Amino Acids (2002) 23: 31–36
DOI 10.1007/s00726-001-0106-6

Amino
Acids
Printed in Austria

# Nefopam, an analogue of orphenadrine, protects against both NMDA receptor-dependent and independent veratridine-induced neurotoxicity

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Received June 29, 2001
Accepted August 6, 2001
Published online June 3, 2002 

**Summary.** Nefopam hyghochloride is a potent analgesic compound commercialized in most Western Europe for 20 years, which possesses a profile distinct from that of opioids or anti-inflammatory drugs. Previous evidence suggested a central action of nefopam but the detailed mechanisms remain unclear. While, nefopam structure resembles that of orphenadrine, an uncompetitive NMDA receptor antagonist, here we report that differently from orphenadrine, nefopam (100 $\mu$M) failed to protect cultured cerebellar neurons from excitotoxicity following direct exposure of neurons to glutamate. Moreover, nefopam failed to displace MK-801 binding to hippocampal membranes. Nefopam effectively prevented NMDA receptor-mediated early appearance (30 min) of toxicity signs induced by the voltage sensitive sodium channel (VSSC) activator veratridine. The later phase (24 h) of neurotoxicity by veratridine occurring independently from NMDA receptor activation, was also prevented by nefopam. Nefopam effect was not mimicked by the GABA receptor agonist muscimol.

**Keywords:** Excitotoxicity – NMDA receptor antagonist – Voltage sensitive sodium channels – Cerebellar neurons in culture

## Introduction

Nefopam hydrochloride is a potent analgesic compound commercialized in most Western Europe for 20 years. Nefopam possesses a profile distinct from that of opioids or anti-inflammatory drugs. It does not cause tolerance, withdrawal reactions or physical dependence, and the potential for its abuse is very low (Heel et al., 1980). Furthermore, nefopam does not produce respiratory depression even in the post-operative period (Gasser and Bellville, 1975; Gerbershagen and Schaffner, 1979). Clinical studies have demonstrated nefopam to be very effective in the prevention of postoperative shivering in patients after general anesthesia (Rosa et al., 1995) without affecting the recovering time between the end of anesthesia and extubation (Piper et al., 1999). Unpleasant adverse effects during therapeutic use have been also reported including dizziness, headache, nausea, vomiting and sweating, consistent with a central mode of action of the drug. Although these side effects are usually minor and not very long lasting, they can probably explain the limited development of nefopam for post-operative use in the last years.

The analgesic properties of nefopam are now being reinvestigated. This drug has been recently demonstrated to induce a rapid and strong depression of the nociceptive flexion ($R_{III}$) reflex in humans (Guirimand et al., 1999), probably through a central mechanism of action (Hunskaar et al., 1987; Fasmer et al., 1987). However, the detailed mechanisms underlying the pharmacological actions of nefopam remain unclear. Evidence exist suggesting a possible action of nefopam on the neurotransmission mediated by glutamate. Thus, nefopam is a cyclic analogue of orphenadrine and diphenhydramine, drugs originally synthesized as central myorelaxants which exert unspecific antagonistic activity at the phencyclidine binding site of NMDA receptors (Kornhuber et al., 1995). Furthermore, nefopam shows pre-emptive analgesic effects in a model of neuropathy (chronic constriction injury of

the sciatic nerve) (Biella et al., 2002) which involves the activation of NMDA receptors.

In this study we tested nefopam for protection against NMDA-receptor mediated neurotoxicity in cultured cerebellar neurons, as well as for affinity to the phencyclidine binding site of the NMDA receptor in rat hippocampal membranes.

## Materials and methods

*Cell cultures*

Primary cultures of rat cerebellar neurons were prepared as previously described (Novelli et al., 1988; 1992). Briefly, cerebella from 8-day-old pups were dissected, cells were dissociated and suspended in basal Eagle's medium with 25 mM KCl, 2 mM glutamine, 100 $\mu$g/ml gentamycin and 10% fetal calf serum. Cells were seeded in poly-L-Lysine coated (5 $\mu$g/ml) 35 mm dishes at $2.5 \times 10^5$ cells/cm$^2$ and incubated at 37°C in a 5% $CO_2$, 95% humidity, atmosphere. Cytosine arabinoside (10 $\mu$M) was added after 20–24 h of culture to inhibit the replication of non-neuronal cells. After 8 days in vitro, morphologically identifiable granule cells accounted for more than 95% of the neuronal population, the remaining 5% being essentially GABAergic neurons. Astrocytes did not exceed 3% of the overall number of cells in culture. Cerebellar neurons were kept alive for more than 40 days in culture by replenishing the growth medium with glucose every 4 days and compensating for lost amounts of water, due to evaporation.

