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1-Methyl-4-Phenylpyridinium-Induced Alterations of Glutathione Status in Immortalized Rat Dopaminergic Neurons

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Abstract

Decreased glutathione levels associated with increased oxidative stress are a hallmark of numerous neurodegenerative diseases, including Parkinson's disease. GSH is an important molecule that serves as an anti-oxidant and is also a major determinant of cellular redox environment. Previous studies have demonstrated that neurotoxins can cause changes in reduced and oxidized GSH levels; however information regarding steady state levels remains unexplored. The goal of this study was to characterize changes in cellular GSH levels and its regulatory enzymes in a dopaminergic cell line (N27) following treatment with the Parkinsonian toxin, 1-methyl-4-phenylpyridinium (MPP⁺). Cellular GSH levels were initially significantly decreased 12 h after treatment, but subsequently recovered to values greater than controls by 24 h. However, oxidized glutathione (GSSG) levels were increased 24 h following treatment, concomitant with a decrease in GSH/GSSG ratio prior to cell death. In accordance with these changes, ROS levels were also increased, confirming the presence of oxidative stress. Decreased enzymatic activities of glutathione reductase and glutamate-cysteine ligase by 20-25% were observed at early time points and partly account for changes in GSH levels after MPP⁺ exposure. Additionally, glutathione peroxidase activity was increased 24 h following treatment. MPP⁺ treatment was not associated with increased efflux of glutathione to the medium. These data further elucidate the mechanisms underlying GSH depletion in response to the Parkinsonian toxin, MPP⁺.

Keywords

MPP⁺; glutathione; Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta. Observed increases in lipid peroxidation and iron accumulation, along with protein and DNA oxidation support the involvement of oxidative stress in PD pathology (Jenner and Olanow, 1996), as do reports of decreased levels of glutathione and other antioxidant defense systems (Buhmann et al., 2004). The degree of glutathione depletion appears to correlate with disease severity and occurs prior to other characteristic changes, such as decreased striatal dopamine levels and diminished

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mitochondrial complex I activity (Jenner, 1998). Several models have shown that depletion of cellular GSH levels by chemical and genetic manipulation sensitizes dopaminergic neurons to oxidative stress (Wullner et al., 1996; Bharath and Andersen, 2005; Chinta et al., 2006a), which can be attenuated by glutathione precursors and thiol reducing agents (Drake et al., 2003; Chinta et al., 2006b). 1-methyl-4-phenylpyridinium (MPP⁺) is the toxic metabolite of MPTP, a merperidine analogue capable of producing a severe Parkinsonian syndrome characterized by many features of PD (Langston et al., 1983). The mechanism of MPP⁺ toxicity is thought to occur by its entry into neurons via the dopamine transporter (DAT) (Javitch et al., 1985), inhibition of complex I of the mitochondrial respiratory chain and consequent production of reactive oxygen species (ROS) levels and ATP depletion (Tipton and Singer, 1993). Because of its ability to selectively damage dopaminergic neurons, MPP⁺ is commonly used as an *in vitro* approach to model cell injury associated with PD (Dauer and Przedborski, 2003). Studies in a variety of *in vitro* models have demonstrated that MPP⁺ decreases glutathione levels in conjunction with increased ROS production (Mithofer et al., 1992; Seyfried et al., 2000; Lee et al., 2003). However, a comprehensive examination of the factors regulating glutathione levels in dopaminergic cells in response to MPP⁺ is lacking in the literature.

