Orphenadrine prevents 3-nitropropionic acid-induced neurotoxicity *in vitro* and *in vivo*

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1 Previous studies indicate that 3-nitropropionic acid (3-NPA) neurotoxicity involves the excitotoxic activation of N-methyl-D-aspartate (NMDA) receptors. Thus, we examined the effect of orphenadrine (an anticholinergic drug with NMDA receptor antagonist properties) on 3-NPA neurotoxicity in both cultured rat cerebellar granule cells (CGCs) and in rats.

2 Orphenadrine protected CGCs from 3-NPA-induced mortality, as assessed by both the neutral red viability assay and laser scanning cytometry, using propidium iodide staining.

3 For rats, two indirect markers of neuronal damage were used: the binding of $[^3H]$-PK 11195 to the peripheral-type benzodiazepine receptor (PBR), a microglial marker, and expression of the 27 kD heat-shock protein (HSP27), a marker of activated astroglia. Systemic administration of 3-NPA (30 mg kg^{-1} per day for 3 days) induced a 170% increase in $[^3H]$-PK 11195 binding, and expression of HSP27.

4 Both the increase in $[^3H]$-PK 11195 and HSP 27 expression were prevented by previous administration of 30 mg kg^{-1} per day of orphenadrine for 3 days. Lower doses (10 and 20 mg kg^{-1}) had no protective effect. Orphenadrine also reduced 3-NPA-induced mortality in a dose-dependent manner.

5 We propose that orphenadrine or orphenadrine-like drugs could be used to treat neurodegenerative disorders mediated by overactivation of NMDA receptors.

British Journal of Pharmacology (2001) **132**, 693–702

Keywords: Orphenadrine; HSP27; peripheral benzodiazepine receptor; N-methyl-D-aspartate; microgliosis; neurodegeneration; laser scanning cytometry

Abbreviations: CGCs, cerebellar granule cells; CNS, central nervous system; HD, Huntington's disease; HSP27, heat shock protein 27; LSC, laser scanning cytometer; NMDA, N-methyl-D-aspartate; 3-NPA, 3-nitropropionic acid; NR, neutral red; Orph., orphenadrine; PBR, peripheral-type benzodiazepine receptor; PMSF, phenylmethylsulphonyl fluoride

Introduction

Administration of 3-nitropropionic acid (3-NPA) to rats induces neuronal damage that mainly affects the striatum and mimics many of the histological and neurochemical features of Huntington's disease (HD) (Wüllner *et al.*, 1994; Borlongan *et al.*, 1995; Dawson *et al.*, 1995; Kodsi & Swerlow, 1997; Sato *et al.*, 1997; Brouillet *et al.*, 1999). 3-NPA inhibits irreversibly succinate dehydrogenase, localized in the mitochondrial inner membrane, and complexes II and III of the respiratory chain and the tricarboxylic acid cycle (Alexi *et al.*, 1998). These effects decrease ATP levels, leading to changes in the neuronal membrane potential. Mg^{2+}, which normally blocks the N-methyl-D-aspartate (NMDA) receptor-coupled channel, is released. Glutamate then activates NMDA receptors and increases intracellular calcium, which initiates an excitotoxic cascade (Zeewalk & Nicklas, 1992; Beal *et al.*, 1993). Studies of experimental models of Huntington's disease in mice (Bogdanow *et al.*, 1998; Levine *et al.*, 1999) and rats (Wüllner *et al.*, 1994) report an increase in the response of striatal neurons to NMDA activation.

Several drugs such as antioxidants, lamotrigine and coenzyme Q10 (Schulz *et al.*, 1996a,b) have been tested to prevent the neuronal damage induced by 3-NPA. Since a link has been suggested between mitochondrial dysfunction, striatal neurodegeneration and NMDA receptor activation, the neurodegenerative process in HD or 3-NPA-induced neurotoxicity could be prevented by blockade of NMDA receptors. MK-801 (dizocilpine), a non-competitive antagonist that blocks the NMDA receptor by binding to a site within its coupled ion channel (the phencyclidine site), protects against 3-NPA-induced neurotoxicity in rat brain striatum (Lee *et al.*, 2000). However, the use of NMDA receptor antagonists *in vivo* causes serious side effects such as hallucinations and psychotomimetic behaviour, mainly due to a complete blockade of the NMDA receptor function.

Orphenadrine is a central nervous system (CNS)-acting antimuscarinic drug used in the treatment of Parkinson's disease that is devoid of psychotomimetic side effects. In addition, orphenadrine acts as a 'mild' non-competitive antagonist of NMDA receptors by binding to the phencyclidine site (Kornhuber *et al.*, 1995). It prevents the neurotoxicity induced by kainic acid in rats, and glutamate-

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induced neuronal cell death in cerebellar granule cells (CGCs) (Sureda *et al.*, 1999). Moreover, some low-affinity channel-blocking antagonists at the NMDA receptor, such as memantine and amantadine, which possess fast strongly voltage-dependent open channel kinetics, are also neuroprotective and well tolerated (Parsons *et al.*, 1999).

After brain damage, proliferation of microglia occurs (Streit *et al.*, 1999). In the brain, peripheral-type benzodiazepine receptors (PBR) are mainly localized in microglia (Stephenson *et al.*, 1995; Kuhlmann & Guilarte, 2000). Therefore, microgliosis is accompanied by an increase in the number of PBRs in the affected area (Benavides *et al.*, 1990; Stephenson *et al.*, 1995) and PBR is thus a suitable indirect indicator of neuronal damage (Benavides *et al.*, 1987). In addition, the 27 kD heat-shock protein (HSP27) is expressed in astroglia after brain damage (Renkawek *et al.*, 1993), and is used as a marker of astroglial stress. We hypothesize that orphenadrine may be suitable for the treatment of neurodegenerative disorders involving excitotoxicity. To test this, we evaluated the protective effect of orphenadrine against 3-NPA-induced neurotoxicity in both CGCs and in rat brain by measuring, respectively, its effect on cell viability and the increase of PBR density and HSP27 expression.

