centrifuged (5 min at 13,000 rpm and 4°C), and the supernatant was removed and supplemented with an equal volume of water

and stored at -80°C until analysis. Before analysis, samples were neutralized with half a volume of $1\text{ M K}_2\text{HPO}_4$. Samples ($100\text{ }\mu\text{l}$) were injected onto an isocratic HPLC system and quantified using absorbance detection. HPLC separation was performed using a 250-mm ACE C18 column (Hichrom) and a mobile phase comprising $50\text{ mM K}_2\text{HPO}_4$ and $50\text{ mM K}_2\text{HPO}_4$, $10\text{ mM tetra-}n\text{-butyl ammonium bromide}$ in 8% (v/v) acetonitrile (pH 7) at a flow rate of 1.2 ml/min . Elution of nucleosides was measured by absorbance at 254 nm using an absorbance reader (Waters 2487 absorbance detector). Nucleoside elution was identified and quantified relative to authentic standards of GTP, GDP, GMP, and ATP (1 mM ; all analytical grade) prepared immediately before analysis and quantified using the peak area using Millennium32 software version 3.2 (Waters). Nucleoside concentration was normalized for protein concentration, as determined by BCA assay.

Results

BE(2)-M17 cells are a dopaminergic cell line expressing markers of neurons vulnerable in PD

The neuroblastoma cell line BE(2)-M17 was characterized and compared to the SH-SY5Y cell line to assess if BE(2)-M17 cells provide a physiologically relevant model of dopaminergic neurons in the SNpc. SH-SY5Y and BE(2)-M17 cell lines were both shown to express DAT, whereas only BE(2)-M17 cells expressed TH, the enzyme responsible for the synthesis of the dopamine precursor L-DOPA (Figs. 1a and c). Furthermore, we confirmed by HPLC with electrochemical detection that undifferentiated BE(2)-M17 cells, but not SH-SY5Y cells, produce dopamine under basal conditions (Fig. 1d). The presence and cellular localization of a number of proteins relevant to PD were confirmed in BE(2)-M17 cells by Western blot (Fig. 1a). It was confirmed that BE(2)-M17 cells express α -synuclein, a protein important in dopamine biology and in modulation of MPP^+ toxicity (Fig. 1c) [36,37]. Furthermore, like the cells specifically vulnerable in the SNpc of PD patients, BE(2)-M17 cells express the dopaminergic marker G-protein-activated inward rectifier potassium channel 2 (GIRK2) (Fig. 1b). Enzymes involved in dopamine synthesis, TH and AADC, as well as VMAT2 and the dopamine receptors D2R and D3R, were shown to be expressed in BE(2)-M17 cells. Activity of the dopamine transporter was confirmed by $[^3\text{H}]\text{DA}$ uptake experiments in the presence and absence of the selective DAT inhibitor mazindol (Fig. 1e).

Given the ability of BE(2)-M17 cells to produce dopamine and express other markers of the dopaminergic neurons that are known to be affected in the SNpc in PD patients, BE(2)-M17 cells were used in preference to SH-SY5Y as a model to investigate the roles α -synuclein plays in modulating MPP^+ -induced mitochondrial dysfunction and also the response of cells to mitochondrial dysfunction.

RNAi-mediated knockdown of α -synuclein protects cells from mitochondrial toxins but does not preserve mitochondrial bioenergetics

Minimal and maximal OCR was monitored in BE(2)-M17 cells sequentially treated with the mitochondrial uncoupling agent FCCP (inducing maximal oxygen consumption), oligomycin (inhibits ATP synthase), and rotenone and antimycin A (which inhibit complexes I and III, respectively). Maximal mitochondrial respiration was determined to lie at

