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Iron mediates neuritic tree collapse in mesencephalic neurons treated with 1-methyl-4-phenylpyridinium (MPP+)

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Abstract Studies in post-mortem tissues of patients with Parkinson's disease (PD) and in mice treated with 6-hydroxydopamine have shown a decrease in the length of axon and dendrites of striatal neurons. However, the etiology of the morphological changes and their relationship to inhibition of mitochondrial complex I and the cellular levels of iron and glutathione (GSH) have not been described. In this study, we characterized the effect of MPP+, an inhibitor of mitochondria complex I, on the integrity of the neuritic tree of midbrain dopaminergic neurons, and determined the influence of iron and cellular levels of GSH on this degeneration. Sub-maximal concentrations of MPP+ induced a drastic dose-dependent reduction of neurites, without modification of the soma or apparent cell death. Concurrent treatment with MPP+ and non-toxic concentrations of iron accelerated the process of degeneration, whereas neurons grown on a medium low in iron showed enhanced resistance to MPP+ treatment. MPP+-induced neurite shortening depended on the redox state of neurons. Pre-treatment with the general antioxidant *N*-acetyl cysteine protected neurons from degeneration. Treatment with sub-maximal concentrations of the inhibitor of GSH synthesis buthionine sulfoximine (BSO), in conjunction with iron and MPP+, produced massive cell death, whereas treatment with BSO plus MPP+ under low iron conditions did not damage neurons. These results suggest that under conditions of inhibition of mitochondrial complex I caused by MPP+, the accumulation of iron and the concurrent decrease in GSH results in the loss of the dendritic tree prior to cell death, of dopaminergic neurons in PD.

Keywords Iron · Parkinson · Glutathione · Complex I · Antioxidants

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Introduction

Most neurons have elaborate dendritic trees that receive thousands of excitatory and inhibitory synaptic stimulus, which are integrated to generate action potentials (Gulledge et al. 2005; Johnston et al. 1996; Vetter et al. 2001). Given their enormous functional importance, it has been postulated that dendrite fragmentation represents an early manifestation of damage that ends up in neuronal death (Fiala et al. 2002; Suzumura et al. 2006).

A number of neurodegenerative diseases involve atrophy in dendrites and axons (Baloyannis 2009; Baloyannis et al. 2006; Takeuchi et al. 2005). In genetic models of Alzheimer's disease, the β-amyloid peptide induces a selective diminution in the size of the dendritic tree in neurons of the cortex and the dentate gyrus (Capetillo-Zarate et al. 2006). Studies in post-mortem tissue of patients with PD and in mice injected with 6-hydroxydopamine, a neurotoxin used as a model of PD, showed that both the length of the dendrites and the density of the synaptic spines diminish significantly in neurons of the prefrontal cortex, the putamen and caudate nucleus (McNeill et al. 1988; Solis et al. 2007; Stephens et al. 2005; Zaja-Milatovic et al. 2005). The molecular mechanism

responsible for the change in the dendritic architecture is unknown, albeit decreased dopaminergic stimuli generated by neurons of the substantia nigra have been postulated as a causative agent (Zaja-Milatovic et al. 2005). Reduction in both the total length of the dendrites and the density of spines is likely to have a deleterious impact on the ability of these neurons to establish synapses, explaining in part the symptoms of the disorder.

Pathognomonic signs of dopaminergic neuron death observed in PD include decreased GSH content and inhibition of mitochondrial complex I (Banerjee et al. 2009; Hattingen et al. 2009; Jenner and Olanow 1998; Mandel et al. 2007; Schapira et al. 1990; Youdim 2008). GSH is the most abundant intracellular reductant in the central nervous system, where it acts as the main antioxidant agent (Meister and Anderson 1983). Initially, post-mortem studies revealed decreased levels of GSH in degenerating substantia nigra of PD patients (Perry et al. 1982; Sian et al. 1994; Sofic et al. 1988). Diminished GSH levels were postulated as an early event in idiopathic PD-associated neuronal death (Fitzmaurice et al. 2003; Jenner and Olanow 2006); the decrease in GSH content establishing a positive feedback loop with complex I inhibition (Chinta et al. 2006; Vali et al. 2007).

Iron accumulation is another element relevant to neuronal death in PD. Iron is an intrinsic ROS producer, so cells must have mechanisms to avoid accumulation of iron above cellular needs. Indeed, in numerous neurodegenerative diseases iron accumulation has been noticed in zones that undergo neuronal death (Berg and Youdim 2006; Perry et al. 2003; Sayre et al. 2000; Weinreb et al. 2010). In particular, iron accumulation has been demonstrated in the melanized dopaminergic neurons of the substantia nigra pars compacta (SNpc) (Kienzl et al. 1999). Taking into account that redox-active Fe^{2+} produces the highly damaging hydroxyl radical (Halliwell 2006), an increase in iron is likely to results in increased oxidative damage. Moreover, there is an inverse relationship between iron and GSH levels: after exposure to increasing concentrations of iron, SH-SY5Y cells undergo sustained iron accumulation and a biphasic change in intracellular GSH levels, increasing at low (1–5 μM) Fe and decreasing thereafter. Cell exposure to high iron concentrations (20–80 μM) markedly decreases the GSH/GSSG molar ratio and the GSH half-cell reduction potential, which results in loss of cell viability (Núñez et al. 2004). The thermodynamic balance of one-electron reactions of iron in the intracellular milieu, indicates that iron in the presence of oxygen and the reductive environment of the cell is a net consumer of GSH and producer of the hydroxyl radical (Borquez et al. 2008).