Methods

Drugs

3-NPA, orphenadrine, neutral red and cell culture salts and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ro 5-4864 was purchased from Fluka Chemie AG (Germany). [^{3}H]-PK 11195 (86 Ci mmol^{-1}) was from New England Nuclear (Wilmington, DE, U.S.A.). Cell culture media were obtained from GIBCO (Life Technologies, Paisley, U.K.). Other chemical reagents were of analytical quality.

Cerebellar granule cell cultures

Primary cultures of CGCs were prepared from 7-day-old Sprague Dawley rat pups using the method of Nicoletti *et al.* (1986). Briefly, cerebella were quickly removed and cleaned of meninges, followed by manual slicing with a sterile blade, dissociation with trypsin and treatment with DNase. Cells were adjusted at 8×10^5 cells ml^{-1} and were plated on both poly-L-lysine-coated 24-well plates and glass coverslips at a density of 320,000 cells cm^{-2}. Cultures were grown in Eagle's medium (BME basal medium) containing 10% FCS, 2 mM L-glutamine, 0.1 mg ml^{-1} gentamicin and 25 mM of KCl. Cytosine arabinoside (10 μM) was added 16–18 h after plating, in order to inhibit the growth of non-neuronal cells. Cultures prepared by this method were more than 95% enriched in granule neurons.

Treatment of CGCs and survival assay

CGCs were used after 7–10 days in culture. 3-NPA was dissolved in culture medium and neutralized with NaOH to pH 7.4 before being added to the cells at different concentrations. To investigate the neuroprotective effect of

orphenadrine, different concentrations of this drug were added to the medium 30 min before 3-NPA. Cell death was determined 24 h after 3-NPA addition using the neutral red assay and laser scanning cytometry.

Laser scanning cytometry

CGCs grown on glass coverslips were stained for at least 10 min with propidium iodide at a final concentration of 10 μg ml^{-1} (Darzynkiewicz *et al.*, 1999). Propidium iodide crosses disrupted cell membranes and stains the DNA of dead cells. Cell viability was measured using a laser scanning cytometer (LSC) (CompuCyte, Cambridge, MA, U.S.A.) with a red fluorescence detector. The laser scanned the coverslip and the cytometer stored the position, scatter and fluorescence of each cell, so that they could be easily localized and observed through the coupled microscope, equipped with a CCD camera. A total number of 2500 cells were analysed per coverslip and representative images were taken with a Leica DMRB fluo microscope using the 20× object lens.

Neutral red viability assay

Twenty-four hours after 3-NPA addition, 10 μl of a neutral red (NR) solution was added to each culture well to give a final concentration of 40 μg ml^{-1} (Babich & Borenfreund, 1991). The NR solution had been preincubated overnight at 37°C and centrifuged (1500 × *g* for 10 min) prior to use to remove fine precipitates of dye crystals. The assay plate was then returned to the incubator for another 3 h to allow for uptake of the supravital dye into the viable CGCs. Thereafter, the media was removed and the cells were rapidly washed with PBS followed by the addition of 0.2 ml of a solution of 1% acetic acid-50% ethanol to extract the dye from the cells. After 10 min at room temperature and gentle shaking, the absorbance of the extracted dye was measured in a microplate spectrophotometer (BioRad 550) equipped with a 540 nm filter. Surviving cells are given as percentage compared with untreated cells (controls). The EC$_{50}$ value for 3-NPA was calculated by non-linear regression using GraphPAD Prism software (GraphPAD Software, San Diego, CA, U.S.A.).

In vivo *drug treatments*

The ethics committee of the Universitat de Barcelona, according to the European Community guidelines, approved the protocol concerning the use of experimental animals carried out in this study. Adult male Sprague Dawley rats (275–300 g) were obtained from Harlan Ibérica (Spain). They were housed at 21±1°C and maintained under a 12 h light/dark cycle. On day 1, 3-NPA was dissolved in saline, adjusted to pH 7.4 with NaOH and administered to a group of 16 rats (3-NPA group) at a dose of 30 mg kg^{-1} i.p. once a day for 3 days. With this dosing regimen the animals displayed the symptoms of 3-NPA toxicity after 3–4 days. Three groups of 10 animals received, respectively, a single dose of 10, 20 or 30 mg kg^{-1} i.p. of orphenadrine (dissolved in saline) 30 min before 3-NPA (30 mg kg^{-1}, i.p.), on each day of treatment. Another group of 10 animals were injected with saline (control group) and three groups of three animals per group received orphenadrine alone at 10, 20 or

30 mg kg^{-1}, i.p., respectively (orphenadrine controls). The animals were weighed on days 1–4 and 8. On day 8, surviving rats were killed by decapitation, and striatum, hippocampus and cerebral cortex were dissected out, frozen on dry ice and stored at $-80°$C.

Tissue homogenate preparation

Tissue samples were thawed on ice, weighed and homogenized in 10 volumes of cold homogenization buffer (5 mM Tris-HCl, 320 mM sucrose, 4.5 μg μl^{-1} aprotinin, 10^{-4} M phenylmethylsulphonyl fluoride (PMSF) and 10^{-3} M sodium orthovanadate, pH 7.4) with a Potter-Elvehjem Teflon-glass homogenizer. The homogenates were centrifuged at 15,000 × g for 30 min at 4°C. The pellets were resuspended in buffer, washed twice and re-centrifuged at 15,000 × g for 30 min. The final pellets were resuspended in Tris-HCl 50 mM buffer (pH 7.4) containing protease inhibitors (4.5 μg μl^{-1} aprotinin, 10^{-4} M PMSF and 10^{-3} M sodium orthovanadate). Striatum preparations from five saline-treated (controls) and four 3-NPA-treated rats were pooled and reserved for saturation binding experiments. The remaining homogenates were aliquoted separately for both binding and Western blot assays. The homogenates were stored at $-80°$C until use. Protein content was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as the standard.

Radioligand binding assays

Equilibrium binding assays were performed at 0–4°C using [^{3}H]-PK 11195. Assays were done in a final volume of 0.25 ml (Tris-HCl 50 mM, pH 7.4), which contained 125 μl of [^{3}H]-PK 11195 (in a concentration range of 0.5–15 nM) and 125 μl of tissue homogenate preparation (100 μg of protein per assay). Specific binding was defined as the difference between the radioactivity bound in the absence (total binding) and in the presence (non-specific binding) of 10 μM of unlabelled Ro 5-4864.