Glutathione is the most abundant intracellular non-protein thiol compound involved in a variety of normal physiological functions (Sies, 1999). Importantly, glutathione functions as an antioxidant in the scavenging of free radical molecules and the detoxification of xenobiotics. Additionally, glutathione can be converted between its reduced (GSH) and oxidized (glutathione disulfide, GSSG) forms, thereby forming a specific redox couple important in maintaining the cellular redox environment. Taken together, GSH and GSSG are the most commonly used biomarkers of oxidative stress and indicators of change in the reducing capacity of the cell (Schafer and Buettner, 2001). Maintaining glutathione levels is important in order to preserve reduced protein-thiol groups, transport and store cysteine, and protect against electrophilic intermediates and free radical species (Bharath et al., 2002). While GSH levels are important for cellular protection, a more critical determinant of cellular viability may be the capacity of glutathione regulatory enzymes to maintain steady state levels during times of increased GSH utilization (Meister, 1991; Fernandes et al., 2002). Therefore, it is not surprising that several enzymes exist to maintain glutathione levels in the cell. Both glutathione peroxidase (GPx) and glutathione reductase (GR) are essential in regulating the glutathione redox cycle. The removal of hydrogen peroxide and repair of oxidized protein thiols is catalyzed by GPx by coupling their reduction with the oxidation of GSH. GR is one of several enzymes in the cell that functions to maintain reduced GSH levels and keep GSSG at relatively low levels, thus establishing redox environment and protecting against oxidative stress. Additionally, both *de novo* and scavenging enzymes exist along with transport systems to control cellular levels of GSH. The measurement of steady state levels takes into account both the cellular concentrations of glutathione as well as the contributions of the enzymes and systems regulating these concentrations to gain a more complete understanding of cell processes than either aspect alone.

Since GSH plays an important role in defense against oxidative stress and is implicated in the pathogenesis of PD, it is critical to fully characterize the glutathione response following MPP⁺ treatment in dopaminergic cells. The 1RB3AN27 (N27) dopaminergic cell line derived from rat fetal midbrain neurons expresses DAT required for the uptake of MPP⁺, tyrosine hydroxylase for dopamine synthesis and several other key features characteristic of dopamine neurons (Clarkson et al., 1998). In this study, we investigated the time-dependent effects of MPP⁺ on GSH/GSSG levels and activity of regulatory enzymes involved in their metabolism in the N27 cell dopaminergic cell line. Additionally, measurements of cell toxicity and ROS production were examined and correlated with alterations in cellular glutathione status.

MATERIALS AND METHODS

Chemicals

Cell culture medium, fetal bovine serum and antibiotics were obtained from Invitrogen (Carlsbad, CA). 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) was obtained from Molecular Probes (Eugene, OR). All other materials were obtained from Sigma (St. Louis, MO). N27 cells were a kind gift from Dr. Wenbo Zhou at University of Colorado Health Sciences Center.

Cell Culture and MPP⁺ Treatment

The T-antigen immortalized N27 cell line described previously (Prasad et al., 1994) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml), and 2 mM L-glutamine. Cells were maintained at 37°C in a 5% CO₂ humidified atmosphere. N27 cells were treated with MPP⁺ and collected at 6, 12, and 24 hr time points by scraping in ice-cold PBS buffer and analyzed immediately or frozen at -80°C. For GSH efflux experiments, medium was supplemented with 100 μM acivicin to prevent the enzymatic breakdown of GSH.

Cellular Viability Assay

Release of lactate dehydrogenase (LDH) enzyme activity was used as a measure of cell death. At indicated time points, media samples from treated cells were withdrawn and remaining viable cells were lysed with 0.1% Triton X-100 in Tris/NaCl buffer. LDH activity was measured spectrophotometrically at 30°C as the amount of pyruvate consumed, by monitoring the decrease in absorbance due to the oxidation of NADH at 340 nm (Vassault 1983). LDH values expressed as percentage of activity in the extracellular medium compared to total activity (media + cell lysate).