Another source of free radicals derives from the non-enzymatic oxidation of dopamine induced by iron, which produces semiquinones and H_2O_2 (Paris et al. 2005; Zoccarato et al. 2005). Thus, iron, both through the Fenton reaction or by dopamine oxidation, is a dangerous pro-oxidant agent. Studies on iron accumulation in PD patients have been performed in tissue from patients who have died after the final steps of the pathology. Accordingly, it remains unclear what is the temporal position of iron toxicity during the progression of PD.

The sequel of degeneration that occurs following administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in animals has provided a useful model of PD because it induces pathologies similar to that seen in man (Przedborski and Vila 2003; Smeyne and Jackson-Lewis 2005). After systemic administration, MPTP has the capacity to cross the blood–brain barrier. Once in the brain, the pro-toxin is metabolized in the glial cells to 1-methyl-4-phenyl-2,3-dihydropyridinium by the enzyme monoamine oxidase B and then to the active toxic compound MPP+ (Przedborski and Vila 2003). Thereafter, MPP+ is released from glial cells and selectively taken up by dopaminergic neurons through the plasma membrane dopamine transporter (Mayer et al. 1986). Once in the cell, MPP+ distributed into cellular compartments: it can be sequestered into secretory vesicles via the vesicular monoamine transporter (Del Zompo et al. 1993) or it can enter into the mitochondria where it interferes with complex I of the electron transport chain, lowering levels of ATP and an increasing oxidative stress (Cleeter and Schapira 1992; Nicklas et al. 1987; Scotcher et al. 1990; Wong et al. 1999).

A still unanswered question regards if the increase in oxidative stress produced by inhibition of complex I is sufficient for the collapse of the dendritic tree observed in PD. Under the hypothesis that the loss of the dendritic tree is an early event derived from the inhibition of complex I, in this work we characterized the effects of MPP+, iron, the antioxidant *N*-acetyl cysteine (NAC) and the inhibitor of glutathione synthesis BSO on the integrity of the neurites of mesencephalic neurons in culture. The results reported here show that iron plays a cardinal role as a mediator of the neurotoxic effects of MPP+.

Methods

Mesencephalic cell culture

Mesencephalic cells were prepared as described (Brouard et al. 1992). On day 14 of gestation, pregnant Sprague–Dawley rats were exposed to CO_2 followed by laparotomy. The fetuses were collected in cold L-15 medium and the brains were isolated. The mesencephalic dopaminergic region (A8, A9, and A10 dopaminergic nuclei) was dissected and dispersed by repeated pipetting in DMEM/F12

medium containing 0.1% bovine albumin, 5 mg/ml insulin, 30 nM L-thyroxin, 20 nM progesterone, 30 nM sodium selenite, 100 U/ml penicillin, 100 mg/ml streptomycin, and 5% fetal bovine serum (FBS). Cells were plated on glass cover slips percolated with 1 mg/ml poly-L-lysine at a density of 55,000 viable cells/cm^2. On the first day in vitro (DIV1) the medium was changed and then half of the medium was changed at DIV3, DIV5, and DIV7. Cells were used at DIV7. Treatment of animals complied with the Animal Care and Use guidelines of the Ethics Committee of the Faculty of Sciences, Universidad de Chile, and experiments were approved by the Ethics Committee.

Immunocytochemistry

Immunocytochemistry was performed as described (Mena et al. 2008). Primary antibodies were anti-tyrosine hydroxylase (TH) (mouse monoclonal and rabbit polyclonal, Sigma Chem. Co.), anti-glial fibrillary acidic protein (GFAP) (mouse monoclonal, Promega) anti-MAP1B (goat polyclonal, Santa Cruz Biotechnology), anti-MAP2 (rabbit polyclonal from Santa Cruz Biotechnology), and anti-β3-tubulin (mouse monoclonal, Sigma Chem. Co.). Secondary antibodies (Invitrogen) were anti-mouse IgG labeled with Alexa Fluor 488 and 546, anti-rabbit IgG labeled with Alexa Fluor 488 and 546, and anti-goat IgG labeled with Alexa Fluor 633. The nucleus was stained with TO-PRO-3 (Invitrogen). Cells were viewed in a Zeiss LSM Meta confocal laser scanning microscope (Carl Zeiss, Göttingen, Germany).

Determination of neurite length and soma morphology

The length of the dendritic tree was determined in TH-positive cells as described (Coombs et al. 2007). Soma size and total neurite length were determined with the LSM5 image browser program (Carl Zeiss, Göttingen, Germany). To determine the soma size, a contour line was drawn around each soma and the area enclosed was calculated.

Iron treatment

The regular iron concentration in the culture medium, 7 μM, was taken as the control concentration. DIV7 cultures were treated for 24 h with modified culture medium containing 2, 7, 40, or 80 μM Fe. The 40 and 80 μM Fe conditions were achieved by adding to the culture medium the corresponding amounts of iron in the form of the Fe-NTA complex. Low iron (2 μM) culture medium was obtained by Chelex treatment of the FBS using a variation of a described method (Núñez and Tapia 1999). Briefly, 20 ml FBS was treated with 1.5 g of Chelex-100 in its sodium form and 2.8 g of Chelex in its hydrogen form. The pH was adjusted to 5.5 and the mixture was incubated overnight at 4°C under mild agitation. The serum was collected by centrifugation and the resin was washed twice with 10 ml of saline. The washes were collected and added to the serum. At this stage, FBS was 50% concentrated and contained 10 μM Fe. Since Chelex also binds Cu, Zn, and Mn, the low iron serum was supplemented with $CuCl_2$, $ZnCl_2$, and $MnCl_2$ to reach concentrations of 5 μM, 30 μM, and 15 nM, respectively. The concentration of iron in the different media was corroborated by atomic absorption spectrometry as described (Arredondo et al. 2006).