After incubation for 120 min, samples were filtered under vacuum on to Whatman GF/B glass fibre filters pre-soaked in 0.5% polyethyleneimine. Filters were rapidly washed three times in 4 ml of ice-cold Tris-HCl (50 mM) and placed in vials containing 10 ml of Cocktail Scharlau Biogreen 1 (FEROSA, Spain). Radioactivity was measured by liquid scintillation spectroscopy in a Beckman LS-1800 with an efficiency of 40%. The specific binding in these conditions was about 90%.

Control 3-NPA and orphenadrine treated animals were compared by measuring specific [^{3}H]-PK 11195 binding at a concentration of 2 nM in the conditions described above.

Data analyses and statistics

Analysis of saturation isotherms (K_d, dissociation constant; B_{max}, maximum density of binding sites) was determined by computer-assisted non-linear regression, using Radlig 4.0 software (Biosoft, Elsevier). Saturation data are expressed as the best fit value ± standard error of the mean (s.e.mean) obtained from three separate experiments carried out in duplicate. Other data are expressed as mean ± s.e.mean from n determinations carried out in duplicate. Multiple mean comparisons were done by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered to be significant at the $P < 0.05$ level.

Western blot analysis

Aliquots of tissue homogenate, containing 30 μg of protein per sample, were placed in sample buffer (0.5 M Tris-HCl pH 6.8, 10% glycerol, 2% (w v^{-1}) SDS, 5% (v v^{-1}) 2-β-mercaptoethanol, 0.05% bromophenol blue) and denaturalized by boiling at 95–100°C for 5 min.

Samples were separated by electrophoresis on 10% acrylamide gels. Subsequently, proteins were transferred to polyvinylidene fluoride sheets (Immobilon^{TM}-P, Millipore Corp., Bedford, MA, U.S.A.) using a transblot apparatus (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Membranes were blocked overnight with 5% non-fat milk dissolved in TBS-T buffer (Tris 50 mM; NaCl 1.5%; Tween 20, 0.05%, pH 7.5). Membranes were then incubated with a primary rabbit polyclonal antibody anti-HSP27 (1 : 5000, SPA-801, StressGen Biotechnologies Corp., Victoria, BC, Canada). After 90 min, blots were washed thoroughly in TBS-T buffer and incubated for 1 h with peroxidase-conjugated anti rabbit IgG antibody (Amersham Corp., Arlington Heights, IL, U.S.A.). Immunoreactive protein was visualized using a chemiluminescence-based detection kit according to the manufacturer's protocol (ECL kit; Amersham Corp., Arlington Heights, IL, U.S.A.).

Results

Neuroprotective effects of orphenadrine against 3-NPA-induced neurotoxicity in cerebellar granule cell cultures

Twenty-four hours exposure to 3-NPA (1–1000 μM) caused a concentration-dependent neuronal mortality, as assessed by a decrease in neutral red accumulation (Figure 1) and an increase in propidium iodide staining (Figure 2). The EC$_{50}$ value for 3-NPA was 144 ± 3 μM ($n = 5$). When CGCs were exposed to 100 μM 3-NPA in the presence of different
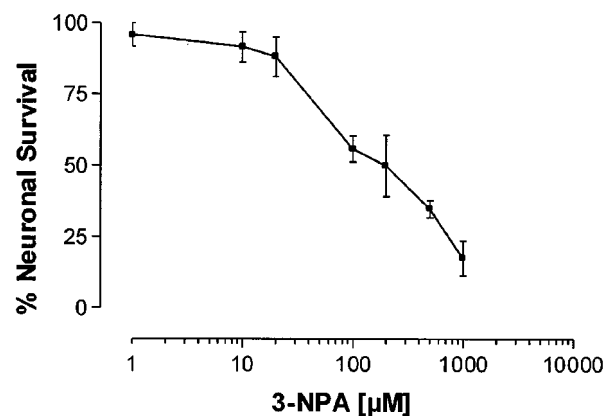

Figure 1 Cerebellar granule cell viability after treatment with 3-NPA. 7–10 days old CGCs were treated with a range of concentrations of 3-NPA (1–1000 μM). Cell viability was assessed 24 h after by neutral red assay. The results are presented as the means ± s.e.mean from three to five separate experiments carried out in quadruplicate.

concentrations of orphenadrine, they were protected from death (Figures 2 and 3). The highest cytoprotective effect of orphenadrine was at a concentration of 6 μM (Figure 3). Doses of orphenadrine above 24 μM failed to prevent 3-NPA toxicity and concentrations above 48 μM potentiated cell death. Orphenadrine alone, at a concentration of 12 μM, had no effect either on cell viability or on morphology but, at a concentration of 48 μM, induced a rapid necrotic death (data not shown). Visually, 3-NPA induced cellular aggregation, volume diminution and neurite fragmentation that were prevented by orphenadrine pre-treatment (Figure 4).

Effect on mortality and loss of body weight

3-NPA-treated animals showed general incoordination, drowsiness and general weakness. Some showed hindlimb paralysis without rigidity, recumbency and some of these died. Mortality in the groups 3-NPA and 3-NPA pre-treated with 10 mg kg^{-1} orphenadrine was 40%. The groups pre-treated with 20 and 30 mg kg^{-1} orphenadrine showed 30 and 10% mortality, respectively.
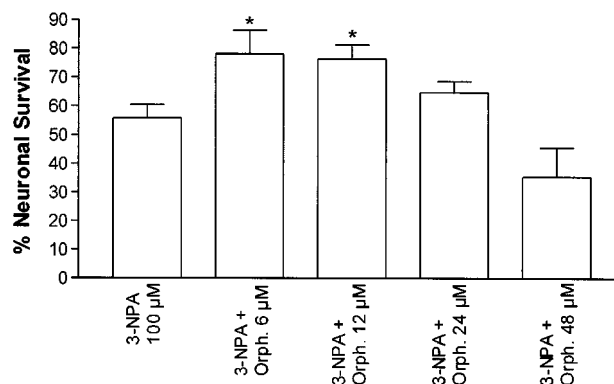

