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Promethazine protects against 3-nitropropionic acid-induced neurotoxicity

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Abstract

Promethazine (PMZ), an FDA-approved antihistaminergic drug, was identified as a potentially neuroprotective compound in a NINDS screening program. It was shown to protect against ischemia in mice, to delay disease onset in a mouse model of amyotrophic lateral sclerosis and to inhibit Ca²⁺-induced mitochondrial permeability transition in rat liver mitochondria. We investigated whether PMZ could protect against the neurotoxic effects induced by 3-nitropropionic acid (3-NP), an inhibitor of the succinate dehydrogenase, used to model Huntington's disease (HD) in rats. Lewis rats receiving chronic subcutaneous infusion of 3-NP were treated with PMZ. The findings indicate that chronic PMZ treatment significantly reduced 3-NP-induced striatal lesion volume, loss of GABAergic neurons and number of apoptotic cells in the striatum. PMZ showed a strong neuroprotective effect against 3-NP toxicity *in vivo*.

Keywords

Huntington; Neurotoxicity; Neuroprotection; Apoptosis; Calbindin; Succinate dehydrogenase; Mitochondria

1. Introduction

Huntington's disease (HD) is an inherited autosomal dominant disorder caused by a mutation in the gene encoding the protein Huntingtin (Htt). HD is associated with preferential degeneration of striatal medium-sized spiny GABAergic neurons that leads to motor deficits (The Huntington's Disease Collaborative Research Group, 1993; Harper, 1991). The protein Htt is associated with outer mitochondrial membranes and the mutated form of the protein is able to promote mitochondrial permeability transition as well as a decrease in the Ca²⁺ threshold to induce it (Panov et al., 2002; Choo et al., 2004).

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A consistent defect in mitochondria complex II–III has been found in the caudate nucleus of patients with HD (Browne et al., 1997). Administration of the succinate dehydrogenase (SDH) inhibitor, 3-nitropropionic acid (3-NP) in rodents replicates many of the characteristics of HD, such as abnormal movements induced by degeneration of striatal GABAergic neurons with a relative sparing of interneurons and afferents, mitochondrial dysfunction, release of proinflammatory cytokines and gliosis (Beal et al., 1993; Brouillet et al., 1999; Blum et al., 2001). It has been shown that there are major strain differences in susceptibility of rats. Although Lewis rats are relatively resistant to 3-NP toxicity as compared to both Fisher 344 and Sprague–Dawley rats, they have been shown to develop much more consistent lesions (Ouay et al., 2000). Lewis rats were, therefore, used in the present studies.

Promethazine (PMZ) was identified as a neuroprotective compound as part of a NINDS program to screen 1040 FDA-approved compounds in a variety of high-throughput assays (Stavrovskaya et al., 2004). PMZ is a phenothiazine derivative possessing antihistaminic and anticholinergic effects, but weak antidopaminergic properties (1/10 that of chlorpromazine). Clinically achievable doses of PMZ were shown to induce protection in different models. *In vitro*, PMZ protected primary cerebrocortical neurons subjected to oxygen/glucose deprivation and, *in vivo* it reduced infarct size and neurological impairments in mice subjected to middle cerebral artery occlusion/reperfusion (Stavrovskaya et al., 2004). PMZ also delayed disease onset in a mouse model of amyotrophic lateral sclerosis (Wang et al., 2007). In experiments with isolated rat liver mitochondria, PMZ which accumulates into mitochondria (Muller, 1996), delayed the Ca^{2+} -induced mitochondrial permeability transition pore (PTP) without impairing mitochondrial physiology (Stavrovskaya et al., 2004). The mitochondrial PTP is the opening of pores in the inner mitochondrial membrane, resulting in free diffusion of solutes <1.5 kD, destroying the proton gradient, generating oxygen species, and releasing direct mediators of downstream caspase-dependant and independent cell death pathways (Stavrovskaya et al., 2004). In isolated mice brain mitochondria, PMZ partially prevented and reversed MPP^{+} -induced depolarization of membrane potential and inhibited the calcium-induced PTP (Cleren et al., 2005). Here, we demonstrate that chronic injections of PMZ in the 3-NP model of HD, induce strong neuroprotective effects. Since PMZ penetrates the blood–brain barrier, is frequently used in patient care, and has a high oral bioavailability, PMZ could readily be moved to late-stage preclinical trials.