Statistical analysis

All experiments were performed at least on three independent occasions. At least ten neurons were measured for each treatment. One-way ANOVA was used to test differences in mean values, and Tukey's post-hoc test was used for comparisons (In Stat, GraphPad Prism, San Diego, CA). Results are expressed as mean ± SEM.

Results

Characterization of the mesencephalic cells culture

Primary cultures from day 14 rat embryo mesencephalic cells contain a mixture of neurons and other types of cells. While most of the neurons were positive for the neuronal marker β3-tubulin, some of them were TH-positive dopaminergic neurons (Fig. 1a). The overall morphology of TH-positive cells was either bi- or multi-polar, showing some variations in the shape of the cell bodies, which were mainly fusiform or polygonal. Additionally, some of the longest and minor neurites displayed abundant branching points. The culture contained a substantial amount of astrocytes, as determined by GFAP staining (Fig. 1b) and β3-tubulin-positive, TH-negative neurons. Nuclei staining with TO-PRO3 (blue) afforded an estimate of the total number of cells. TH-positive neurons were also positive for the neuritic marker MAP1B (Fig. 1c) and the dendritic marker MAP2 (Fig. 1d). The branching was not restricted to either MAP1B or MAP2 positive compartments.

The relative abundance of TH-positive neurons was 0.7%, as estimated by the ratio of TH-positive cells to TO-PRO3 positive cells. This value is in accordance with the one reported by Brouard et al. (Brouard et al. 1992) in the original method. Non-dopaminergic neurons corresponded to 62% of the culture and astrocytes to 5%. The rest of the cells was not characterized, probably corresponding, in part, to oligodendrocytes (Engele et al. 1996).

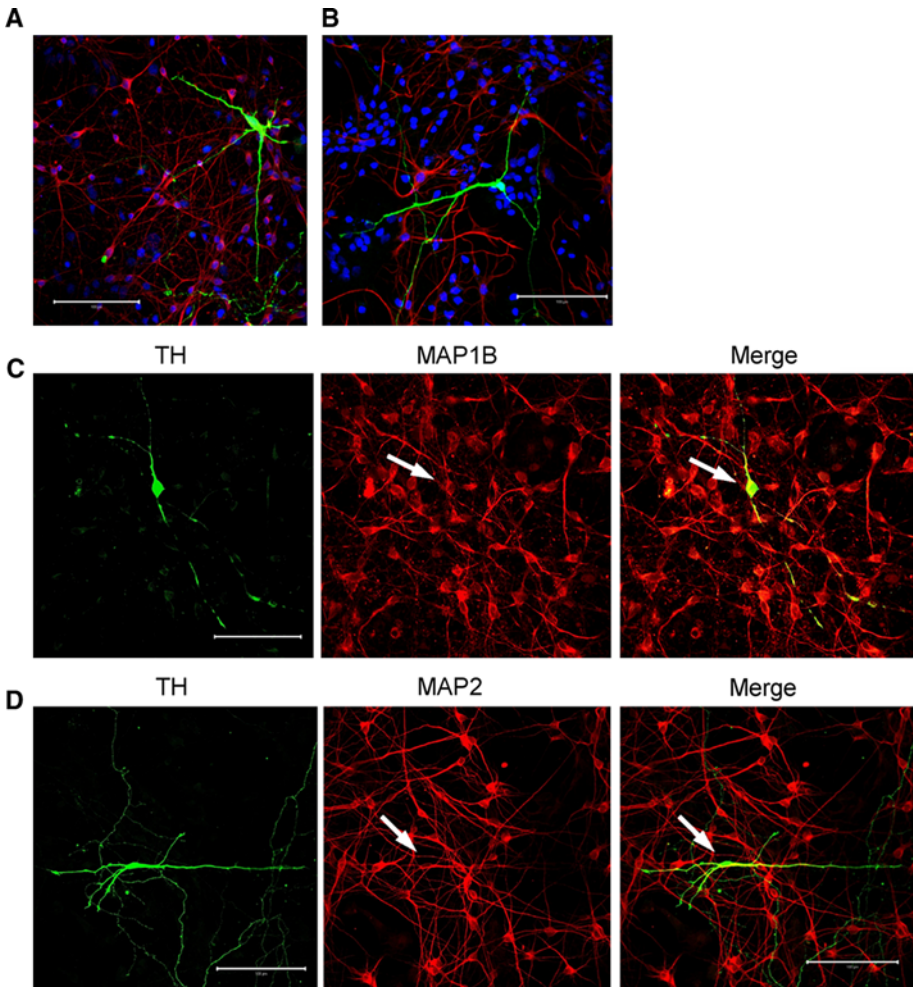


Fig. 1 Immunostaining characterization of mesencephalic cells. **a** Image of a dopaminergic neuron detected with anti-TH antibody (*green*). Other neuronal types were labeled with the axonal marker anti-β3-tubulin antibody (*red*), and nuclei were stained with TO-PRO3 (*blue*). Calibration *bar*: 100 µm. **b** Presence of astrocytes. Dopaminergic neurons labeled with anti-TH antibody (*green*); astrocytes labeled with anti-GFAP antibody (*red*) and nucleus stained with TO-PRO3 (*blue*). Calibration *bar*: 100 µm. **c** Neurites were labeled with anti-MAP1B antibody (*red*) and dopaminergic neurons labeled with anti-TH antibody (*green*). The *arrows* indicate the position of the TH-positive neuron. Calibration *bar*: 100 µm. **d** Co-staining for TH (*green*) and dendritic tree marker MAP2 (*red*). The *arrows* indicate the position of the TH-positive neuron. Calibration *bar*: 100 µm