approximately 140% basal OCR levels (Fig. 2a). Nonmitochondrial respiration was determined to be approximately 22% of basal OCR levels. Treatment of BE(2)-M17 with 100 μ M MPP⁺ led to a rapid inhibition of cellular OCR: $t_{1/2}$ = 34.4 min (Fig. 2b). Maximal inhibition of cellular OCR (mitochondrial respiration) was observed after approximately 80 min, after which a plateau effect was observed. No significant decrease in non-MPP⁺-treated cells was observed during this time period. Pretreatment of BE(2)-M17 with the DAT inhibitor mazindol resulted in a significantly attenuated MPP⁺-induced mitochondrial impairment, confirming the DAT dependence of MPP⁺ toxicity (Fig. 2c). The VMAT2 inhibitor reserpine did not significantly alter the kinetics of MPP⁺-induced mitochondrial impairment at the concentration used (10 μ M), indicating that sequestration of MPP⁺ into vesicles is not protective in MPP⁺-induced mitochondrial impairment in this cell culture model.

It has been previously shown that inhibition of oxidative stress and the knockdown of α -synuclein protein levels by RNAi are each able to protect cells from MPP⁺-induced cytotoxicity [16,30,38,39]. We therefore investigated if the mechanism of this protection was through preservation of mitochondrial respiration. RNAi-mediated knockdown of α -syn reduced protein expression by 80% compared to cells transfected with the control oligomer MUT12, which differs from the SNCA1 oligomer only by one base (Fig. 3a and inset) [30]. Knockdown of α -syn resulted in significantly increased cell survival after 48 h MPP⁺ treatment, as assessed by cell counts and fewer necrotic cells, as determined by PI staining (P <0.05; Figs. 3b and c).

Knockdown of α -syn using siRNA did not significantly affect the kinetics of MPP⁺-induced mitochondrial impairment compared to either untransfected cells or cells incubated with the MUT12 control oligomer (Fig. 3d). Preincubation of BE(2)-M17 for 2 h with the NOS inhibitor L-NAME, the antioxidant ascorbic acid, or BH4 (an antioxidant known to be essential for NOS coupling and MPP⁺-induced cell death) did not significantly inhibit MPP⁺-induced decrease in cellular OCR (Fig. 3e). Incubation with modulators of oxidative stress/NOS activity did not inhibit mitochondrial impairment, indicating that oxidative stress and NOS activation/uncoupling does not contribute to the initial mitochondrial impairment observed in the MPP⁺ model.

These results suggest that both antioxidants and α -syn knock-down exert their protective effects on MPP⁺-treated cells after mitochondrial inhibition. It has been previously shown that RNAi-mediated knockdown of α -syn reduces DAT function by approximately 50%; however, it appears that this decreased DAT function does not significantly retard initial MPP⁺-induced mitochondrial inhibition. Given this observation we sought to further investigate the role of α -syn in oxidative stress and NOS activation down-stream of mitochondrial activity.

Mitochondrial dysfunction decreases cellular BH4 synthesis

Given our previous observation that α -syn knockdown attenuates MPP⁺-induced NOS activation [16], we investigated the effects of MPP⁺ on levels of the NOS cofactor and antioxidant, BH4. Treatment of BE(2)-M17 cells with MPP⁺ at both 50 and 100 μ M resulted in a significant decrease in cellular BH4 levels after 24 h and reduced the oxidized biopterin

ratio (BH4:(BH2+B)). A more pronounced decrease in BH4 levels was observed after 48 h (Figs. 4a and d). Intracellular BH4 levels were decreased by approximately 25% after 24 h and 75% after 48 h treatment with 100 μ M MPP⁺ (both $P < 0.001$, compared to untreated cells) (Fig. 4a). The cellular BH4:(BH2 + B) ratio was significantly decreased at both time points, with the ratio decreasing by between 25 and 40% after 24 h and 60 and 75% after 48 h. Surprisingly, given the large decreases in BH4 levels, a concomitant increase in BH2 or biopterin levels was not observed (Figs. 4b, c, and e). This indicates both that the decrease in the BH4:(BH2 + B) ratio was driven solely by a decrease in cellular BH4 levels and that BH4 levels are due to decreased BH4 synthesis and not directly to an oxidative mechanism.

