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Phenothiazines interfere with dopaminergic neurodegeneration in *Caenorhabditis elegans* models of Parkinson's disease

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ABSTRACT

Oxidative stress is involved in the pathogenesis of various neurodegenerative disorders, conventional antioxidant strategies have yet been of limited success. We have employed transgenic *Caenorhabditis elegans* expressing DsRed2 in dopaminergic neurons and CFP pan-neuronally, to characterize in larval and adult animals the effects of rotenone and 1-methyl-4-phenyl-pyridinium (MPP^+) on the dopaminergic system. Investigating the antioxidant phenothiazine and different derived antipsychotic drugs, it was found that free phenothiazine exerted strong neuroprotection at the cellular level and resulted in a better performance in behavioral assays, whereas apomorphine and other dopamine agonists only rescued adult locomotor parameters. Phenothiazine antipsychotics with dopamine antagonist properties were likewise not cytoprotective, but even induced motor deficits by themselves. Beyond phenothiazine, other tricyclic imines elicited significant neuroprotection at considerably lower doses than different natural antioxidants. Mitochondrially targeted antioxidants were more potent than these untargeted natural antioxidants, yet not as potent as the untargeted compound phenothiazine. Thus, dopaminergic toxicity of rotenone and MPP^+ *in vivo* can be forestalled by nanomolar concentrations of certain chain-breaking antioxidants irrespective of dopamine receptor modulation or mitochondrial targeting.

Introduction

Substantial evidence suggests the involvement of mitochondrial dysfunction and oxidative stress in the development of Parkinson's disease (PD) (Abou-Sleiman et al., 2006; Sayre et al., 2008). Therefore, the identification of novel antioxidants to prevent damage from oxidative stress could be a promising strategy for this neurodegenerative condition. Various studies in animal models of PD have demonstrated beneficial effects of certain antioxidant compounds, e.g. preventing dopaminergic cell death in rodents treated with MPTP or 6-hydroxydopamine (Moosmann and Behl, 2002; Weber and Ernst, 2006; Kamat et al., 2008). However, neuroprotective effects of low-molecular mass antioxidants have often been realized only at rather high and toxicologically critical doses. In humans, merely one compound, the mitochondrial redox cofactor ubiquinone, has appeared to provide therapeutic benefit in early stages of PD and other neurodegenerative conditions (Shults et al., 2002; Cooper et al., 2008). Hence, there is an urgent need for the development of novel compounds with high antioxidant potency, calling for reliable *in vivo* disease models that may be adapted to automated pharmacological screening procedures.

Experimental animal models of PD based on MPP^+ or rotenone toxicity are valuable tools for the development of new therapeutic strategies. Following systemic administration, these substances induce dopaminergic neurodegeneration in a specific fashion and in diverse organisms such as *Caenorhabditis elegans* (Nass and Blakely, 2003; Braungart et al., 2004), *Drosophila melanogaster* (Coulom and Birman, 2004), *Mus musculus*, *Rattus norvegicus*, and different primates (Beal, 2001). Occasional clinical observations and epidemiological studies have revealed the specificity and reproducibility of such drug-induced dopaminergic neurodegeneration also in humans (Langston et al., 1983; Liou et al., 1997; Petrovitch et al., 2002; Di Monte, 2003).

A number of clinically used phenothiazine derivatives acting as histamine and dopamine receptor antagonists have been published to exert antioxidant effects *in vitro* (Jeding et al., 1995). Amongst these, promethazine was identified as a neuroprotective compound in mice subjected to ischemic reperfusion injury (Stavrovskaya et al., 2004) and MPTP toxicity (Cleren et al., 2005). However, phenothiazine derivatives belonging to the so-called "typical" antipsychotics that primarily act as dopamine receptor antagonists, may in fact exacerbate the pathogenesis of PD in spite of potential antioxidant activities. After all, these compounds are well-known for their induction of PD-like extrapyramidal side-effects by virtue of their dopaminergic antagonism (Weiden, 2008). In previous studies, we have observed that free phenothiazine possessed an exceptionally high antioxidant efficacy against MPP^+ and rotenone neurotoxicity *in*

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vitro, whereas N-substituted phenothiazines with dopamine antagonist activity had no cytoprotective effects (Hajieva et al., 2009). Intriguingly, free phenothiazine is known to be devoid of significant dopamine receptor binding (Andersen et al., 1985).

In the present study, we have further developed the MPP^+- and rotenone-based *C. elegans* model of dopaminergic neurotoxicity initially described by Braungart et al. (2004), to perform a systematic analysis of the neuroprotective and behavioral effects of the phenothiazines and other dopamine receptor modulators. The basic characteristics of the nematode's dopaminergic system, including the presence of postsynaptic dopamine receptors, appear to make it a suitable *in vivo* model of PD amenable to automated screening procedures (Sanyal et al., 2004; Schmidt et al., 2007).

Our results demonstrate that only antioxidant, free phenothiazine shows significant cellular and behavioral protection from MPP^+ and rotenone toxicity at nanomolar (100–500 nM) concentrations, whereas dopamine agonists merely preserved behavioral parameters, while dopamine antagonists had no effect or even exacerbated both outcomes.

Materials and methods

Materials

Unless otherwise stated, all laboratory chemicals as well as reagents for the cultivation and maintenance of *C. elegans* were purchased from Sigma-Aldrich at the highest available purity. MitoQ ([10-(3,6-dihydroxy-4,5-dimethoxy-2-methylphenyl)decyl] triphenylphosphonium bromide; a mixture of quinol and quinone form) was a kind gift from Michael P. Murphy (MRC Mitochondrial Biology Unit, Cambridge, UK). The tetrapeptide SS-31 (D-Arg-Dmt-Lys-Phe-NH_2) was purchased from Bachem.