Determination of the total length of the neuritic tree

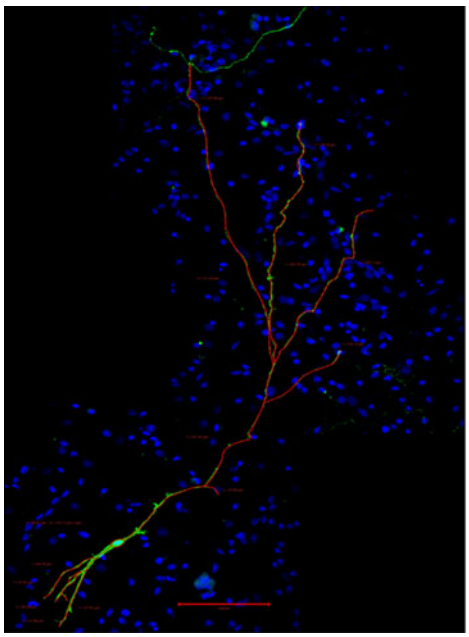
The total neuritic length of TH-positive cells was calculated as the sum of all neurite (axon and neurites) length including primary, secondary (branching from primaries) and tertiary neurites, shown as red lines drawn over the green processes in Fig. 2a. The total length of the neuritic tree of DIV7 dopaminergic neurons was relatively constant, 1,947 ± 90 µm (mean ± SEM; $n = 27$) and the area of the soma was 120 ± 9 µm^2 (mean ± SEM; $n = 23$).

Effect of MPP+ on neurite morphology

MPP+ is neurotoxin known to inhibit mitochondrial complex I and cause dopaminergic cell death. When tested in mesencephalic cell cultures, MPP+ shortened the average length of dopaminergic neurons in a dose and time dependent manner. TH-positive neurons treated with 0.1 µM MPP+ for 24 h showed a drastic shortening of the neuritic tree. Figure 3a shows a control TH-positive neuron, with a long axon and numerous neurites, and a neuron after incubation with 0.1 µM MPP+ showing a decreased dendritic tree. Quantification of the effect revealed that 0.1 µM MPP+ decreased the neuritic tree length to 50 and 65% of control, at 12 and 24 h, respectively (Fig. 3b). This effect was accentuated to 75% decrease by 1 µM MPP+. None of the treatments modified the soma area (Fig. 3c). No indication of cell death of TH-positive neurons was evident as determined by the ratio of TH-positive neurons to overall cells, specified by TO-PRO3 staining. In the experiment shown in Fig. 3, the percent ratio of TH-positive cells/total cells was 0.73 ± 0.03 and 0.67 ± 0.07 for control and MPP+-treated cells, respectively. Similarly, non-dopaminergic neurons did not show any evidence of change in cell morphology, a demonstration of the specificity of the MPP+ treatment.

Combined effects of MPP+ and Fe on dendritic morphology

Iron is elevated in SNpc neurons. Since this elevation is thought to be instrumental in neuronal death, it was of interest to study the combined effects of MPP+ and Fe on neuritic tree morphology. Treatment of 7 DIV primary



	Primary processes	Secondary processes	Tertiary processes
	1012.56	22.89	
		116.16	
		250.13	
		378.05	
	127.81	58.18	21.81
		84.34	12.08
Sum	1140.37	909.75	33.89
Total length	2084.01		

Fig. 2 Total length determination of dendritic processes of dopaminergic neurons. **a** Representative image of a TH-positive neuron (*green*) under control conditions. The image corresponds to a composite of 4 picture frames joined to show the extensive branching of the neuron. Overexposure of soma fluorescence was necessary to detect the milder fluorescence of the processes. Partial lengths, obtained with the LSM5 image browser program, are shown in *red*. Calibration *bar*: 100 μm. **b** Worksheet used to determine total length of the dendritic tree, which in this neuron was 2,084 μm

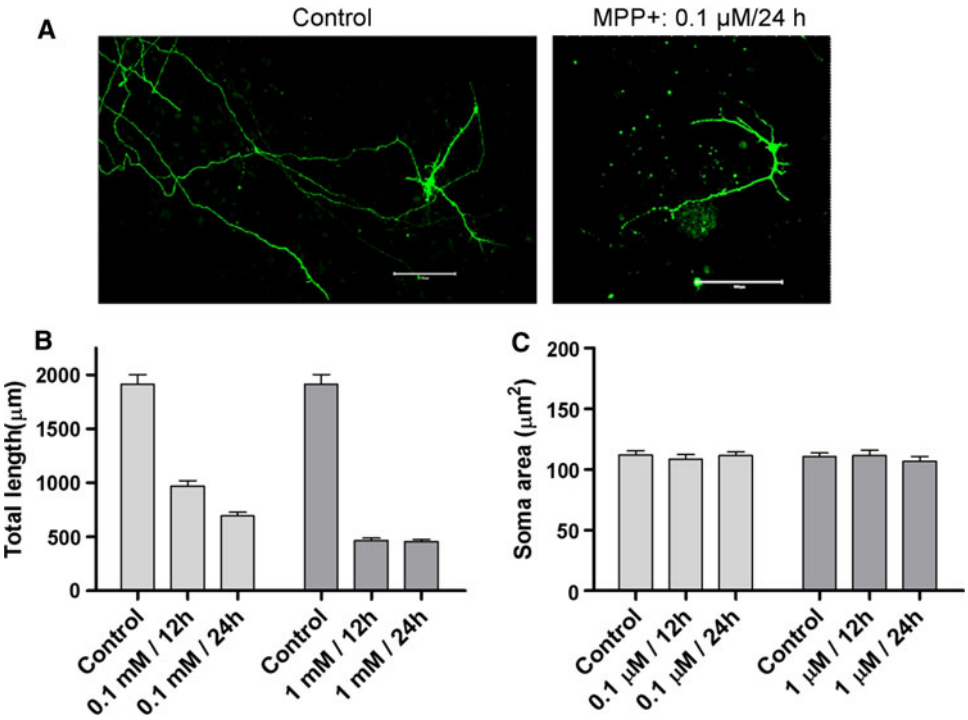


Fig. 3 Effect of MPP+ on the integrity of the neuritic tree. **a** Mesencephalic cells were treated without (control) or with 0.1 μM MPP+ for 24 h and then immunostained for TH (*green*). Shown are representative images. **b** Cells were treated for 12 or 24 h with either 0.1 or 1 μM MPP+. Cells were then immunostained for TH and dendritic tree length of TH-positive neurons was determined as described. Data represent mean ± SEM, $n = 10$ for each condition. **c** Soma area determination of cells described in **b**. Data represent mean ± SEM, $n = 10$ for each condition. No significant differences in soma area were noted