Measurement of GSH and GSSG via HPLC methods

GSH and GSSG levels were measured by HPLC with electrochemical detection (HPLC-EC) following minor modifications to the method described previously (Lakritz et al., 1997). Analytes were detected using a CoulArray system (Model 5600, ESA, Chelmsford MA) on two coulometric array cell modules, each containing four electrochemical sensors attached in series. Electrochemical detector potentials were 150/300/450/580/700/820 mV vs. Pd. Frozen cell samples were sonicated in 0.1 N perchloric acid prior to thawing to prevent artificial formation of thiol disulfide during preparation. Homogenates were centrifuged at 12,000 × g for 15 minutes at 4°C. Aliquots of supernatant were injected into HPLC and separated on a 5 μm, 150 × 4.6 mm YMC ODS-A column (Waters, Milford MA). Mobile phase was composed of 125 mM sodium phosphate, 1% methanol at pH 3.0 with flow rate of 0.35 ml/min.

For measurement of GSH efflux, media samples at given time points were collected and diluted with an equal volume of KPBS buffer (50 mM potassium phosphate buffer, 17.5 mM EDTA, 50 mM serine, 50 mM boric acid, pH 7.4). Reduced des-gly-glutathione was added as an internal standard to each sample. Samples were then treated with 5 mM monobromobinane and incubated in the dark at room temperature for 30 minutes. 70% perchloric acid was used to terminate the derivatization reaction and samples centrifuged at 16,000 × g for 10 minutes and an aliquot of supernatant was used for analysis. Samples were analyzed on HPLC (Hitachi, San Jose CA) equipped with a fluorometric detector (Model L-2840) and eluted with a Synergi 4 μm Hydro-RP 80A C18 column (150 × 4.6 mm; Phenomenex, Torrance CA) using a mobile phase of 1% acetic acid and 7% acetonitrile, pH 4.25 at a flow rate of 1.0 ml/min. Detector excitation and emission wavelengths were 390 and 480 nm, respectively.

Measurement of glutathione peroxidase and glutathione reductase activities

Frozen cells were sonicated in 0.1% Triton X-100 in 100 mM Tris-HCl buffer, pH 8.0 and centrifuged at $12,000 \times g$ for 15 minutes. Glutathione peroxidase (GPx; EC 1.11.1.9) and glutathione reductase (GR) activities (EC 1.6.4.2) were determined by the oxidation of NADPH at 340 nm, as described (Wendel, 1981; Carlberg and Mannervik, 1985). 100 μ g of protein extract was added in each reaction mixture and activity expressed as units/mg protein and then percent control.

Measurement of glutamate-cysteine ligase and glutathione synthetase activities

Samples were prepared as described above. Glutamate-cysteine ligase (GCL; EC 6.3.2.2) and glutathione synthetase (GS; EC 6.3.2.3) activities were determined by the oxidation of NADH using a coupled assay with pyruvate kinase and lactate dehydrogenase (Zhou and Freed, 2005). The reaction was initiated by adding 100 μ g of protein extract and absorbance at 340 nm was monitored. The specific activity is expressed as units/mg protein and values expressed as a percent of control values.

Measurement of Intracellular ROS levels

H₂DCFDA, which enters and becomes oxidized in the cell to the fluorescent product, 2',7'-dichlorofluorescein (DCF), was used to measure intracellular ROS levels. Following treatment, N27 cells were incubated with 2 μ M H₂DCFDA for 25 minutes in the dark at 37°C. Cells were resuspended in PBS and fluorescence recorded on the FL-1 channel of FACScan (Becton Dickinson, Franklin Lakes NJ). Propidium iodide (PI) was used as a cell viability stain and measured on the FL-2 channel of the flow cytometer. Cells with positive fluorescence for PI, indicating cell death, were excluded from cell counts. Data acquisition and analysis was performed using Cell Quest software (Becton Dickinson).

Statistical Analysis

One-way analysis of variance (ANOVA) with Newman-Kuels multiple comparisons post-test was performed using GraphPad Prism version 4.0 software (GraphPad Software, Inc., San Diego CA). Two-way ANOVA was performed where indicated with the Bonferroni post-test. *p* values < 0.05 were considered statistically significant.