*Neurotoxicology*

Neurons were used between 14–20 days in culture. Drugs were added into the growth medium at the indicated concentrations, and neuronal cultures were observed for signs of early neurotoxicity at 30 min., as well as for neuronal survival 24 h thereafter, by phase contrast microscopy. To quantify neuronal survival cultures were stained with fluorescein diacetate and ethidium bromide (Novelli et al., 1988; Fernández et al., 1991), photographs of three randomly selected culture fields were taken and live and dead neurons were counted. Results were expressed as percentage of live neurons Total number of neurons per dish was calculated considering the ratio between the area of the dish and the area of the picture (~3,000).

*Receptor binding*

Male Sprague-Dawley rats (200–250 g) were decapitated and their brains were removed rapidly. Hippocampi were dissected and pooled. Tissue preparation was performed according to the method of Wong and collaborators (Wong et al., 1986) with minor modification. In brief, hippocampi were homogenized in 9 volumes of ice-cold 0.32 M sucrose by nine strokes with a Teflon/glass homogenizer at 500 rpm. The homogenate was centrifuged at 1,000 × g, and the supernatant was recentrifuged at 10,000 × g for 20 min at 4°C. The pellet was suspended in assay buffer (Tris-HCl 5 mM, pH 7.4) and incubated at 23°C for 20 min prior to final centrifugation at 10,000 × g at 4°C. The pellet was resuspended in assay buffer (55 ml/gram of original tissue) and frozen rapidly at −80°C until use.

On the day of assay the membranes were thawed and displacement studies were carried out using an incubation volume of 1 ml containing 0.4–0.5 mg membrane protein, 5 mM Tris-HCl buffer (pH 7.4), 10 nM [$^3$H]-MK801 and increasing concentrations of MK801 and nefopam (from $10^{-10}$ to $10^{-5}$ M and from $10^{-7}$ to $10^{-2}$ M respectively).

The mixture was incubated for 45 min at 23°C and then subjected to rapid filtration through Whatman GF/B filters, which were washed immediately with two 5 ml portions of ice-cold assay buffer in a Millipore filter system. The time required for the complete filtration and washing procedure was less than 10 seconds. Radioactivity retained on the filters was determined by using a conventional liquid scintillation counter (Packard, Meriden, CT, USA). Protein concentration were determined according to the method of Lowry et al. (1951).

All experiments were carried out in duplicate.

*Data presentation and analysis*

For statistical analysis the one-way or the two-way analysis of variance (ANOVA) was used to identify overall treatment effects, followed by the unpaired two-tailed Student's t-test for selective comparison of individual data groups. Only significances relevant for the discussion of the data are indicated in each figure.

*Materials*

[$^3$H]MK801 (22.5 Ci/mmol) was purchased from DuPont/NEN (Boston, MA, USA). Unlabeled (+)-10,11-dihydro-5-methyl-5H-dibenzo-[a,d]-cyclohepten-5,10-imine hydrogen maleate (MK-801) was obtained from RBI (Natick, MA, USA) as well as from Sigma. Saxitoxin was a generous gift of Dr. V. Zitko of St. Andrews Biological Station, N.B. (Canada). Veratridine, L-glutamate, and orphenadrine were from Sigma.

## Results

The exposure of cultured cerebellar neurons to glutamate results within 30 min in swelling and darkening of cell bodies and appearance of varicosities in neurites (Novelli et al., 1988). These signs could be completely prevented by the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801. Thus, this is a convenient paradigm for testing the effectiveness of drugs against the early onset of excitotoxicity, and we used it for testing the potential anti-excitotoxic properties of nefopam as compared to those of orphenadrine. As reported in Table 1, orphenadrine (100 $\mu$M) effectively prevented the appearance of early excitotoxicity signs, while nefopam (100 $\mu$M) did not.