cultures for 24 h with increasing concentrations of Fe in the 2–40 μM range produced no effect on the total neuritic tree length, while treatment with 80 μM Fe significantly reduced this morphometric parameter (1,947 ± 90 vs. 951 ± 73 μm, $P < 0.001$) (Fig. 4a). No changes in soma morphology were observed for the complete iron range studied. Treatment with 0.1 μM MPP+ plus 40 μM Fe for 24 h induced greater shortening of the extensions as compared with MPP+ alone (692 ± 34 vs. 422 ± 25 μm, $P < 0.05$). On the contrary, low (2 μM) Fe substantially decreased the shortening induced by MPP+ to 1,082 ± 77 μm ($P < 0.01$) (Fig. 4b). Thus, iron seems to be an intrinsic component of MPP+-induced neurodegeneration since low concentrations prevented and high concentrations increased the effect of MPP+ on neuritic tree length.

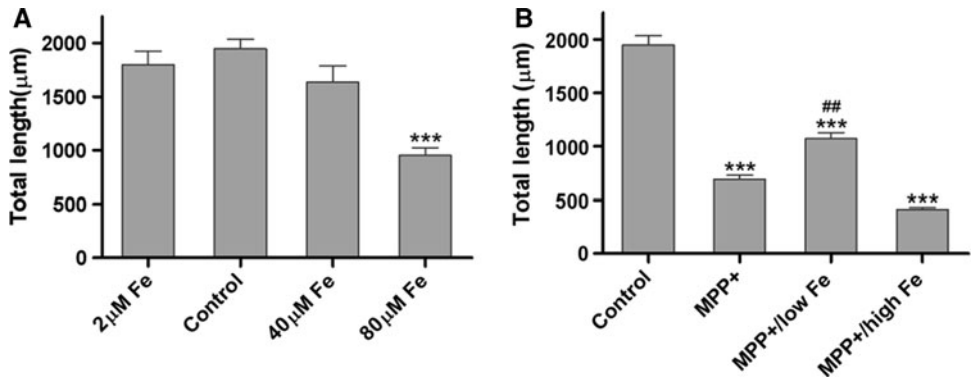


Fig. 4 Additive effects of iron and MPP+ on dendritic tree length. **a** Mesencephalic cells were incubated for 24 h with different amounts of iron in the culture media. The control culture media contained 7 μM Fe, while low iron medium contained 2 μM Fe. Cells were then immunostained for TH and dendritic tree length of TH-positive cells was determined. **b** Additive effects of iron and MPP+. Cells were incubated for 24 h in culture media containing 40 μM Fe with or without 0.1 μM MPP+. Cells were then immunostained for TH and total length of the dendritic tree of TH-positive cells was determined. Values represent mean ± SEM, $n = 10$. $^{***}P < 0.001$; *ns* not significant respect to control. $^{\#\#}P < 0.01$ respect to MPP+ and MPP+/40 μM Fe treatments

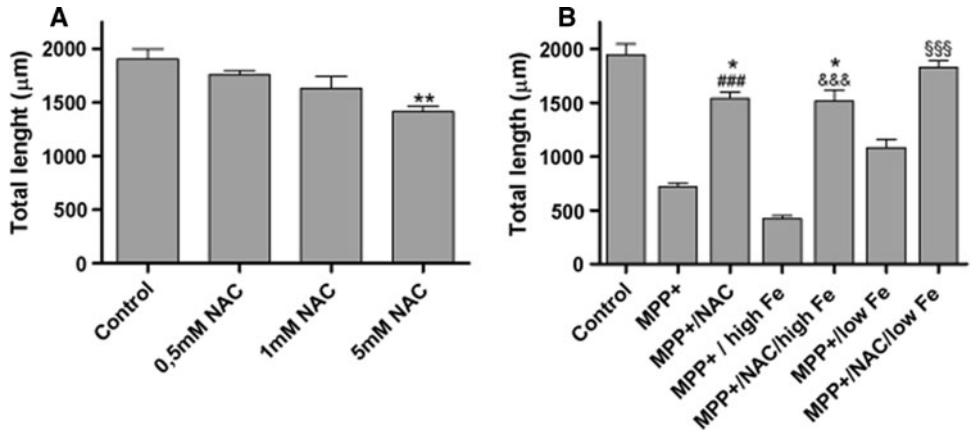


Fig. 5 The antioxidant NAC protects TH-positive cells from MPP+-induced shortening of the dendritic tree. Cells were incubated for 24 h with 0, 0.5, 1, or 5 μM NAC added to the culture medium and then immunostained for TH. **a** Total length of TH-positive cells. **b** Combined effect of MPP+, Fe, and NAC. Cells were incubated for 24 h under the conditions stated in this figure. NAC and MPP+ concentrations were 0.5 mM and 0.1 μM, respectively. High Fe was 40 μM and low Fe was 2 μM. Values represent mean ± SEM, $n = 10$. $^{*}P < 0.05$, $^{**}P < 0.01$ respect to control. $^{\#\#\#}P < 0.001$ respect to MPP+ treatment, $^{\&\&\&}P < 0.001$ respect to MPP+/high Fe treatment, and $^{\S\S\S}P < 0.001$ respect to MPP+/low Fe treatment