RESULTS

Effects of MPP⁺ on N27 cell viability

A time course and concentration-response of MPP⁺ neurotoxicity was conducted in N27 dopaminergic cells to determine optimal concentrations and time points for glutathione measurement (Fig. 1). LDH enzyme leakage into the media reflects cellular damage and was used as a measure of viability. At indicated time points, enzymatic activity in media samples was compared to the sum of LDH activity from media and cell lysate samples. Significant increases in LDH release were initially observed at 24 h after MPP⁺ additions, but only at concentrations of 300 μ M – 3 mM. As expected, neurotoxicity increased with further MPP⁺ incubation with most concentrations exhibiting significant cell damage at the 48 h time point compared to controls. To minimize any effects due to loss of cell viability, MPP⁺ concentrations of 100 μ M and 1 mM (low and high concentration groups, respectively) with incubation times no greater than 24 h were used for all subsequent experiments.

Effects of MPP⁺ on cellular glutathione levels

GSH and GSSG levels were measured using HPLC-EC methods described in *Materials and Methods*. Surprisingly, a statistically significant increase in GSH levels was observed at 24 h in both 100 μ M and 1 mM MPP⁺ groups (approximately 20% compared to controls, Fig. 2A).

As expected, GSSG increased to a greater extent than GSH. GSSG levels were increased by 70% and 279% following incubation of N27 cells with 100 μ M and 1 mM MPP⁺, respectively at the 24 h time point (Fig. 2B). Concomitant with these changes, GSH/GSSG ratios were decreased 24 h following MPP⁺ in a concentration-dependent manner (Fig. 2C).

Because GSH levels were unexpectedly increased at the 24 h time point, time course experiments were performed to characterize changes in GSH (Fig. 3). Decreases in GSH were initially observed at the 6 h time point, reaching significance after 12 h following incubation with 100 μ M and 1 mM MPP⁺. Two-way ANOVA indicated that time, but not dose had a significant effect on the observed changes in GSH (Time: [F(3,60) = 23.04, p < 0.0001]; Dose: [F(2,60) = 0.66, p < 0.5203]). As described in Fig. 2A, GSH recovered to levels greater than controls by 24 h. As expected, GSSG levels increased while GSH/GSSG ratios decreased steadily throughout the time course (data not shown). However, changes observed at earlier times (6 and 12 h) were lower in magnitude compared with a later time point (24 h), indicating a time-dependent change in redox state following MPP⁺ treatment. These data indicate significant changes in both reduced and oxidized forms of glutathione prior to loss of cell viability. Additionally, decreased GSH/GSSG values suggest that the cellular redox environment becomes more oxidized following MPP⁺ treatment. Lastly, increased levels of GSH at 24 h suggest that an adaptive response may be initiated to overcome oxidative stress.

Effect of MPP⁺ on intracellular ROS levels

To verify the occurrence of MPP⁺-induced oxidative stress in N27 cells, intracellular ROS levels were measured using DCF fluorescence via flow cytometry. Representative histograms showing DCF staining in N27 cells incubated with vehicle or MPP⁺ are shown in Fig. 4A. The percentage of cells staining positive for DCF was significantly greater than controls 6 h after incubation of N27 cells with MPP⁺ (117% and 275% following low and high concentrations, respectively, Fig. 4B). Similar values were also observed at the 12 h time point, but by 24 h a decrease in ROS levels was observed reaching significance in the 1 mM group, although still increased compared to vehicle-treated controls. These data indicate increased levels of ROS following MPP⁺ treatment, confirming the presence of oxidative stress in this model.

Effect of MPP⁺ on glutathione regulatory systems

Significant changes in GSH and GSSG levels (Figs. 2 and 3) suggest an alteration in one or more factors that regulate cellular glutathione levels. Since GSH was initially decreased at early time points (6 and 12 h) following MPP⁺, the role of glutathione efflux from cells under these conditions was examined. Previous *in vitro* studies suggest that MPP⁺-induced efflux of GSH underlies its ability to deplete intracellular GSH (Di Monte et al., 1987; Mithofer et al., 1992; Hirrlinger et al., 2002). The γ -glutamyl transpeptidase inhibitor, acivicin, was added to the cell culture medium to prevent the extracellular breakdown of GSH. Acivicin had no effects on cell viability or changes in cellular glutathione levels at the concentration (100 μ M) used in these experiments (data not shown). No changes in extracellular GSH or GSSG concentrations were observed following MPP⁺ treatment (data not shown). Thus, an efflux mechanism does not appear to account for the observed GSH depletion in this model.