α -Synuclein regulates basal BH4 levels in dopaminergic cells through modulation of GTPCH activity

siRNA-mediated knockdown of α -syn resulted in a significant increase in basal cellular BH4 levels with an increase of approximately 40% observed compared to untransfected cells (Fig. 5a; $P < 0.01$ vs untransfected cells/control oligo). However, knockdown of α -syn did not significantly attenuate the decrease in cellular BH4 levels induced by MPP⁺ treatment (Fig. 5a).

Based on these observations, the mechanism underlying the increase in basal levels of BH4 after α -syn knockdown in cells was investigated. Knockdown of α -syn resulted in a significant increase in basal GTPCH activity, compared to untreated cells and cells transfected with a control siRNA oligomer, by approximately 1.75- and 1.4-fold, respectively ($P < 0.05$) (Fig. 5c), whereas no significant difference was observed in GTPCH activity between untreated cells and cells transfected with a control siRNA oligomer. Treatment of BE(2)-M17 cells with MPP⁺ did not result in significant changes to GTPCH activity, independent of RNAi-mediated knockdown of α -syn. In addition, the levels of GTPCH protein expression were assessed and no significant differences were observed after α -syn knockdown or MPP⁺ treatment, indicating that BH4 levels are regulated directly by changes in enzymatic activity rather than by protein expression (Figs. 5b and d).

Decreased cellular BH4 synthesis is driven by MPP⁺-induced bioenergetics changes

The previously observed MPP⁺-induced decrease in BH4 activity could not be rescued using guanosine, indicating the mechanism of BH4 decrease is downstream of Inosine-5'-monophosphate dehydrogenase (IMPDH) (Fig. 6a). It was confirmed that GTP, ATP, GDP, and GMP could be resolved and quantified using HPLC (Fig. 6b). MPP⁺ was found to induce large (up to 70%) decreases in cellular GTP and ATP levels (both $P < 0.05$ vs non-MPP⁺ - treated cells; Figs. 6c and d). No statistically significant increases were observed in cellular GDP or GMP after MPP⁺ treatment (Figs. 6e and f). Knockdown of α -syn did not significantly alter cellular BH4 levels under basal conditions, or preceding MPP⁺ treatment, indicating that decreasing α -syn levels increases basal BH4 levels through modulation of GTPCH activity rather than through modulation of cellular GTP levels (Figs. 6c–f). In addition, these data show that mitochondrial dysfunction decreases cellular BH4 levels through bioenergetic manipulation, via decreased GTP levels.

Discussion

We have previously reported that knockdown of the Parkinson-associated protein α -syn in neuronal cells confers protection against the neurotoxin MPP⁺ [16,30]. The α -syn knockdown resulted in decreased DAT activity, decreased NOS activity, and decreased cell death, but no change in superoxide production. Here we elucidate the mechanism, using real-time kinetic analysis of mitochondrial bioenergetics, by which α -syn knockdown confers protection from MPP⁺ toxicity. We demonstrate that reduction of cellular α -syn levels results in increased BH4 levels in cells through increased GTPCH activity in a dopaminergic cell line. We find that protection is not conferred through reduced DAT function, MPP⁺ uptake, or maintenance of mitochondrial respiration. Finally, we demonstrate that mitochondrial dysfunction, as induced by MPP⁺, results in decreased BH4 synthesis and not by direct oxidation of BH4 to BH2 or biopterin and that this decrease is independent of modulation of GTPCH enzymatic activity or protein levels or via IMPDH inhibition (summarized in Fig. 7).

The observation of a direct, real-time switch of cells from complex I-dependent oxidative phosphorylation to mitochondrial-independent glycolysis after MPP⁺ treatment is interesting. However, as ATP depletion is a major mechanism of MPP⁺-induced toxicity, glycolysis is clearly unable to meet the ATP requirements of the cell in the absence of oxidative phosphorylation. This is also shown by the acidification of the cell culture medium after MPP⁺ treatment. Indeed, the glycolytic inhibitor 2-deoxyglucose exacerbates ATP loss in MPTP-treated mice [40] and supplementation with D-glucose confers protection from MPP⁺ in an astroglial cell line [41]. We observe that pharmacological inhibition of VMAT2 by reserpine does not affect the kinetics of MPP⁺-induced mitochondrial impairment in this model of MPP⁺ toxicity, although VMAT2 overexpression has, however, been shown in vivo to be protective against MPTP toxicity. Overall, both MPP⁺ dose and uptake kinetics play roles in determining the contribution of MPP⁺ sequestration to ameliorating toxicity. The lack of effect of α -syn knockdown-mediated DAT impairment on MPP⁺-induced mitochondrial inhibition in this model reflects the potency of MPP⁺ rather than the physiological role of α -syn for DAT regulation [30,42,43].