Figure 3 Orphenadrine protects cerebellar granule cells from 3-NPA neurotoxicity. Cerebellar granule cells were pre-treated with orphenadrine previously to 3-NPA (100 μM). Cell survival was assessed after 24 h by neutral red assay. The results are given as the percentage of absorbance of treated cells referred to that obtained in control cells. The results are presented as the means$\pm$s.e.mean from three to five separate experiments carried out in quadruplicate. Statistical analysis was performed using ANOVA. (*$P < 0.05$ vs 3-NPA group).
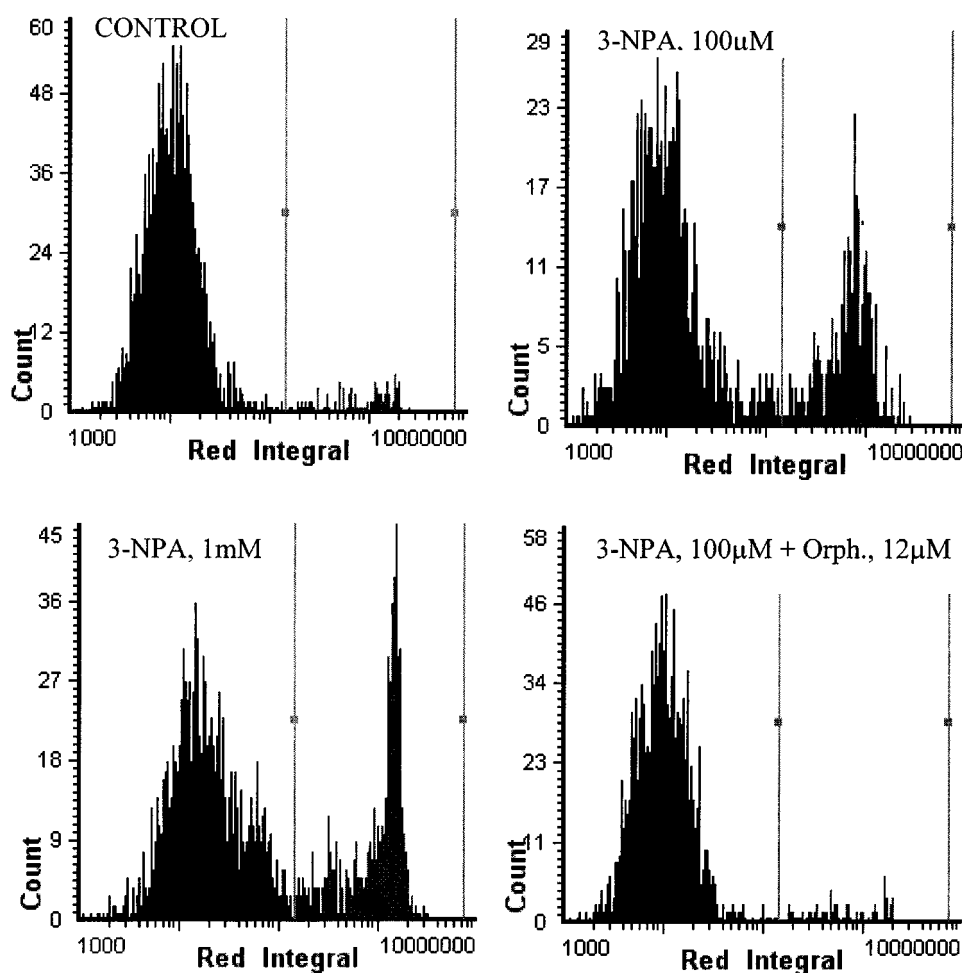

Figure 2 Laser scanning histograms showing the effect of orphenadrine against 3-NPA induced toxicity in CGCs. Pre-treatment of CGCs with orphenadrine induced a diminution of propidium iodide staining (cells between vertical bars), indicating a neuroprotective effect. The ordinate is the number of cells at each propidium iodide fluorescence intensity. Histograms are each one representative of those obtained from three different experiments carried out in triplicate.

All the groups of 3-NPA treated rats suffered a significant loss of body weight (Figure 5), which continued until day 4, when it was maximum. After day 4, a partial recovery of body weight was observed, although control values were not reached. Although the di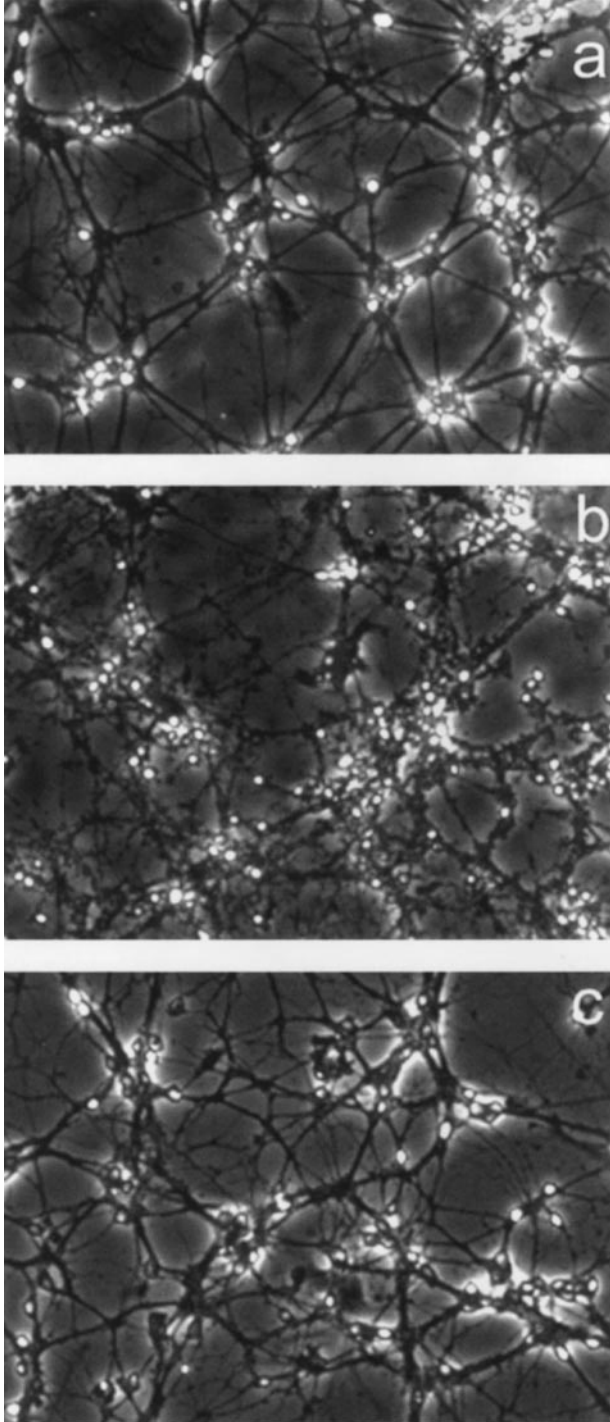fferences were not statistically significant, rats receiving 30 mg kg^{-1} orphenadrine before 3-NPA showed a slightly faster recovery of body weight.