To determine if MPP⁺ altered glutathione regulation, the enzymatic activities of GR and GPx were measured. GR activity was significantly decreased by approximately 20% in both low and high concentration groups at 6 h (Fig. 5A). Decreased activity continued at 12 h in the 100 μ M group, while GR activity was restored to near control levels with 1 mM treatment at the same time. At the 24 h time point after MPP⁺, GR activity recovered to control levels. By contrast, GPx activity remained unchanged at early time points following MPP⁺ treatment (Fig. 5B). However, increased GPx activities of 31% and 39% above controls were observed at the 24 h time point following 100 μ M and 1 mM MPP⁺, respectively. These data suggest a change

in glutathione regulatory enzyme activities during the time course of MPP⁺ incubation that may account for some of the observed changes in glutathione levels.

Effect of MPP⁺ on glutathione synthetic capacity

Activities of the enzymes responsible for glutathione synthesis were measured to determine potential alteration in GSH production capacity by MPP⁺. Two ATP-dependent enzymes sequentially catalyze synthesis: GCL combines glutamate and cysteine to form γ -glutamylcysteine, to which glycine is added by GS to form GSH. Significant decreases in GCL activity were observed 6 and 12 h following incubation with 100 μ M and 1 mM MPP⁺, respectively compared to controls (Fig. 6A). Activities were slightly decreased after 12 h compared to 6 h, while GCL activity recovered to control levels by 24 h following both concentrations. By contrast, no significant changes in GS activity were observed with MPP⁺ treatment (Fig. 6B). These data suggest that a decrease in GCL enzyme activity can affect glutathione synthetic capacities leading to decreased GSH levels in the cell following MPP⁺ treatment.

DISCUSSION

A detailed examination of the cellular glutathione status and the enzymes responsible for maintaining steady state levels in response to the Parkinsonian toxin MPP⁺ in N27 dopaminergic cells show that: (1) MPP⁺-induced decreases in GSH/GSSG redox state were due to initial depletion of GSH as well as increased GSSG levels prior to overt loss of cell viability, (2) MPP⁺ did not alter GSH transport or efflux to the media, and (3) GR and GCL activities were decreased at early time points (6 and 12 h), while GPx activity was increased only 24 h after MPP⁺ incubation.

Initial changes in GSH levels included depletion to approximately 80% of control values 12 h following incubation with low (100 μ M) or high doses (1 mM) of MPP⁺, in parallel with increased GSSG levels. However, after 24 h incubation, GSH levels recovered to values greater than controls, while GSSG continued to increase. As a result of these changes, decreases in GSH/GSSG ratios also occurred indicating a shift towards a more oxidized redox environment. Interestingly, the cause of the observed effects of GSH/GSSG ratios appears to be quite different depending on the duration of MPP⁺ treatment. Our results regarding changes in GSH and GSSG are in accordance with other models of MPP⁺ treatment (Desole et al., 1993; Seyfried et al., 2000), however our study also addressed the enzyme systems regulating such changes.