The lack of protection of mitochondrial bioenergetics by α -syn knockdown or by administration of antioxidants agrees with our previous observation that α -syn knockdown does not affect super-oxide levels, as mitochondria are likely to be the main source of superoxide production in the MPP⁺ model. Overall, we conclude that reduction of α -syn expression by RNAi confers protection downstream of the inhibition of complex I by MPP⁺. Although numerous mechanisms have been proposed for MPP⁺ toxicity, modulation of the cofactor and antioxidant BH4 potentially links key processes implicated in the death of dopaminergic neurons in the SNpc. The observed increase in cellular BH4 after α -syn knockdown is potentially protective owing to the roles of BH4 as an antioxidant and in maintenance of NOS coupling. In dopaminergic and noradrenergic neurons the relative demand for BH4 is high because of the presence of tyrosine and tryptophan hydroxylases. In addition, these neurons may be more susceptible to fluctuations in cellular BH4 levels because of increased demand and/or perturbations in oxidative stress and GTP availability, as induced acutely by MPP⁺ in this model. The observed increase in cellular BH4 levels

after α -synuclein knockdown stems from modulation of GTPCH activity although whether this is a result of direct α -syn–GTPCH interaction remains to be investigated.

It has been previously shown that incubation of cells with the iron chelators HBED and deferoxamine was able to attenuate (but not completely inhibit) the MPP⁺-induced BH4 decrease in cells [29]. The implication of these data is that an oxidative mechanism would be responsible for BH4 conversion to BH2/biopterin. However, we demonstrate that oxidation of BH4, driven by mitochondrial ROS or intracellular iron accumulation, is secondary to the ATP/GTP depletion we have shown to occur in the MPP⁺ model. The loss of BH4 synthesis in response to MPP⁺ cannot be rescued by the addition of guanosine to cells, indicating MPP⁺ confers its effects downstream of IMPDH/HGPRT. The observations that GTPCH activity or protein levels are not affected by MPP⁺ treatment mean that the loss of BH4 is a result of inhibition of either the synthesis of the GTPCH substrate GTP or the enzymes that act post-GTPCH: PTPS and sepiapterin reductase (Fig. 7). However, given the previous observation that supplementation of MPP⁺-treated cells with sepiapterin increases BH4 levels and inhibits cytotoxicity [29] and the gross changes in cellular bioenergetics reported in the present study, the most likely source of this inhibition would be the decrease of cellular GTP levels. Together, these data suggest a further biochemical benefit conferred by the reduction of α -syn levels in the upregulation of BH4, a crucial molecule in maintenance of redox control and NOS coupling as well as in the production of the neurotransmitters affected in PD. The apparent convergence of cellular α -syn and BH4 levels in dopaminergic cells indicates an interesting new avenue for future investigation in PD patients and models.

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Abbreviations

PD	Parkinson disease
SNPc	substantia nigra pars compacta
α-syn	α -synuclein
GTPCH	GTP cyclohydrolase I
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP⁺	1-methyl-4-phenylpyridinium
NOS	nitric oxide synthase
DAT	dopamine transporter
BH4	5,6,7,8-tetrahydrobiopterin

BH2	7,8-dihydrobiopterin
B	biopterin
PI	propidium iodide
OCR	oxygen consumption rate
ECAR	extracellular acidification rate
GIRK2	G-protein-activated inward rectifier potassium channel 2

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Fig. 1.