2. Materials and methods

2.1. Reagents

Reagents were purchased from Sigma (St. Louis, USA). PMZ was dissolved in phosphate buffered saline (PBS). 3-NP was dissolved in distilled water and the pH was adjusted to 7.4. 2,3,5-Triphenyl tetrazolium chloride (TTC) was diluted (2%) in distilled water.

2.2. Animals

Male Lewis rats (12 weeks old) were maintained in a temperature/humidity-controlled environment under a 12 h light/dark cycle with free access to food and water. Rats were

anesthetized with isoflurane during the subcutaneous implantation of osmotic minipumps (2ML1, Alzet) containing 3-NP (54 mg/kg/day, for 6 days) or distilled water. Constant subcutaneous infusion of Lewis rats with 3-NP, through osmotic minipumps, induces no death, motor symptoms and striatal degeneration with a similar time course in all rats, which results in less variability in lesion sizes between rats (Brouillet et al., 2005). Beginning 3 h before the surgery, rats were injected with PMZ (5, 10, 20 or 40 mg/kg, i.p. each 12 h $\times$ 13 doses) or PBS. Rats were sacrificed 6 days after the surgery. The experiments were carried out in compliance with the National Institute of Health guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Cornell University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. Immunohistochemistry

Fresh brains were immerse-fixed in 4% paraformaldehyde overnight at 4 °C. Prior to sectioning, the tissues were placed in 30% sucrose overnight at 4 °C for cryoprotection. Serial coronal sections (50 μ m thick) were cut through the striatum using a cryostat. Free-floating sections were processed for NeuN or calbindin (both from Chemicon, Temecula, CA; 1:1000) immunohistochemistry. *In situ* demonstration of apoptotic cells was performed using the ApopTag Peroxidase Apoptosis Detection Kit (Chemicon, Temecula, CA, USA). In addition to TTC staining for assessing lesion volume, neuron loss in the medial striatum outside the lesion was analyzed. Calbindin-positive neurons and NeuN-positive neurons were quantified within a 1 mm² rectangular area per section in the medial striatum outside the 3-NP lesion (see asterisks in Figs. 2 and 3). For consistency, the medial side of the rectangle was placed 100 μ m from the edge of the striatum lining the lateral ventricle, and placed in the middle of the dorsoventral axis of the lateral ventricle. For each rectangular area, 20 counting grids were examined using a system consisting of a Nikon Eclipse E600 microscope equipped with a computer-controlled LEP Biopoint motorized stage, a DEI-750 video camera, a Dell Dimension 4300 computer, and the StereoInvestigator (v. 4.35) software program (Microbrightfield, Burlington, VT, USA). The use of the stereology software ensured unbiased sampling within the areas examined. Three sections through the caudate putamen per animal were used for quantification (bregma 1.2 mm; bregma 0.48 mm; bregma -0.26 mm). Results are expressed as the number of positive cells/200 μ m². Tissue sections were examined using a Nikon Plan Apo 100 $\times$ objective lens with a 1.4 numerical aperture. The size of the x-y sampling grid was 140 μ m and the counting frame thickness was 14 μ m. For the quantification of apoptotic cells, since the cells were concentrated within the lesion site, the counts are expressed as positive cells/section.