[^{3}H]-PK 11195 binding

Specific binding of [^{3}H]-PK 11195 to homogenate preparations of striatum was saturable and of high affinity (Figure 6). Using non-linear regression analysis, our data were fitted by a single-site model, which yielded a K_d of 1.7 ± 0.2 nM ($n=3$) for control and of 4.7 ± 1 nM ($n=3$) for 3-NPA rats ($P<0.01$). Maximal binding site densities were respectively 400 ± 37 fmol mg^{-1} protein and 843 ± 45 fmol mg^{-1} protein ($P<0.0001$). All subsequent assays were performed using 2 nM [^{3}H]-PK 11195.

The density of [^{3}H]-PK 11195 (2 nM) binding sites in striatum is given in Figure 7a. 3-NPA (30 mg kg^{-1} per day

Figure 4 Phase-contrast micrographs of cerebellar granule cells injured by 3-NPA and protected by orphenadrine. 3-NPA induced cellular aggregation, volume diminution and neurite fragmentation that were prevented by orphenadrine pre-treatment. Control cells (a), cells treated with 3-NPA 100 μM (b) and 3-NPA 100 μM plus 12 μM orphenadrine (c) are shown.
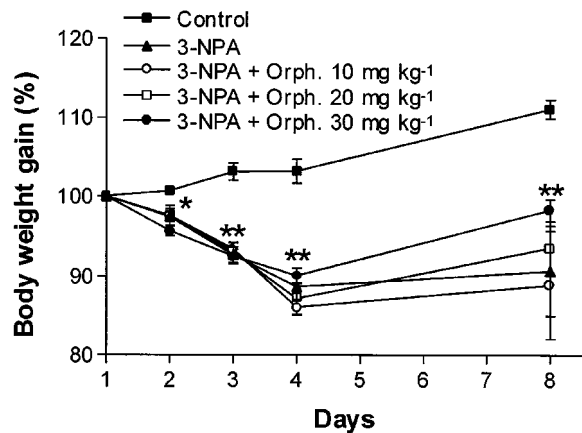
Figure 5 Temporal evolution of rats' body weight during (days 1–3) and after (days 4–8) treatment with 3-NPA or orphenadrine plus 3-NPA. Data are expressed as means $\pm$ s.e.mean of the percentage of initial body weight (100%) for the surviving rats of each group (*$P<0.05$ and **$P<0.01$, for all the groups treated with 3-NPA vs control group).
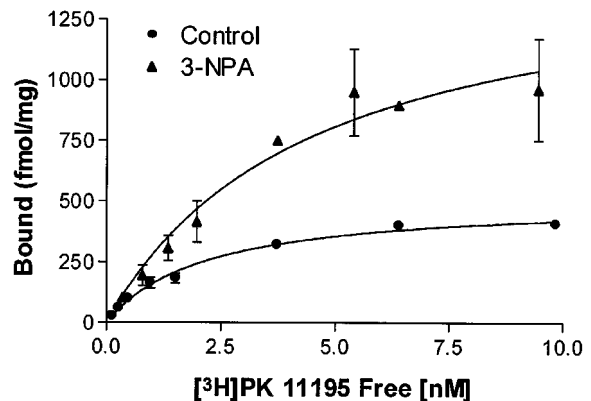
Figure 6 Saturation curves of [^{3}H]-PK 11195 binding to striatum. Rats received daily 30 mg kg^{-1} 3-NPA during 3 days and were sacrificed at day 8 from the start of treatment. Specific binding at increasing concentrations of [^{3}H]-PK 11195 was measured to homogenates from control and 3-NPA-treated rats. Results are means $\pm$ s.e.mean of data obtained from three separate experiments carried out in duplicate. Details of the binding procedure are described in Methods.

for 3 days) induced a significant 170% increase in PBR (171 ± 16 fmol mg^{-1}, $n = 5$, for control compared with 463 ± 57 fmol mg^{-1}, $n = 6$, for 3-NPA-treated rats, $P < 0.05$). Pre-treatment with 30 mg kg^{-1} orphenadrine per day prevented the 3-NPA-induced PBR increase to a value not different from control values (244 ± 9 fmol mg^{-1}, $n = 9$, $P < 0.05$ vs 3-NPA group; $P > 0.05$ vs control group). Lower doses of orphenadrine had no effect on the 3-NPA-induced increase in PBR. Orphenadrine alone had no effect on PBR levels (data not shown).

3-NPA increased PBR levels in the hippocampus of three animals (193 ± 13 fmol/mg for control, $n = 5$, and 443 ± 118 for 3-NPA treated, $n = 3$, $P < 0.05$). Figure 7b shows the mean PBR levels of all the treated animals. The PRB levels in the hippocampus from animals pre-treated with 30 mg kg^{-1} orphenadrine per day were not different from control (249 ± 17 fmol mg^{-1}, $n = 9$, $P > 0.05$ vs control; $P < 0.05$ vs

3-NPA). No increase in PBR levels was found in cortex of 3-NPA rats (224 ± 44 fmol mg^{-1}, $n = 6$, vs 205 ± 29 fmol mg^{-1}, $n = 5$, control group, $P > 0.05$).