BE(2)-M17 cells are a dopaminergic cell line expressing markers of dopaminergic neurons. (a and b) Representative western blots showing (a) a comparison of relative DAT and TH expression levels in SH-SY5Y and BE(2)-M17 and (b) a number of proteins involved in dopamine synthesis and homeostasis and the dopaminergic marker GIRK2 in BE(2)-M17 cell lysate (5 μg). (c) Immunocytochemistry showing cellular localization of α-syn, DAT, VMAT2, and TH (green) of fixed BE(2)-M17. Cell nuclei were stained with DAPI (blue). (d) Representative HPLC chromatogram showing catecholamine content in BE(2)-M17 (solid line) and SH-SY5Y (dotted line). Cells were harvested in perchloric acid (100 mM) and separated by reverse-phase HPLC with electrochemical detection. Inset: quantification of noradrenalin, L-DOPA, and dopamine content in BE(2)-M17 and SH-SY5Y cells, normalized for protein content ($n=3$) (e) [³H]DA uptake in BE(2)-M17 cells in the presence

or absence of the DAT inhibitor mazindol. Cells were incubated with [^3H]DA (10 nM) for time periods from 0 to 15 min either with or without mazindol (10 μM). Uptake was quantified using scintillation counting and normalized to protein content in each sample ($n=3$, $\pm$ SEM). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Fig. 2. MPP⁺ rapidly induces mitochondrial impairment in BE(2)-M17 cells in a DAT-dependent manner. (a) Stress test of BE(2)-M17 cells using mitochondrial electron transport chain uncoupling agents, identifying minimum and maximal oxygen consumption rates in cells. The OCR was measured in BE(2)-M17 cells treated with 300 nM FCCP or 2 μ M oligomycin (injection A) and then with 300 nM FCCP or 3 μ M rotenone and antimycin A (injection B). OCR was normalized to the OCR in the well immediately before injection (OCR %). Data points represent means of at least six determinations. (b) MPP⁺ rapidly impairs the cellular OCR of BE(2)-M17 cells. Oxygen consumption rate was measured in cultured BE(2)-M17 cells (1.5×10^4 cells/well) before the injection of 100 μ M MPP⁺ or medium alone (control). The change in the oxygen consumption rate (OCR %) was monitored for 270 min post-injection of MPP⁺ in a minimum of six wells. Curves were fitted using nonlinear regression analysis: $t_{1/2} = 34.4$ min for MPP⁺-treated cells, error bars represent $\pm$ SD ($n = 3$). (c) MPP⁺ induced mitochondrial impairment in a DAT-dependent manner. BE(2)-M17 cells were pretreated with dopamine (30 nM), reserpine (10 μ M), or mazindol (100 μ M) for 2 h before injection of MPP⁺. Cellular OCR was monitored for 180 min post-MPP⁺ injection ($n = 3$).

Fig. 3.

MPP⁺-induced mitochondrial impairment is independent of α -synuclein knockdown or manipulation of oxidative stress and NOS activity. (a) siRNA transfection reduces α -syn expression in cells by up to 80%. Knockdown of α -synuclein was assessed by Western blot and expression levels were normalized to actin expression ($n = 3$). (b and c) Knockdown of α -syn protects cells from MPP⁺ toxicity. Cells were transfected with SNCA or control oligo for 36 h before treatment with MPP⁺ (100 μ M) for 48 h. (b) Total cell numbers were assessed by bright-field microscopy. Bars represent mean cell death compared to non-MPP⁺-treated controls ($\pm$ SEM; $n = 3$). (c) Necrotic cells were stained for 30 min with propidium iodide and counted by fluorescence microscopy. Bars represent the mean percentage of propidium iodide-positive cells ($\pm$ SEM; $n = 3$). (d) OCR was measured in BE(2)-M17 cells transfected with an siRNA targeting α -synuclein or a control oligo for 48 h before treatment with MPP⁺ (100 μ M). The effects of MPP on OCR were monitored for 70 min post-MPP⁺ treatment. Data points represent mean OCR values $\pm$ SD of triplicate values obtained from three independent transfections. (e) Attenuation of oxidative stress or NOS activation/uncoupling does not attenuate MPP⁺-induced mitochondrial impairment. BE(2)-M17 cells were preincubated with compounds known to attenuate MPP⁺-induced cell death by inhibiting oxidative stress or NOS activation/uncoupling. BE(2)-M17 cells were pretreated with L-NAME (1 mM), BH4 (10 μ M), or ascorbic acid (100 μ M) for 2 h before cellular OCR was monitored for 90 min in the presence/absence of MPP⁺ (100 μ M; $n = 3$). * $P < 0.05$ vs control oligo, # $P < 0.05$ vs untransfected cells.