Next we tested whether nefopam could be effective in preventing the development of excitotoxicity in the 24 h following the exposure to glutamate. As shown in Fig. 1, nefopam did not reduce glutamate toxicity. We also tested the possibility that Nefopam may require a longer incubation time before exposure to glutamate for maximum effectiveness. However, neither early sign of neurotoxicity nor neuronal survival after 24 h were ameliorated following pre-incubation with nefopam for up to 3.5 h before expose to glutamate (data not shown). Higher concentrations of nefopam

**Table 1.** Effect of nefopam and orphenadrine on excitotoxicity

| | Toxicity signs at 30 min. | | | |
|---|---|---|---|---|
| | NONE | MK-801 | Orphenadrine | Nefopam |
| NONE | − | − | − | − |
| VTD | + | − | − | − |
| GLU | + | − | − | + |

Cerebellar neurons in primary culture were exposed to the indicated drugs. Nefopam, MK-801 and orphenadrine were added 5 min. before other drugs. Drugs were used at the following concentration: veratridine (*VTD*), 10 $\mu$M; glutamate (*GLU*), 40 $\mu$M; Nefopam, 100 $\mu$M; orphenadrine, 100 $\mu$M; MK-801, 2 $\mu$M. The presence (+) or the absence (−) of signs of early neurotoxicity such as darkening and swelling of cell bodies were evaluated at 30 min. (n.d)– not done.

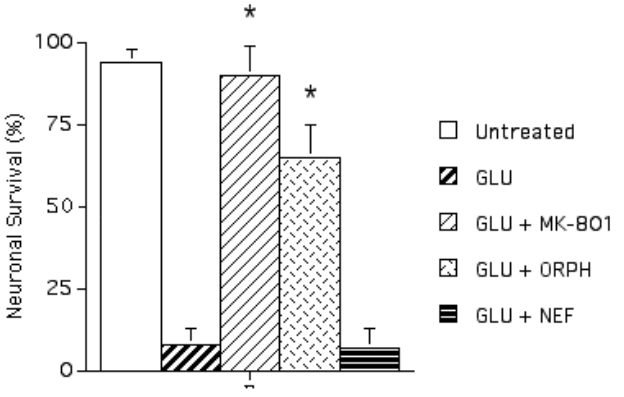


**Fig. 1.** Orphenadrine and nefopam effect on glutamate toxicity. Neuronal cultures were exposed to the indicated drugs and neuronal survival was determined 24 h later. MK-801 (1 $\mu$M), orphenadrine (*ORPH*, 100 $\mu$M) and nefopam (*NEF*, 100 $\mu$M), were added 5 min before glutamate (*GLU*, 40 $\mu$M). Values represent the mean ± SD from at least two independent experiments. * $P < 0.01$ vs. GLU

(1 mM) were toxic to neurons after 24 h exposure (data not shown).

In order to establish whether nefopam possess any affinity for the phencyclidine binding site of the NMDA receptor, where orphenadrine is known to bind (Kornhuber et al., 1995), we tested the effectiveness of nefopam in displacing MK-801 binding from rat hippocampal membranes. As shown in Fig. 2, 100 $\mu$M nefopam did not significantly displaced MK-801 binding to hippocampal membranes, whereas approximately 1 mM nefopam was necessary to displace MK-801 binding by 50%.

We then tested whether nefopam could have any effect on glutamatergic neurotransmission by modulating glutamate release. For this purpose, we induced glutamate release from cerebellar neurons in culture

**Table 2.** Muscimol does not reduce neither early nor late phases of veratridine neurotoxicity

| | Toxicity signs at 30 min. | | Neuronal survival at 24 h (%) | |
|---|---|---|---|---|
| | NONE | VTD | NONE | VTD |
| NONE | − | + | 94 ± 4 | 10 ± 5[a] |
| MUSCIMOL | − | + | 95 ± 6 | 12 ± 6[a] |
| NEFOPAM | − | − | 96 ± 5 | 92 ± 8[b] |

Cerebellar neurons in primary culture were exposed to the indicated drugs. Nefopam, and Muscimol were added 5 min. before other drugs. Drugs were used at the following concentration: veratridine (VTD), 10 $\mu$M; Nefopam, 100 $\mu$M; Muscimol, 100 $\mu$M. The presence (+) or the absence (−) of signs of early neurotoxicity such as darkening and swelling of cell bodies were evaluated at 30 min. Neuronal survival was determined 24 h later as indicated in the text. Values represent the mean ± SD (n = 2–6).