Western blot analysis

The Western blot analysis of stress protein in the striatum showed a marked expression of HSP27 in 3-NPA rats, whereas no expression was detected either in control or in animals treated with orphenadrine alone. Pre-treatment with 30 mg kg^{-1} orphenadrine before 3-NPA administration inhibited HSP27 expression. Lower doses of orphenadrine had no effect on HSP27 expression after 3-NPA administration. Only three of the rats treated with 3-NPA alone expressed HSP27 in the hippocampus but none of the rats pre-treated with 30 mg kg^{-1} of orphenadrine expressed this protein in this area ($n = 6$ for each group). Figure 8 shows representative Western blots of striatum and hippocampus originating from the same rats. The animals showing the highest levels of PBR also showed the highest HSP27 expression.

Discussion

This study demonstrates that orphenadrine protects *in vitro* and *in vivo* against 3-NPA-induced neuronal damage because it prevents cell mortality and the expression of two markers of neuronal damage (HSP27 and increase in PBR number) in the striatum of rats.

Histological and neurochemical analyses have demonstrated that systemic administration of 3-NPA to mature rats induces striatal damage (Beal *et al.*, 1993; Miller & Zaborszky, 1997; Lee *et al.*, 2000). Microgliosis accompanies the neuronal degeneration induced by several neurotoxins in rat brain. As in the brain PBRs are concentrated in the microglia, their increase is widely being used as an indirect
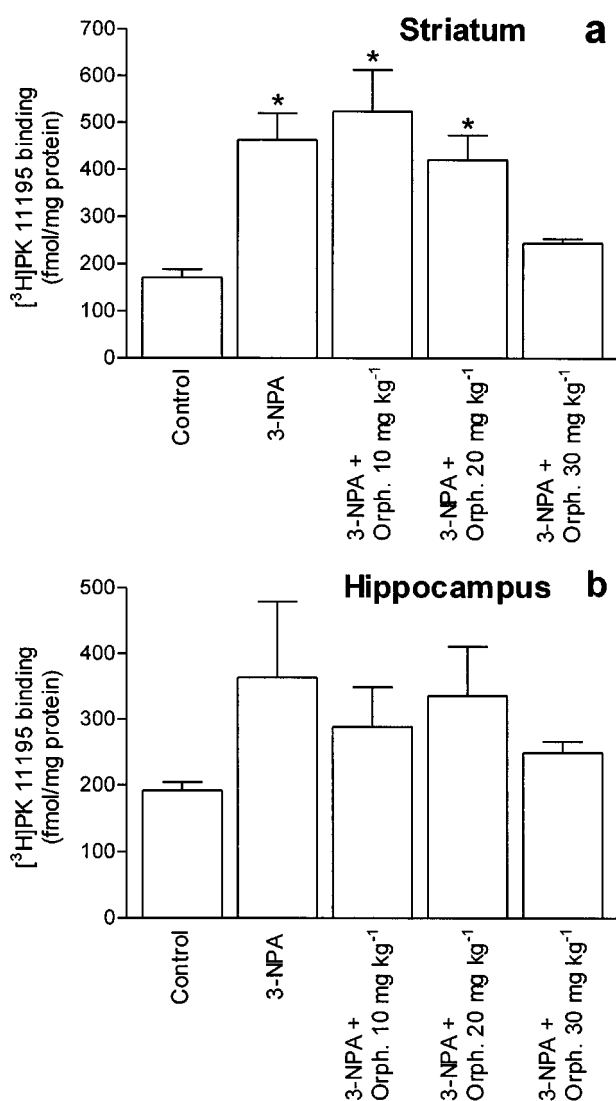

Figure 7 Effect of pre-treatment with orphenadrine on 3-NPA-induced increase in PBR density. [³H]-PK 11195 binding (2 nM) was performed to homogenate preparations of striatum (a) and hippocampus (b) of individual rats, as described in Methods. Values are expressed as means ± s.e.mean of these obtained from five to nine animals. (*$P < 0.05$ vs control).
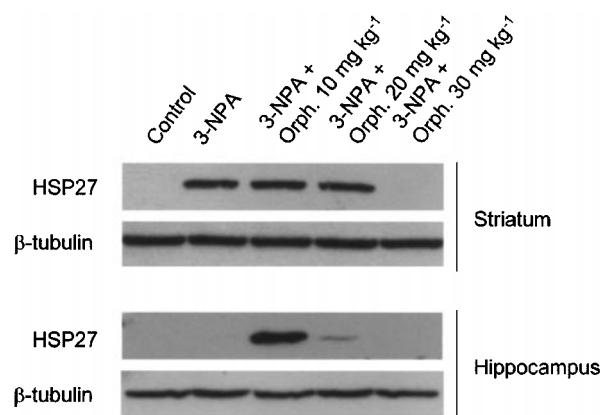

Figure 8 Representative Western blots showing HSP27 expression in striatum and hippocampus of the same rats after treatment with 3-NPA, alone or preceded by different doses of orphenadrine. Pre-treatment with 30 mg kg^{-1} orphenadrine prevented HSP27 expression induced by 3-NPA treatment in the striatum. Owing to the preference of 3-NPA lesions for the striatum, not all the rats treated with 3-NPA alone expressed this protein in the hippocampus (second lane). Western blots were performed in duplicate, with samples originating from at least four animals of each group of treatment. β-tubulin expression was used as a gel load control.

marker of neuronal damage (Benavides *et al.*, 1990; Stephenson *et al.*, 1995). For example, density of PBR increases in brain regions injured by excitotoxicity (Shoemaker *et al.*, 1982; Camins *et al.*, 1998) or focal ischemia (Benavides *et al.*, 1990). In our experiments, 3-NPA administration to rats increased both B_{max} and K_d for $[^3H]$-PK 11195 binding. Therefore, these results indicate that the damage induced by 3-NPA is also accompanied by an increase in $[^3H]$-PK 11195 binding to striatum. This increase is prevented by orphenadrine at a dose of 30 mg kg^{-1} before 3-NPA administration, indicating a protective effect. The mitochondrial damage induced by this neurotoxin could lead to an alteration of mitochondrial membrane structure that could be responsible for the decrease of the $[^3H]$-PK 11195 affinity for the peripheral benzodiazepine receptor in the damaged striatum.