Effect of the antioxidant NAC on neurite morphology

Since increased oxidative stress is a landmark of PD neurons, it was of interest to test the effect of antioxidants on the neuritic tree collapse induced by MPP+. To that end, primary cultures of rat mesencephalus were incubated for 24 h with 0.1 μM MPP+ in the presence of the glutathione precursor NAC, a powerful antioxidant and free radical scavenger, and varied concentrations of iron. Prior to this, a concentration of 0.5 mM NAC was determined to produce no noticeable effect on the overall neuritic tree length or on the soma area (Fig. 5a). Incubation of cultures with 0.5 mM NAC protected against MPP+-induced neurodegenerative damage (Fig. 5b). Compared to the average length of untreated neurons, MPP+ treatment in the presence of NAC shortened the extensions only by 20.9%, unlike the treatment with MPP+ alone that produced a shortening of 65.1%. Moreover, NAC largely reversed the combined effect of MPP+ plus 40 μM Fe, and the combined effect of NAC and a low concentration of iron completely abolished the effect of MPP+ (Fig. 5b). These results strongly suggest that inhibition of complex I by MPP+ does not produce shortening of the neuritic tree if low iron and sufficient antioxidant defenses are present.

Inhibition of GSH synthesis accentuates the effects of MPP+ and Fe

GSH is the main antioxidant present in neurons, and decreased GSH levels have been reported as an early event

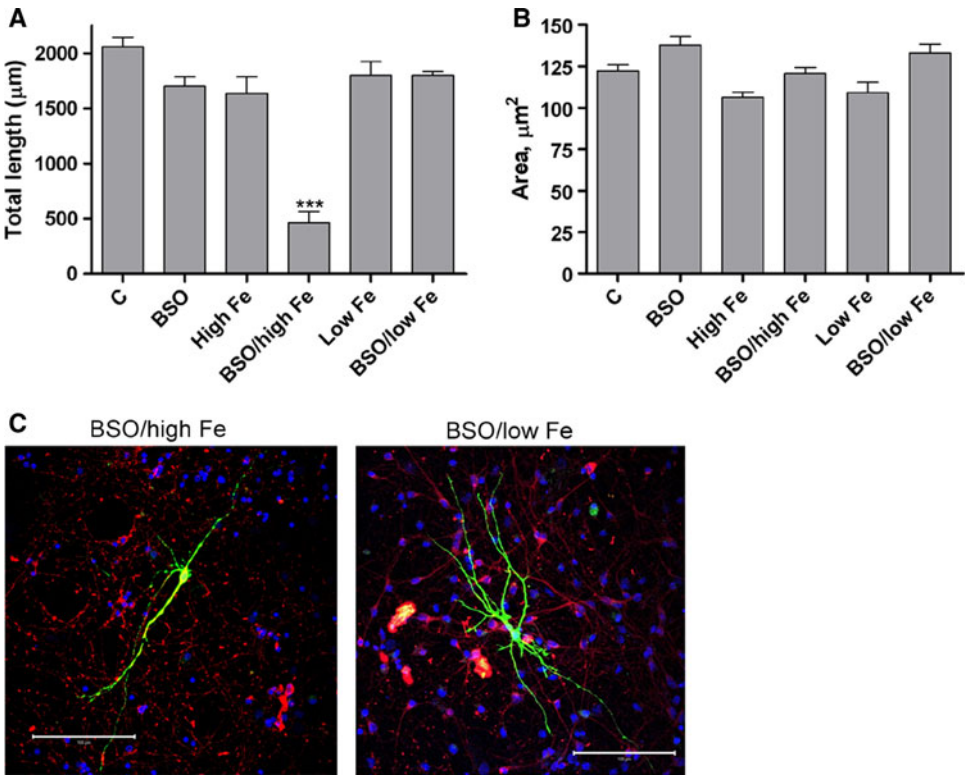


Fig. 6 Combined effects of BSO and Fe on neuritic tree length. **a** Mesencephalic cell cultures were treated for 24 h with 50 μM BSO and different concentrations of iron: control (7 μM), low (2 μM), and high (40 μM) after which the dendritic tree length was determined. The data represent mean ± SEM, $n = 10$. ***$P < 0.001$. **b** Soma area determination of cells described in **a**. Data represent mean ± SEM, $n = 10$ for each condition. No significant differences in soma area were noted. **c** Images of representative cells treated for 24 h with 50 μM BSO and high (40 μM) or low (2 μM) Fe. *Scale bar* 100 μm

leading to neurodegeneration. To evaluate the relevance of GSH on neuritic tree length, BSO, the irreversible inhibitor of gamma-glutamyl cysteine ligase, was used to inhibit GSH biosynthesis. Initial experiments determined 50 μM BSO as the concentration to use in ensuing experiments, since it did not produce neuritic tree shortening (Fig. 6a). Although neither 50 μM BSO nor 40 μM Fe significantly shortened the length of the neuritic tree, the combined treatment of BSO and Fe produced a marked shortening to 466 ± 99 μm, an effect that was not observed when the medium had low (2 μM) Fe (Fig. 6a, c). None of the conditions tested produced changes in soma morphology (Fig. 6b, c).