[a] $P \leq 0.01$ vs. NONE in the same line.

[b] $P \leq 0.01$ vs VTD in the same column.

by depolarizing them with veratridine (Gallo et al., 1982). Activation of voltage sensitive sodium channels (VSSC) by veratridine (10 $\mu$M) results within 30 min in swelling and darkening of cell bodies and appearance of varicosities in neurites (Diaz-Trelles et al., 2000). These signs are similar to those elicited by exposure of cultures to toxic concentrations of exogenous glutamate (Novelli et al., 1988), and they could be completely prevented by the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 (1 $\mu$M) (Diaz-Trelles et al., 2000). Exposure of cultures to nefopam (100 $\mu$M) for 5 min before veratridine stimulation completely prevented the appearance of veratridine-induced NMDA receptor-dependent excitotoxicity (Table 1).

Exposures to veratridine for 24 h led to a significant reduction in neuronal survival that occurred in the presence of MK-801 (Diaz-Trelles et al., 2000). As shown in Fig. 3, nefopam prevented neurodegeneration following 24 h exposure to veratridine to the same extent as the VSSC blocker saxitoxin (50 nM) (Terlau et al., 1991).

In order to exclude that nefopam may protect from veratridine–induced neurotoxicity by activating GABA receptors, we pretreated cultures with muscimol before the addition of veratridine. As shown in Table 2, neither the early NMDA receptor-dependent nor the later NMDA receptor-independent phase of veratridine-induced neurotoxicity were reduced by the presence of muscimol.

*Discussion*

The chemical analogy of orphenadrine, a non competitive antagonist at the NMDA receptor (Kornhuber et al., 1995), with nefopam, suggest the possibility of a similar action for the latter drug. Our results demonstrate that unlike orphenadrine, nefopam was not useful in preventing NMDA-receptor mediated neurotoxicity following the direct exposure to glutamate. When comparing nefopam to orphenadrine for neuroprotection against glutamate toxicity, it should be considered that: 1) approximately 1 mM nefopam was required to inhibit by 50% MK-801 binding to rat hippocampal membranes (Fig. 2), while no displacement of Mk-801 binding was observed at the concentration of nefopam used in neuronal cultures; 2) approximately 20 $\mu$M orphenadrine was reported to inhibit by 50% MK-801 binding to human prefrontal cortex membranes (Kornhuber et al., 1995); 3) MK-801 binding in the forebrain of human and rodents possess a Kd of similar magnitude (Quarum et al., 1990); 4) the Kd for MK-801 binding in the rat cerebellum is approx. one order of magnitude lower than in the forebrain (Reynolds and Palmer, 1991; Ebert et al., 1991). Thus, it appears reasonable that the concentration of orphenadrine we used in this study may have provided significant protection from excitotoxicity, while nefopam did not.

On the other hand, our data indicate a novel effect of nefopam on VSSC. The capability of depolarizing

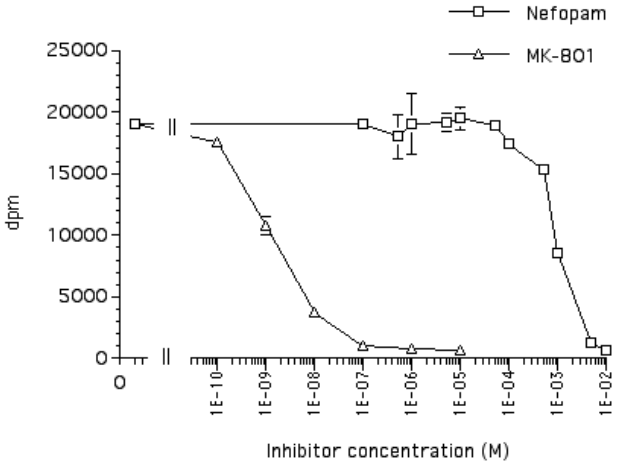


**Fig. 2.** Inhibition of [3H]MK-801 binding to rat hippocampal membranes by unlabelled MK-801 and nefopam. The details of the experimental procedure are reported in the methods. Values are mean ± SD of duplicate determinations from one representative experiment that has been repeated with very similar results