Fig. 4.

MPP⁺ induces decreased cellular BH4 levels through reduction of BH4 synthesis. MPP⁺ treatment of cells results in decreased BH4 levels. BE(2)-M17 cells were treated with 50 or 100 μM MPP⁺ for 24/48 h before harvest. Intracellular levels of (a) BH4, (b) BH2, (c) biopterin, and (d) total BH4, BH2, and biopterin (BH_x) were measured by HPLC coupled with both electrochemical and fluorescence detection, and (e) BH4:(BH2 + B) was calculated. Bars represent the mean + SD of triplicate determinations (n = 3) *P < 0.05, **P < 0.01, ***P < 0.001 vs untreated cells.

Fig. 5.

Knockdown of α -synuclein increases basal cellular BH4 levels through increased GTPCH activity. 36 h post-siRNA transfection, cells were treated with 100 μ M MPP⁺ for 48 h before harvest. (a) Cellular BH4 levels are significantly increased by α -syn knockdown as measured by HPLC with electrochemical detection ($n = 3$; + SEM). ** $P < 0.01$ vs untransfected cells/control oligo, # $P < 0.05$ vs untreated cells, ## $P < 0.01$ vs untreated cells. (b) α -Syn knockdown increases basal GTPCH activity. Activity was assessed in cell lysates by incubation with GTP, as described under Materials and methods. Bars represent mean GTPCH activity normalized for protein content by BCA assay, normalized to untreated cells ($n = 5$; + SEM). * $P < 0.05$. (c and d) α -Syn knockdown and MPP⁺ treatment do not significantly affect GTPCH expression in cells. (c) Expression levels of GTPCH in cell lysate (10 μ g), as assessed by Western blot. (d) Densitometric analysis of GTPCH expression relative to actin expression in cell lysates (10 μ g), normalized to untreated cells. Bars represent mean band intensity, relative to actin intensity, normalized to untreated cells ($n = 4$; + SEM).

Fig. 6. MPP⁺ reduces cellular BH₄ synthesis through depletion of cellular GTP/ATP levels. (a) Guanosine supplementation does not rescue MPP⁺-induced BH₄ decreases. Cells were treated with 100 μM MPP⁺ for 48 h with or without the addition of 10 μM guanosine for 20 h before harvest. Bars represent mean BH₄ levels + SD (*n* = 3). (b–f) MPP⁺ treatment disrupts cellular bioenergetics independent of α-synuclein levels. (b) HPLC chromatogram showing elution of indicated phosphorylated nucleosides in untreated and MPP⁺-treated cells, as detected by absorbance at 254 nm. Cellular levels of (c) GTP, (d) ATP, (e) GDP, and

(f) GMP after 24 h MPP⁺ treatment in cells with and without knockdown of α -syn with SNCA oligo ($n = 3$; + SEM). ** $P < 0.01$, *** $P < 0.001$ vs untreated cells.

Fig. 7.

Schematic representation of the interplay between α -syn and tetrahydrobiopterin after MPP⁺ treatment. (1) MPP⁺ enters dopaminergic cells through the DAT transporter and (2) accumulates in mitochondria, inhibiting complex I. Although α -syn knockdown reduces DAT activity and MPP⁺ import, α -syn knockdown does not impair complex I inhibition. (3) Complex I inhibition induces both ROS production and ATP depletion. ATP depletion results in decreased BH₄ synthesis, exacerbating oxidative stress and increasing cell death. α -Syn knockdown increases GTPCH activity under basal conditions resulting in increased BH₄ levels, increasing antioxidant capacity and decreasing cell death.