2.4. TTC staining

Two percent TTC (2,3,5-triphenyltetrazolium chloride) solution was made with distilled water and kept in brown bottles at 4 °C until use. One-millimeter-thick fresh brain slices were cut with a rat brain matrix (Harvard Apparatus, Holliston, MA, USA) and promptly dipped in dishes with 2% TTC for 30 min in a dark cabinet until the intact tissue of the slice was stained to deep purple and the lesioned area of the striatum was noted by pale staining. Stained slices were then kept in 4% paraformaldehyde in the dark at 4 °C not more than 2 weeks before analysis. The lesioned surface of the striatum was measured on both sides of

each slice where the striatum was visible, using a microcomputer imaging device (MCID; Imaging Research Inc., St. Catharine, Ont., Canada). The striatal lesion volume was calculated by averaging the striatal lesion surface on both sides of each slice, multiplying it with the 1 mm section thickness, and summing the values of all the lesioned sections. TTC measurements directly correlate with measurements made on adjacent Nissl-stained sections (Schulz et al., 1995). Different brains were used for TTC staining and immunohistochemistry.

2.5. Succinate dehydrogenase (SDH, EC 1.3.99.1) activity measurement

Cortex tissue was frozen at -80°C , thawed and homogenized thoroughly in 10 mM HEPES (pH = 7.4). Tissue homogenate was centrifuged at $1000 \times g$ during 5 min; pellet was discarded and the activity of SDH was measured in the supernatant. The SDH activity was measured spectrophotometrically as described previously (Arrigon and Singer, 1962; Klivenyi et al., 2004). The reaction medium was composed of 50 mM KCl, 10 mM HEPES (pH = 7.4), 10 mM succinate, 2 mM KCN, 1×10^{-6} M rotenone, 0.050 mM 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone, 0.1 mM 2,6-dichlorophenol indophenol and 0.02 mM EDTA. The reaction was monitored at 600 nm, and the activity was calculated employing $E \text{ (mM)} = 21 \text{ cm}^{-1}$ for 2,6-dichlorophenol indophenol.

2.6. Statistical analysis

Data were computed in a database (Graphpad Instat[®] software) and expressed as mean $\pm$ SEM. Differences between groups and interaction between treatments were assessed by a Student's *t*-test or by a one-way analysis of variance followed, when appropriate, by a Student–Newman–Keuls post hoc test. A probability level of 5% ($p < 0.05$) was considered significant.

3. Results

3-NP treatment induced striatal lesion volumes of approximately 14 mm^3 , as shown by TTC staining (Fig. 1). Chronic PMZ treatment (5 mg/kg, i.p. each $12 \text{ h} \times 13$ doses) reduced this lesion volume by 71% (Fig. 1, $p < 0.01$) and the 10, 20 and 40 mg/kg regimens totally prevented the appearance of a lesion (data not shown, $p < 0.01$). The PMZ regimen 20 mg/kg (i.p. each $12 \text{ h} \times 13$ doses) was chosen to determine PMZ neuroprotective effects in the following experiments.

In addition to the analysis of the volume of the well-demarcated lesion in the dorsolateral striatum induced by 3-NP as revealed by TTC staining, we also examined the medial striatum outside the lesion for depletion of medium-sized spiny neurons. Calbindin immunohistochemistry revealed that 3-NP treatment induced a significant loss of medium-sized spiny neurons in the medial striatum outside the TTC-stained lesion (Table 1: 4.3 ± 0.9 calbindin-positive cells/ $200 \mu\text{m}^2$) compared to control animals (7.78 ± 0.2 ; $p < 0.01$). Thus, 3-NP-induced neuron loss occurred not only within the TTC-stained lesion site but also in the medial striatum outside the area of severe lesion. Within the core of the lesion site, calbindin-positive neurons were virtually absent (Fig. 2). The PMZ treatment strongly prevented 3-NP-induced GABAergic neurodegeneration in the medial striatum (Table 1: 6.8

± 0.3 calbindin-positive cells/200 μm^2 ($p < 0.05$) and Fig. 2, right panel). To test whether actual loss of neurons in 3-NP-treated rats occurred in the medial striatum, sections adjacent to those used for calbindin staining were immunostained with an antibody against the neuron marker NeuN (Fig. 3). Administration of 3-NP significantly reduced the number of NeuN-positive neurons in the medial striatum (Table 1: 6.7 ± 1 NeuN-positive neurons/200 μm^2) compared to control (11.45 ± 0.5 ; $p < 0.05$). This result suggests that the reduction of calbindin-positive neurons reflects neuronal death, and not mere downregulation of calbindin expression. PMZ treatment significantly mitigated the 3-NP induced NeuN-positive neuron loss (Table 1: 9.86 ± 1 ; $p < 0.05$, vs. 3-NP).