Several reports show that acute intraperitoneal administration of 3-NPA can damage cerebral areas other than the striatum, such as the hippocampus and the cortex (Beal *et al.*, 1993; Miller & Zaborsky, 1997; Brouillet *et al.*, 1999; Lee *et al.*, 2000). Our results show that, in some of the animals, 3-NPA can induce an increase of PBR levels in the hippocampus. This increase was prevented by pre-treatment with 30 mg kg^{-1} orphenadrine. As we did not detect a significant increase in $[^3H]$-PK 11195 binding in the cortex, this corroborates the reported selectivity for 3-NPA-induced damage, which affects mainly the striatum and, in some cases, can spread to the hippocampus.

On the other hand orphenadrine, at a dose of 30 mg kg^{-1}, prevented the expression of HSP27 in the striatum after 3-NPA administration. Kato *et al.* (1994; 1995) described that the distribution of HSP27 corresponds to reactive astroglial cells. Moreover, its expression is increased after ischemia (Kato *et al.*, 1994; 1995), administration of kainic acid (Plumier *et al.*, 1996) and in Alzheimer's disease (Renkawek *et al.*, 1993). In addition, astrocytosis is prominent in both Huntington's disease (Vonsattel *et al.*, 1985) and in chronic experimental 3-NPA intoxication (Beal *et al.*, 1993). Therefore HSP27 expression is a useful parameter to measure the extent of the 3-NPA toxicity in the striatum and, as it was prevented by orphenadrine (30 mg kg^{-1}), indicates a neuroprotective effect of this drug. Owing to the reported preference of 3-NPA-induced lesions for the striatum, not all the rats treated with 3-NPA alone expressed HSP27 in the hippocampus. This is in agreement with the findings obtained by Beal *et al.* (1993), who reported that only some of the animals that received 3-NPA showed gliosis and neuronal loss in the hippocampus, in addition to striatal lesions. Thus the expression of HSP27 in the hippocampus is not reliable for measuring neuroprotective effects against 3-NPA as it does not follow the same pattern than the striatum. However, the fact that none of the rats pre-treated with orphenadrine 30 mg kg^{-1} expressed HSP27 in the hippocampus gives support to the protective effects observed in striatum.

HSP27 has a key role in reactive astrocytes by regulating the dynamics of actin filaments, probably during structural remodelling after neuronal degeneration (Plumier *et al.*, 1996). Transfection of HSP27 has an antiapoptotic effect in neurons through an increase in the levels of glutathione (Guénal *et al.*, 1997; Mehlen *et al.*, 1999; Wagstaff *et al.*, 1999). It remains to be determined whether the increase in HSP27 expression in astrocytes *in vivo* is also neuroprotec-

tive. In this context, the increase in HSP27 expression found after 3-NPA administration could be a response to neural injury. Moreover, the prevention of 3-NPA-induced HSP27 expression by orphenadrine contributes to the neuroprotective effect of this drug in this experimental model of Huntington's disease.

Following 3-NPA administration, all groups of rats suffered a loss of body weight, which was slightly recovered after discontinuing 3-NPA. Both central and peripheral effects of 3-NPA could explain this effect. The striatal lesions and bradykinesia (Guyot *et al.*, 1997) could be partly responsible for reducing rat appetite and food intake. However, peripheral effects such as the impairment of energetic metabolism, mobilization of energy stores and lipid peroxidation by nitroanions (Fu *et al.*, 1995) would be the main cause of weight loss. The rats pre-treated with 30 mg kg^{-1} orphenadrine also suffered from loss of weight and, although they recovered slightly better than the other groups, they did not reach control levels. This observation supports that the protective effect of orphenadrine takes place mainly in the CNS, where NMDA receptors are located.

Several hypotheses have been proposed to explain the mechanism of neuronal damage induced by 3-NPA. Among them, indirect excitotoxic lesion by NMDA receptor activation is the most widely described (Wüllner *et al.*, 1994) although currently it is more accepted that 3-NPA neurotoxicity is the result of cooperative pathways between excitotoxicity, metabolic compromise and oxidative stress (reviewed by Alexi *et al.*, 1998). 3-NPA, by inhibiting succinate dehydrogenase, a key enzyme of the Kreb's cycle, causes an energetic impairment which depletes ATP. The lack of ATP causes the failure of ATP-dependent ion pumps and channels which results in depolarization of glutamaceptive neurons and relief of the Mg^{2+} block in the NMDA receptor channel. Then physiological concentrations of glutamate can activate the receptor channel, leading to Ca^{2+} influx. This Ca^{2+} influx cannot be reversed because of the depleted ATP, leading to Ca^{2+} overload and excitotoxicity. On the other hand, Ca^{2+} activates enzymes such as nitric oxide synthase, which produces NO and free radicals that induce oxidative stress together with those produced by impaired mitochondrial function. In addition, dopamine, which has been found to increase after 3-NPA systemic administration, can also lead to the production of free radicals (Nishino *et al.*, 1997). Moreover, energy collapse can by itself cause cell death due to inhibition of vital protein synthesis, which depends on ATP.

In view of the several pathways implicated, several strategies have been adopted to prevent 3-NPA-induced neuronal damage. Schulz *et al.* (1996a,b) assayed *in vivo* several compounds acting at these different pathways, that is: free radical trappers (S-PBN), coenzyme Q_{10}, which improves mitochondrial function, and compounds that inhibit glutamate release (lamotrigine) or block NMDA receptors (MK-801, memantine). All these compounds showed a protective effect that was potentiated when they were associated with another compound acting at a different point. The protective effects of MK-801 and lamotrigine have also been assessed *in vivo* through magnetic resonance imaging and proton magnetic resonance spectroscopy (Lee *et al.*, 2000). On the other hand, the key role for free radicals in 3-NPA toxicity has been demonstrated in transgenic mice expressing

increased free radical scavenging activity (Beal *et al.*, 1995) as they were more resistant than wild-type to 3-NPA neurotoxicity. Fu *et al.* (1995) found an increase in cerebral activity of superoxide dismutase and content of malonaldehyde that indicates a defensive response to oxidative stress, as well as an increased lipid peroxidation in liver. A recent study (La Fontaine *et al.*, 2000) has measured brain protein oxidation in rats after 3-NPA treatment and provides evidence that a widespread oxidative damage occurs prior to the appearance of morphologic lesions.