We then tested the effects of the combination of MPP+, BSO, and iron, a condition that mimics the findings in terminal PD neurons. The neuritic tree of dopaminergic neurons treated with MPP+, BSO, and 40 μM Fe underwent profound shortening to 13% of the length of control neurons (Fig. 7a). With this condition all of the minor neurites collapsed, while the longest neurite, presumably the axon, was severely shortened (Fig. 7b). This condition was the most drastic condition tested, inducing the degeneration not only of dopaminergic neurons but also of TH-negative neurons (Fig. 7b). Again, low iron afforded a neuroprotective effect, since co-incubation with MPP+ plus BSO under low iron conditions produced only a moderate shortening of the dendritic tree to 71% of the control length. Interestingly, BSO treatment showed a paradoxical protective effect against MPP+, since co-incubation with MPP+ and BSO resulted in smaller shortening of the neuritic tree when compared with MPP+ alone (control: 1,947 ± 90 μm; MPP+: 693 ± 35 μm; MPP+/BSO: 1,235 ± 111 μm). Again, low iron protected against neurite degeneration: treatment with MPP+ and BSO under low iron conditions shortened extensions of dopaminergic neurons only by 13.1%, to 1,691 ± 53 μm (Fig. 7a).

Discussion

Studies in post-mortem tissue reveal reduction in spine density and dendrite length in neurons of the caudate and putamen of patients with PD compared with controls (Baloyannis 2009; Baloyannis et al. 2006; McNeill et al. 1988; Stephens et al. 2005; Takeuchi et al. 2005; Zaja-Milatovic et al. 2005). Loss of the dendritic tree may be a consequence of the inhibition of complex I, since inhibition of complex I by MPP+ in alterations in fast axonal transport (Morfini et al. 2007). Indeed, MPP+ application to isolated squid axoplasm results in increased dynein-dependent retrograde transport and diminishes the kinesin-1 dependent anterograde transport (Morfini et al. 2007), eventually causing a degeneration called "dying back". The dying back-type neuropathies are characterized by a sequence of events that starts with the loss of synaptic

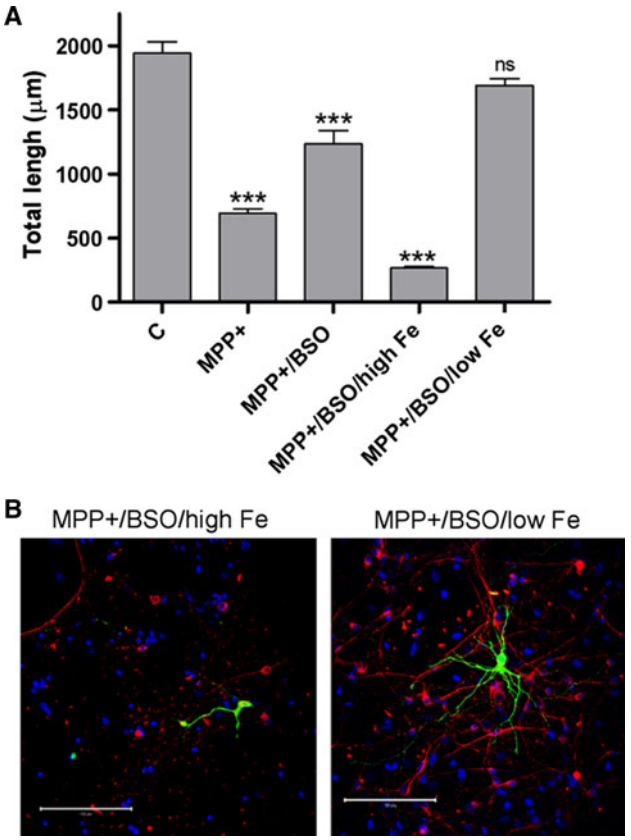


Fig. 7 MPP+ accentuates the combined effects of BSO and Fe. **a** Mesencephalic cell cultures were treated for 24 h with 0.1 μM MPP+ in combination with 50 μM BSO and low (2 μM) or high (40 μM) Fe after which they were immunostained for TH (*green*), *β*-3-tubulin (*red*) and nuclei (*blue*). The dendritic tree length of TH-positive neurons were then determined. The data represent mean ± SEM, $n = 10$. ***$P < 0.001$. **b** Image of a culture treated with MPP+, BSO, and Fe, in which loss of dopaminergic and non-dopaminergic neurons is evident. *Scale bar* 100 μm

function, followed by distal axonopathy and ending with neuronal death (Bossy-Wetzel et al. 2004; Coleman 2005).

Increased oxidative damage and increased iron levels have been found in the substantia nigra and the lateral segment of the globus pallidus of parkinsonian tissue (Berg and Youdim 2006; Jenner and Olanow 2006; Kienzl et al. 1999; Mandel et al. 2007; Meister and Anderson 1983; Sian et al. 1994; Sofic et al. 1988; Vali et al. 2007; Youdim 2008; Youdim and Riederer 1997). Most probably, mitochondrial dysfunction and oxidative damage underlie the final events that lead to neuronal death during PD. In this work, we evaluated the effect of MPP+ the shortening of the neuritic tree of dopaminergic neurons derived from the mid brain of rat embryos, and the additional effects induced by iron and the depletion of GSH, as well as the effects of the antioxidant NAC.

The morphological changes produced by treatment with MPP+ in these neurons were remarkable. Untreated neurons showed a large number of axonal and neuritic branches, with an average length of about 2,000 μm. In contrast, neurons treated with 0.1 μM MPP+, a dose that did not produce cell death, showed a dramatically shortened neuritic tree without loss of cell viability. Iron, at a concentration that per se did not produce shortening of the neuritic tree, accentuated the effect of MPP+, while under low iron conditions the effect of MPP+ was largely abolished. These results suggest that iron plays a crucial role in the neuritic degeneration induced by MPP+, acting as transducer of the damage induced by this neurotoxin. Given that in the intracellular cell environment iron is a net ROS producer (Borquez et al. 2008; Halliwell 2006), its cellular accumulation probably enhances the oxidative load generated by MPP+.