3-NP induced apoptosis (79.3 ± 16 apoptotic cells per section) within the lesion site of the rat striatum (Fig. 4, left panel). Very few apoptotic cells (19.5 ± 2 apoptotic cells per section) were found in the striatum of 3-NP-lesioned rats treated with PMZ (Fig. 4, right panel). In contrast to PBS/3-NP rats, the degree of apoptosis in the striatum of PMZ/3-NP treated rats was insufficient to induce a large lesion. Altogether, these data show that clinically achievable doses of PMZ are strongly neuroprotective and capable of attenuating tissue damage induced by chronic 3-NP in rats. Although the chronic PMZ treatment was clearly and strongly neuroprotective in our 3-NP toxicity model and, despite the fact PMZ by itself slightly increased succinate dehydrogenase activity ($p < 0.05$, Fig. 5), PMZ did not reduce the strong inhibition of succinate dehydrogenase activity ($p < 0.001$, Fig. 5) induced by 3-NP in rat cortex. We also found that 3-NP induced activation of calpain, as assessed by fodrin degradation, was significantly attenuated by PMZ treatment (data not shown).

4. Discussion

3-NP, a suicide inhibitor of the mitochondrial enzyme succinate dehydrogenase (Alston et al., 1977), produces a well characterized animal model of HD (Beal et al., 1993; Ouayri et al., 2000), in which there is excitotoxicity, oxidative damage, and increased susceptibility to apoptosis (Beal et al., 1995; Sugino et al., 1997; Bogdanov et al., 1999). In the present study, PMZ treatment prevented 3-NP-induced GABAergic neurotoxicity. 3-NP treatment resulted in massive apoptosis in the striatum, which was significantly attenuated in PMZ-treated animals. This result is consistent with the observation that PMZ prevented caspase 3 activation in stroke-related pathology (Narayanan et al., 2004).

We and others previously showed that caspase inhibitors protect against 3-NP toxicity (Yang et al., 2004; Bizat et al., 2005). The neuroprotective effect of zVAD involves inhibition of calpain. 3-NP treatment of hippocampal neurons results in a massive elevation of both mitochondrial and cytosolic Ca^{2+} , a depletion of ATP, decreased mitochondrial membrane potential, and activation of calpain but not caspase activity (Nasr et al., 2003). Similarly, 3-NP administration *in vivo* results in cytochrome c redistribution, transient caspase-9 processing, and activation of calpain, however levels of active caspase 3 remain low (Bizat et al., 2003a). The low levels of active caspase-3 are a consequence of calpain activation, since pharmacologic blockade of calpain increases levels of processed caspase 9 and caspase 3.

Calpain cleaves the p20 subunit of active caspase-3 near its catalytic site. Calpain inhibition reduces 3-NP induced striatal lesions and DNA fragmentation (Bizat et al., 2003b). In the present experiments, promethazine had no effect on 3-NP induced inhibition of succinate dehydrogenase. Consistent with an effect on the mitochondrial PTP and an attenuation of calcium dysregulation, the activation of calpain as assessed by fodrin degradation was attenuated in the PMZ-treated rats. Other studies showed that 3-NP induced mitochondrial defects amplify intra-cellular calcium dysregulation, although Ca^{2+} entry due to NMDA receptor activation was not exacerbated (Jacquard et al., 2006). Using a kinetic model it was shown that promethazine delays calcium efflux from mitochondria, which reduces calcium cycling and activation of the PTP (Baranov et al., 2008).