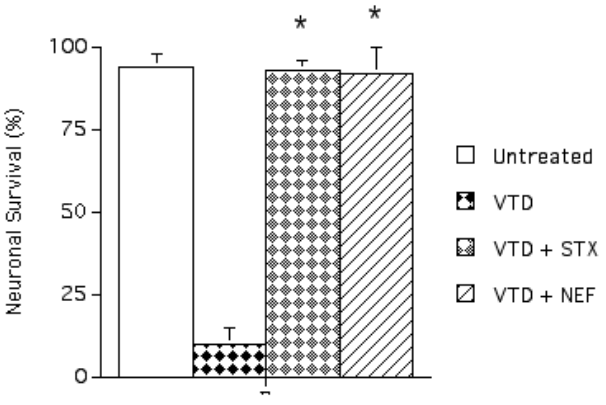


**Fig. 3.** Nefopam protects neurons from NMDA receptor-independent component of veratridine toxicity. Neuronal cultures were exposed to veratridine (*VTD* 10 $\mu$M) for 24 h. Nefopam (*NEF*, 100 $\mu$M) or Saxitoxin (*STX*, 50 nM) were added 5 min prior to VTD. Values represent the mean ± SD from three independent experiments (n = 6). * $P < 0.01$ vs.VTD

stimuli such as veratridine to release glutamate from cultured cerebellar neurons has been demonstrated previously (Gallo et al., 1982), and the amount of glutamate released is sufficient to activate NMDA receptors, to stimulate cGMP synthesis (Fernández-Sánchez et al., 1993), and to induce excitotoxicity (Diaz-Trelles et al., 2000). Thus, our data provide novel evidence suggesting that nefopam may modulate glutamatergic neurotransmission.

The early (30 min) MK-801-sensitive excitotoxic component of veratridine toxicity was important in determining the speed of the neurodegenerative process, but it had a very minor contribution to the overall toxicity by veratridine after 24 h (Diaz-Trelles et al., 2000). This late, MK-801-independent toxicity, may be attributed to the large influx of sodium through the persistently activated VSSC and the activation of biochemical pathways, such as oxygen radical formation, leading to neuronal death (Fernández-Sánchez et al., 2002).

Cultured cerebellar neurons express GABA receptors (Gallo et al., 1984), and GABAergic neurons account for approximately 3% of total neuronal population in these cultures (Nicoletti et al., 1986). Therefore, it is possible to speculate that nefopam could mimic the action of GABA receptor agonists and protect neurons by increasing GABAergic inhibitory neurotransmission. However, neither the NMDA receptor-mediated nor the NMDA receptor-independent component of veratridine-induced toxic-

ity, were reduced by the GABA receptor agonist muscimol, rendering such possibility unlikely.

It is tempting to speculate that the actions on VSSC we report may play a significant role in the analgesic effects of nefopam. Several lines of evidence suggest sodium channel inhibition to be a mechanism for analgesia (Besson, 1999). The sodium channel blocker carbamazepine has been shown to be effective in the treatment of neuropathic pain (Rizzo, 1997; Harke et al., 2001). Intrathecal administration of the sodium channel blocker lamotrigine produced a spinal long-lasting antihyperalgesic effect in short- and long-term neuropathic models of hyperalgesia (Klamt, 1998). Also, an inhibition of veratridine-induced sodium influx has been found for antidepressants and neuroleptics used in chronic pain (Deffois et al., 1996). Considering that NMDA receptor activation appears to be critical for the development and maintenance of the centrally mediated events in the course of this neuropathy (Kim et al., 1997; Kuwamata and Omote, 1996), our evidence that nefopam may modulate depolarization-dependent glutamate release, is consistent with nefopam capability to strongly reduce behavioral and electrophysiological signs of the neuropathy induced by ligature of the sciatic nerve (Biella et al., 2002).

In conclusion, this study demonstrates the analgesic compound nefopam hydrochloride to reduce neuronal death following the activation of voltage-operated sodium channels in cultured cerebellar neurons. This novel action of nefopam may be of interest in reducing the excessive release of endogenous glutamate involved in some neurological and neurodegenerative disorders.

## Acknowledgements

This work was supported by CICYT, Grants SAF98-0171 and HI1998-0188, by the University of Oviedo, Grant NP-00-530-27, and by CNR, Grant CT98.01049. CT04.115.30823

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