Initial GSH depletion 6 and 12 h following MPP⁺ treatment could be partly attributed to an increase in GSSG levels over the same time course due to actions of GPx and other peroxidases. However, another mechanism has been proposed in isolated hepatocytes in which MPP⁺ increases cellular GSH efflux (Di Monte et al., 1987; Mithofer et al., 1992). Our data do not show an increase in extracellular GSH at time points corresponding to decreased intracellular levels suggesting alternate mechanism of MPP⁺-induced GSH depletion in neuronal cells. Following MPP⁺ treatment, both GCL and GR activities were decreased at early time points by approximately 20-25% in all but one group (12 h GR activity with 1 mM MPP⁺). Both GR and GCL are important in maintaining reduced glutathione levels; GR acts to recycle GSSG back to the reduced form, while GCL is the rate-limiting enzyme in GSH synthesis. As these enzyme activities are diminished during MPP⁺ treatment, the cell's capacity to produce and maintain GSH is compromised. Therefore, as GSH consumption continues by a variety of mechanisms, its production may not be maintained leading to the observed depletion of GSH. While our study suggests that decreased activities of both regulatory and synthetic enzymes may be a plausible cause of MPP⁺-induced changes in GSH and GSSG, several possibilities contributing to initial GSH depletion not addressed by these experiments may also exist. One

such possibility is the formation of protein-glutathione mixed disulfides. These compounds are often formed as a consequence of treatment with oxidizing agents and play a significant role in the defense against oxidative damage (Hurd et al., 2005). Additionally, MPP⁺ and lipid peroxide products produced by increased ROS levels are substrates for direct reaction with GSH or substrates for conjugation with GSH via the action of glutathione-S-transferases. Each of these events may be contributing to GSH depletion in response to MPP⁺ incubation, in addition to decreases in GR and GCL activities that were observed here.

However, data from the later 24 h time point of MPP⁺ incubation are much different from that discussed at earlier time points (6 and 12 h). GSH levels recover to concentrations 20% greater than controls, therefore indicating an adaptive response by cells to the oxidative stress resulting from toxic insult. Activities of GCL and GR also returned to control levels, while GPx activity was slightly increased. Despite these changes, GSSG levels increased with a corresponding decline in GSH/GSSG ratio which leads to the observed loss of cell viability at this and later time points. As opposed to data from 6 and 12 h in which GSH depletion played a role, the major cause of the change in glutathione status at the 24 h time point may be a drastic increase in cellular GSSG levels.

As expected, ROS levels measured by DCF fluorescence were also increased in N27 cells following MPP⁺ treatment. Additionally, the levels of ROS correspond inversely with changes in GSH over the time course of MPP⁺ incubation. For example, at early time points when GSH is declining, there are increased ROS levels. However, when GSH levels recovered at the 24 h time point, a decrease in ROS levels was observed. There are several potential explanations of this phenomenon. First, the production of ROS may be attributed primarily to MPP⁺-induced inhibition of complex I activity of the mitochondrial electron transport chain and interactions with nitrogen-based radicals produced by nitric-oxide synthase (Dauer and Przedborski, 2003). Complex I contains sensitive thiol groups and iron sulfur clusters (Bharath et al., 2002) and selective inhibition of activity has been demonstrated following GSH depletion in PC12 dopaminergic cell lines (Jha et al., 2000). Therefore, GSH may be acting to 1) scavenge ROS and reactive electrophiles both by enzymatic and non-enzymatic mechanisms, or 2) repair damaged thiols in complex I, thus reducing inhibition of the complex. Both mechanisms would decrease ROS levels, one via anti-oxidant scavenging and the other by reducing the production of ROS, respectively.

An interesting finding of these studies is the effect of acute MPP⁺ treatment on GCL activity. Typically, an up-regulation of GCL mRNA levels and activity is observed in response to oxidative stress (Kondo et al., 1999). However, in this study a transient but significant decrease in GCL activity was observed at early time points following oxidative stress induced by MPP⁺ treatment. Following a prolonged incubation (24 h) of N27 cells with MPP⁺, GCL enzymatic activity recovered to control levels. Recently, several models have demonstrated that over-expression of GCL can protect against oxidative stress (Fernandes et al., 2002; Robertson et al., 2005) further supporting the importance of GSH synthetic processes. Additionally, administration of γ -glutamylcysteine ethyl ester that feeds into the synthetic pathway downstream of GCL was able to provide neuroprotection against MPTP/MPP⁺ toxicity (Chinta et al., 2006b). Therefore, addressing events such as GCL inhibition involved in glutathione depletion may be an effective therapeutic strategy in toxin-induced models of PD.