Treatment with 0.5 mM NAC substantially protected dopaminergic neurons from damage induced by MPP+ and Fe, significantly reducing the neuritic degeneration produced by them. Interestingly, almost complete protection against MPP+-induced neurite degeneration was achieved by NAC under low iron conditions. It is possible that the damage caused by MPP+ under a low oxidative load is more easily repaired compared with a condition of higher concentrations of iron, where a heavier oxidative burden is expected. These results further suggest that oxidative stress and iron are at the core of both axonal and dendrite degeneration of mesencephalic dopaminergic neurons.

The GSH synthesis inhibitor BSO in conjunction with Fe produced severe damage to the neural architecture, dramatically shortening the processes of dopaminergic neurons treated. This damage did not occur in cells treated separately with Fe or BSO. A possibility is that the damage was the result of increased free radical production catalyzed by iron and decreased protection against this oxidative challenge as a result of reduced of glutathione synthesis.

Treatment with MPP+ in the presence of sub-maximal concentrations of BSO produced less shortening of neurites than that observed in cells treated only with MPP+. This is a paradoxical result considering that both compounds should increase oxidative stress and thus neurite damage. It has been reported that BSO treatment increases the production of the main anti-apoptotic protein Bcl-2 in cells that survived BSO treatment (D'Alessio et al. 2004). Conversely, MPP+ is a neurotoxin that induces apoptosis in treated cells (Kalivendi et al. 2003; Morfini et al. 2007). Consequently, BSO at sub-maximal concentrations could be inducing an anti-apoptotic response that protects neurons from death.

Treatment with BSO and MPP+ under high iron conditions generated massive death of all the cells in the culture. Since MPP+ does not affect non-dopaminergic cells, the toxicity in these cells was most probably due to

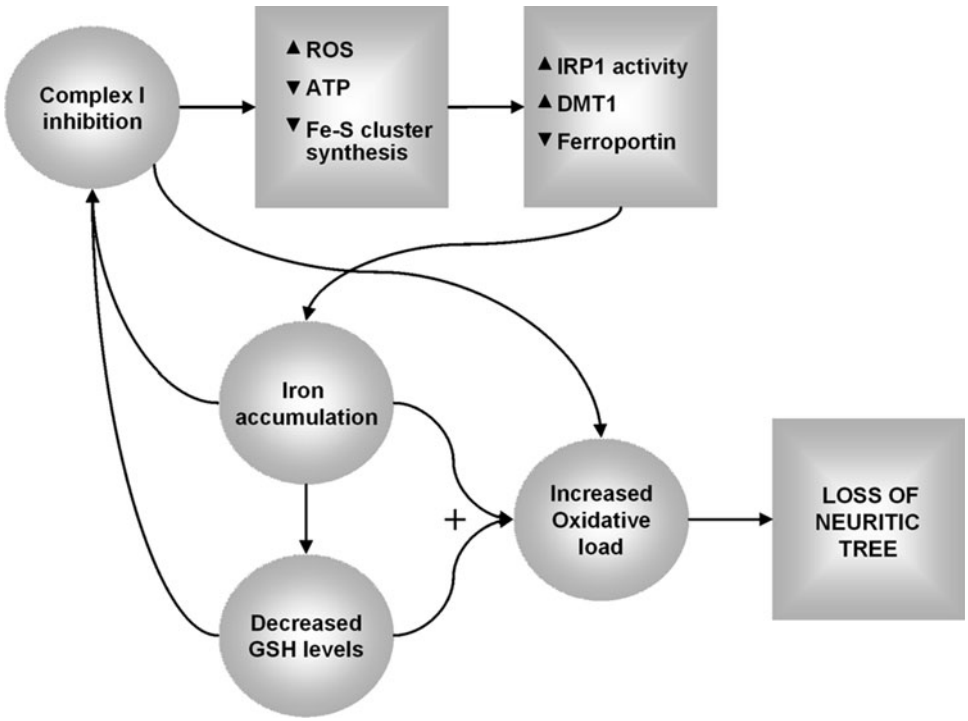


Fig. 8 Hypothetical sequence of events leading to neuritic tree degeneration. Inhibition of mitochondrial complex I leads to increased oxidative stress, decreased mitochondrial function, expressed in decreased levels of ATP and decreased Fe–S cluster synthesis. Decreased Fe–S cluster synthesis will result in increased IRP1 activity, with the consequent increase in DMT1, transferrin receptor and iron entry into the cells. In turn, increased cellular iron levels will result in decreased GSH content

the strong oxidant condition generated by the combination of BSO and iron.

An elevated concentration of intracellular iron was required for massive cell death, since decreasing the concentration of iron in the culture completely protected neurons from the combined effects of BSO and MPP+.

Figure 8 shows a hypothetical sequence of events leading to neuritic tree degeneration, in which inhibition of mitochondrial complex I leads to dysregulation of iron homeostasis and decreased GSH content. The cycle has a positive feedback loop between its components, thus any of them could initiate it. For example, diminished synthesis of GSH will result in increased oxidative stress, increased inhibition of complex I and activation of IRP1, with the consequent increase in DMT1 expression and iron uptake. Similarly, increased iron uptake will result in increased GSH consumption and the ensuing oxidative stress will inhibit complex I, resulting in decreased levels of ATP and Fe–S cluster synthesis.

In summary, we found that iron plays an essential role in MPP+-induced degeneration of the neuritic tree of dopaminergic neurons. We suggest that the accumulation of iron under decreased activity of mitochondrial complex I is a condition that results in loss of function that precedes death of mesencephalic dopaminergic neurons. Our findings highlight the pivotal roles of iron and oxidative stress in neurodegeneration and point to the use of antioxidants and iron chelating molecules specifically targeted to dopaminergic neurons in the SNpc as possible pharmacological therapies against the progression of Parkinson's disease.

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