Such cooperative neurotoxic pathways have also been evidenced *in vitro*. It has been demonstrated that 3-NPA is toxic to CGCs through a mechanism involving NMDA receptors activation because both competitive (AP5) and non-competitive (MK-801) NMDA receptor antagonists prevent its neurotoxic effect (Weller & Paul, 1993). Similar results have been reported for hippocampal neurons (Pang & Geddes, 1997). But in addition to NMDA receptor activation there is an increased Ca^{2+} influx and formation of free radicals because free radical scavengers protect CGCs from 3-NPA-induced damage (Olsen *et al.*, 1999). Also, there exists a synergism between the toxicities of 3-NPA and NMDA in striatal cultures (Greene *et al.*, 1998), indicating that excitotoxicity and metabolic impairment act coordinately. In fact, energy-compromised neurons are more vulnerable to excitotoxicity of glutamate (Novelli *et al.*, 1988). As far as the resulting cell death pathway is concerned, some authors (Weller & Paul, 1993; Pang & Geddes, 1997) reported that, in neuronal cultures, 3-NPA toxicity could be prevented after a short exposure (until 24 h) to this toxin with NMDA receptor antagonists but, after longer periods of exposure, these compounds were unable to prevent cell death. These observations pointed to the existence of an acute excitotoxic cell death and a slowly evolving apoptotic death that cannot be attenuated by NMDA receptor blockade, as a result of metabolic impairment. Our experiments were performed at 24 h exposure to 3-NPA and therefore the death was mostly excitotoxic. Under these circumstances, NMDA receptor blockade by orphenadrine could be protective, as shown by our results. We used CGCs after 7–10 days in culture, when the neurite networking was complete. Older cells were not assayed because an increased sensibility of CGCs to 3-NPA toxicity after day 13 in culture has been reported (Weller & Paul, 1993).

Orphenadrine, in addition to its antimuscarinic properties, acts as a non-competitive NMDA receptor antagonist, and inhibits [^{3}H]-MK-801 binding to the phencyclidine (PCP) site at the NMDA receptor complex (Kornhuber *et al.*, 1995). Thus, protection by orphenadrine may be due to the blockade of NMDA receptors. Indeed, orphenadrine protects CGCs from glutamate neurotoxicity and protects rat brain from the excitotoxic lesion induced by kainic acid (Sureda *et al.*, 1999). Similarly, in the present study we have found that orphenadrine protects CGCs from 3-NPA-induced toxicity.

High-affinity NMDA receptor channel blockers such as MK-801 and PCP produce severe psychotomimetic side effects owing to a persistent blockade of the receptors and cannot be used therapeutically (see Parsons *et al.*, 1999 for a review). Their slow unblocking kinetics and low voltage dependency make them unable to leave the channel even when the neuron depolarizes after a physiological stimulus by glutamate (mM concentrations). Under pathological conditions, NMDA receptors are activated by lower (μM) concentrations of glutamate than under physiological conditions, but for much longer periods of time (Mitani *et al.*, 1992). Thus, there would be of interest NMDA receptor channel blockers that could discriminate between physiological and pathological activation and would block only under pathological circumstances. Memantine and amantadine are compounds that block the NMDA receptor channel at low micromolar concentrations (K_i 0.5 and 10 μM, respectively, whereas MK-801 and PCP show nanomolar affinity) and show fast, strongly voltage-dependent open channel kinetics (Sobolevsky *et al.*, 1998; Parsons *et al.*, 1999). This means that, under resting conditions, both Mg^{2+} and these compounds occupy the channel. Likewise, both are able to leave the channel upon strong synaptic depolarization (physiological conditions) due to their pronounced voltage-dependency and rapid unblocking kinetics. However, memantine and amantadine do not leave the channel so easily as Mg^{2+} upon moderate prolonged depolarization (pathologic). These features make these compounds able to permit physiological glutamatergic transmission and to block excitotoxic stimulus.

Orphenadrine possesses similar NMDA receptor antagonistic properties to amantadine and memantine. This drug has low micromolar affinity for the PCP site (K_i about 6 μM) and similar fast unblocking kinetics as memantine, both with a K_{off} value about 0.2 s^{-1} (Kornhuber *et al.*, 1995; Parsons *et al.*, 1999). Therefore orphenadrine would be expected to have similar neuroprotective effects. In fact, compounds such as amantadine, memantine, budipine and orphenadrine, which are well tolerated clinically and have been used for their anti-parkinsonian effects, have been proposed as candidates for clinical trials on the neuroprotective efficacy of NMDA receptor antagonism (Lange *et al.*, 1997).

Although orphenadrine showed some toxic effect on CGCs at concentrations above 24 μM, no toxicity was observed *in vivo* at the neuroprotective dose of 30 mg kg^{-1}. The behaviour of the rats treated with orphenadrine alone did not show any apparent disturbance compared with saline-treated rats. In humans, micromolar serum concentrations are reached under therapeutic doses (Altamura *et al.*, 1986; Contin *et al.*, 1987), which are enough for NMDA receptor blockade. Although orphenadrine has complex pharmacokinetics and some cases of serious adverse effects have been reported in Parkinson's patients (Gjerden *et al.*, 1998), this drug is being widely used for the treatment of parkinsonism and muscular distonias. The existing clinical experience with orphenadrine allows control and prevents the adverse effects. Therefore, while other safer open-channel kinetics NMDA receptor blockers are being developed and tested in clinical trials, orphenadrine could be considered as a preventive treatment for neurodegenerative disorders involving excitotoxicity.

Although these neuroprotective effects of orphenadrine are promising, more studies are necessary to evaluate its therapeutic safety in the treatment of neurodegenerative diseases.

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