This study demonstrates the importance of regulatory enzymes in maintaining glutathione levels in dopaminergic cells upon MPP⁺ treatment and subsequent oxidative stress. Prolonged MPP⁺ exposure to cells initiates an adaptive response despite loss of cell viability. This suggests that processes other than oxidative damage may also be contributing to cell toxicity. However, the importance of glutathione in the process cannot be overlooked, as evidenced by the many

studies demonstrating that altered GSH levels can mediate cell death in PD models (Wullner et al., 1996; Bahat-Stroomza et al., 2005; Chinta et al., 2006a; Chinta et al., 2006b). The measure of steady state levels has become an important tool to observe differences between the production and utilization of glutathione to ascertain cellular health and response following oxidative insult. In fact, the cell's capacity to regulate glutathione synthesis and consumption under such conditions may be a more critical determinant of cell viability than glutathione levels alone (Meister, 1991; Fernandes et al., 2002). Therefore, this study provides additional analysis of changes in steady state glutathione levels in response to MPP⁺ treatment in dopaminergic cells. Although caution may be applied in extrapolating results obtained from an immortalized cell line to *in vivo* processes, several key features of this cell line allowed us to mechanistically evaluate MPP⁺ neurotoxicity *in vitro*. In summary, our results provide a detailed analysis of MPP⁺-induced changes in glutathione status in dopaminergic cells and further characterize the neurotoxic mechanism of MPP⁺.

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Figure 1.

Effect of MPP⁺ on N27 cell viability after 24 (■) and 48 (▲) h incubations. Data presented as percentage of media LDH activity compared to total (media + cell lysate) LDH activity versus the log of MPP⁺ concentration (M). Data expressed as mean ± SE. *p < 0.05 versus time-matched controls, one-way ANOVA, n=6 per group.

Figure 2.

Cellular GSH (A), GSSG (B) and GSH/GSSG (C) levels in N27 cells following MPP⁺ treatment for 24 h. Bars represent mean (% control) + SE. Bars with different letters were statistically different from one another ($p < 0.05$, one-way ANOVA, $n=6$ per group).

Figure 3.

Cellular GSH levels in N27 cells following MPP⁺ treatment for 6, 12, or 24 h. Data points for control (■), 100 μ M MPP⁺ (▲), and 1 mM MPP⁺ (▼) represent mean (% control) $\pm$ SE. * p < 0.05 versus controls, two-way ANOVA with Bonferroni post-test, $n=6-7$ per group.

Figure 4.

Intracellular ROS levels measured by DCF fluorescence via flow cytometry in N27 cells following MPP⁺ treatment. (A) Representative histograms of DCF fluorescence in vehicle- and 1 mM MPP⁺-treated N27 cells at 6 h. Numbers represent the percentage of cells with DCF positive staining compared to unlabeled controls. (B) ROS levels in N27 cells following treatment with 100 μM or 1 mM MPP⁺ for 6, 12, and 24 h. Data expressed as percentage of cells staining positive for DCF compared to controls. Bars represent mean (% control) + SE. Bars with different letters were statistically different from one another ($p < 0.05$, one-way ANOVA, $n=3$ per group).

Figure 5. Glutathione reductase (GR, A) and glutathione peroxidase (GPx, B) enzyme activities in N27 cells following MPP⁺ treatment for 6, 12, and 24 h. Bars represent mean (% control) + SE. Bars with different letters were statistically different from one another ($p < 0.05$, one-way ANOVA, $n=3$ per group).

Figure 6. Glutamate-cysteine ligase (GCL, A) and glutathione synthetase (GS, B) enzyme activities in N27 cells following MPP⁺ treatment for 6, 12, and 24 h. Bars represent mean (% control) + SE. Bars with different letters were statistically different from one another ($p < 0.05$, one-way ANOVA, $n=3$ per group).