Maintenance and generation of transgenic C. elegans

The N2 Bristol strain and transgenic derivatives of this strain were cultivated and maintained on nematode growth medium (NGM) plates (Brenner, 1974) in the presence of *Escherichia coli* strain HB101 at 20 °C.

The *C. elegans* tyrosine hydroxylase promoter ($P_{cat\text{-}2}$) was amplified from genomic DNA according to a previously established procedure (Lints and Emmons, 1999) using the following primers: forward: GATCTCCAATAACCCGGAAA; reverse: CAGCTCTGCCGAGTGAATTA. Discosoma red fluorescent protein 2 (DsRed2) was amplified from a pDsRed2-N1 vector (Clontech) with the following primers: forward: AGTTAATTCACTCGGCAGAGCTGGCCTCCTCCGAGAACG; reverse: CTCTACAAATGTGGTATGGCTGAT. Both sequences were fused following an established PCR protocol (Hobert, 2002) employing forward: CTGCAGGGATCTCCAATAAC and reverse: CTGATTATGATCTAGAGTCGC primers. The fusion product ($P_{cat\text{-}2}$::DsRed2) was cloned into a TOPO-TA vector following the manufacturer's instructions (TA Cloning Kit from Invitrogen), amplified, and subcloned into a pRL1899 vector (a kind gift from Rueyling Lin, UTSW Medical Center, Dallas, USA) using Gateway LR ClonaseTM II Enzyme Mix (Invitrogen). The dopaminergic reporter $P_{cat\text{-}2}$::DsRed2 and the pan-neuronal reporter $P_{Sng\text{-}1}$::CFP (# 1727, a kind gift from Rudolf Leube, University of Mainz, Germany) were mechanically injected (60 ng) into N2 worms. Integration of the injected constructs was achieved by UV treatment. Two independent lines were outcrossed for at least three times each.

Assessment of larval development

Gravid adult worms were treated with alkaline sodium hypochlorite (Emmons et al., 1979) to obtain synchronized L1 larvae. The L1 larvae were distributed in 48 well plates (40 worms/well) and cultivated in 0.5 ml liquid culture medium (Lewis and Fleming, 1995). At this point, 1.5 mM MPP^+ or 1.5 µM rotenone and the indicated concentrations of the compounds to be investigated were added to the cultures. The experiment was stopped after 72 h (3 days). The developmental status of either the N2 worms or the transgenic animals was microscopically investigated, and the worms were photographed using an inverted Axiovert 200 microscope (50–100× magnification). The transgenic individuals were further explored for fluorescence signals of DsRed2 (553 nm/610 nm filters) and CFP (cyan fluorescent protein; 490 nm/510 nm filters). To this end, triplicates of 10–20 worms per treatment were transferred to a 2% agar pad, and the fluorescent signal was examined with a Zeiss Axiophot fluorescence microscope (200× magnification). The intensity of the DsRed2 signal in the head region of the worms was quantified and scored as a positive signal for the integrity of the dopaminergic system. As an independent inducer of larval arrest, 1 ng/ml 5-fluoro-2′-deoxyuridine (FdU) was employed. Quantification of fluorescence intensity was performed with MetaVue 6.2r4 software (Universal Imaging).

Cultivation and treatment of adult worms

Adult, late L4 transgenic worms were treated with 2 mM MPP^+ or 1.5 µM rotenone and the indicated concentrations of the compounds to be tested for 5–9 days. The culture medium was changed every other day. At the end of the experiment, the worms were anesthetized with 0.1 g/ml levamisole. Dopaminergic DsRed2 and pan-neuronal CFP signals were analyzed by fluorescence microscopy as described above. Triplicates of 10–20 worms were evaluated in each treatment group.

Thrashing assay

Adult, late L4 worms were treated with 2 mM MPP^+ or 1.5 µM rotenone in the presence or absence of the investigated compounds for 5 days. To examine the animals' spontaneous motor activity, the "thrashing assay" was performed according to a previously described procedure (Miller et al., 1996; Thomas and Lockery, 1999). To this end, the worms were transferred into a drop of M9 buffer on a standard microscope slide, and the number of body deflections within 30 s of observation was counted for each individual. In each treatment group, approximately 30–60 animals were evaluated.

Nose-touch assay

Adult N2 nematodes were treated with 2 mM MPP^+ with or without 500 nM of the investigated compounds and examined after 5 days by means of a "nose-touch assay" (Sanyal et al., 2004). Worms were placed on NGM plates without food and subsequently challenged with a series of repeated touches. As standard behavior, nematodes respond to the stimulus and move backwards. Here, each individual animal was repeatedly tested until it ceased to respond to the stimulus. Nematodes with faster irresponsiveness to the nose-touch were considered to be defective in sensory-stimulated motor behavior. Approximately 30–60 animals were analyzed per treatment group.

Immunoblotting

Toxin-induced oxidative stress in adult *C. elegans* was measured as rise in protein carbonyl immunoreactivity using a standard protocol (Hajieva et al., 2009). For sample preparation, 40 adult worms that had been treated with 2 mM MPP^+ or 1.5 µM rotenone for 7–9 days were collected, washed with M9 buffer to remove any residual culture medium, and centrifuged at 200 g for 4 min. After transfer to lysis buffer [20 mM Tris–HCl, pH 6.8; 2% SDS; 3% sucrose; 1 mM DTT], the worms were sonicated for 2 min. The resulting protein lysate was derivatized with 2,4-dinitrophenyl hydrazine (DNPH, 10 mM in 2 M