The calcium dysregulation induced by 3-NP leads to generation of oxidative stress, since free radical scavengers, and agents which activate the Nrf2/ARE-transcriptional pathway to induce antioxidant enzymes, are protective against 3-NP induced toxicity (Yang et al., 2005, 2009a; Shih et al., 2005). We also showed that both coenzyme Q10 and creatine protect against 3-NP toxicity, and that administration of both agents together produces additive neuro-protective effects against 3-NP toxicity, and improves motor performance and survival in a transgenic mouse model of HD (Yang et al., 2009b).

In summary, PMZ significantly reduced striatal lesion volume, loss of GABAergic neurons and protected against apoptotic cell death induced in rats by 3-NP treatment. We believe that the neuroprotection is due to inhibition of the PTP, resulting in lowered cytosolic calcium, and reduced activation of calpain, which is linked to cell death. PMZ did not prevent 3-NP induced inhibition of succinate dehydrogenase, and other experiments on isolated rat liver mitochondria and brain mitochondria, showed that PMZ delayed the Ca^{2+} -induced mitochondrial permeability transition pore (PTP) without impairing mitochondrial physiology (Stavrovskaya et al., 2004; Cleren et al., 2005). Mitochondrial dysfunction and impaired calcium regulation have been shown in both HD transgenic mice as well as in lymphoblasts from patients (Panov et al., 2002; Choo et al., 2004). The strong neuroprotection PMZ induced in our rodent model of HD suggests that this drug could be efficacious in preventing and/or delaying the onset of HD. This is of great interest, since PMZ is well-tolerated and could readily be moved to preclinical trials.

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Fig. 1.
Volume of 3-NP striatal lesion as assessed by TTC staining, ** $p < 0.01$.

Fig. 2. Calbindin immunoreactivity in the rat striatum showing attenuation of 3-NP-induced loss of calbindin-positive neurons by promethazine. Broken line separates the area of severe calbindin-positive neuron loss from relatively spared area of the caudate putamen. Boxed areas are magnified in the lower panel. Asterisks indicate the region analyzed for calbindin-positive neuron counts outside the lesion. ec, external capsule and CPu, caudate putamen.

Fig. 3. NeuN immunoreactivity in the rostral rat striatum showing attenuation of 3-NP-induced loss of NeuN-immunoreactive neurons by promethazine. Boxed areas are magnified in the lower panel. Asterisks indicate the region analyzed for NeuN-positive neuron counts outside the lesion. ec, external capsule and CPu, caudate putamen.

Fig. 4.

In situ detection of apoptosis in the striatum of 3-NP-lesioned rats with or without chronic PMZ treatment. Numerous apoptotic cells (brown) occurred in the 3-NP-treated striatal section but virtually absent after PMZ treatment. Sections were counterstained with methyl green giving non-apoptotic cells green coloration. Inset shows apoptotic cells (brown) in the positive control section from rat mammary gland. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

Fig. 5. Succinate dehydrogenase activity in rat cortex, * $p < 0.05$ and *** $p < 0.001$ as compared to control (PBS/PBS).

Table 1

Number of striatal calbindin-positive neurons at the lesion periphery (100 μm from the lateral ventricle) and number of NeuN-positive neurons in the medial striatum, in control (PBS/PBS) and 3-NP-lesioned rats treated with PBS or PMZ.

	Control	PBS/3-NP	PMZ/3-NP
Number of calbindin-positive neurons (0.2mm ²)	7.8 $\pm$ 0.2 <i>n</i> =5	4.3 $\pm$ 0.9 <i>p</i> < 0.01/control <i>n</i> = 5	6.8 $\pm$ 0.3 (<i>p</i> < 0.05/PBS/3-NP <i>n</i> =5)
Number of NeuN positive neurons (0.2 mm ²)	11.5 $\pm$ 0.5 <i>n</i> =5	6.7 $\pm$ 1.0 <i>p</i> < 0.05/control <i>n</i> = 5	9.9 $\pm$ 1.0 <i>p</i> < 0.05/PBS/3-NP <i>n</i> =6