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Nefopam is more potent than carbamazepine for neuroprotection against veratridine *in vitro* and has anticonvulsant properties against both electrical and chemical stimulation

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Summary. Nefopam (NEF) is a known analgesic that has recently been shown to be effective in controlling both neuropathic pain and convulsions in rodents. In this study we compared nefopam to carbamazepine (CBZ), a reference antiepileptic drug (AED), for their ability to protect cerebellar neuronal cultures from neurodegeneration induced by veratridine (VTD). Furthermore, we tested nefopam for protection against both, maximal electroshock-induced seizures (MES), and isoniazid-induced seizures in mice. Both NEF and CBZ were effective in preventing both signs of excitotoxicity and neurodegeneration following exposure of cultures to 5 μM veratridine for 30 min and 24 h, respectively. Concentrations providing full neuroprotection were 500 μM CBZ and 50 μM NEF, while the concentration providing 50% neuroprotection was 200 μM for CBZ and 20 μM for NEF. Neither NEF nor CBZ reduced excitotoxicity following direct exposure of cultures to glutamate, but CBZ failed to reduce increases in intracellular calcium following stimulation of L-type voltage sensitive calcium channels. *In vivo*, NEF (20 mg/kg i.p.) significantly reduced MES and fully prevented MES-induced terminal clonus (TC). In comparison, NEF was significantly more effective than CBZ in preventing MES, although both drugs were equally effective against MES-induced TC. Furthermore, nefopam provided protection against isoniazid-induced seizures at doses similar to those protecting against MES.

Keywords: Nefopam – Carbamazepine – Cultured cerebellar neurons – Seizures – Behaviour

1. Introduction

Nefopam hydrochloride is a potent analgesic compound commercialized in most of Western Europe for almost 30 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; Villier and Mallaret, 2002). This drug has been 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). 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 recovery time between the end of anesthesia and extubation (Piper et al., 1999). Furthermore, nefopam has recently been used to separate the modulation of vasoconstriction from shivering (Alfonsi et al., 2004). Unpleasant adverse effects consistent with a central mode of action of the drug have also been reported during therapeutic use and include dizziness, headache, nausea, vomiting and sweating. However, the detailed mechanisms underlying the pharmacological actions of nefopam remain unclear. It was initially studied for its muscle relaxant properties (Tobin and Gold, 1972), and the involvment of dopamine reuptake in its mechanism of action (Koe, 1976; Esposito et al., 1986; Rosland and Hole, 1990). A mechanism of action

similar to that of some antidepressants, has also been demonstrated in animals (Fuller and Snoddy, 1993). Evidence exists suggesting a possible action of nefopam on the neurotransmission mediated by glutamate. Nefopam is a benzoxazocine and is considered as 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., 2003) which involves the activation of NMDA receptors.

However, two independent groups have recently shown that nefopam may bind to voltage sensitive sodium channels (VSSCs), and block sodium influx that follows their activation, while it does not possess a pharmacologically relevant affinity for glutamate receptors (Fernández-Sánchez et al., 2001, 2002; Verleye et al., 2004). More recently, nefopam has been shown to reduce calcium influx, cGMP formation, and NMDA receptor-dependent neurotoxicity, induced by the activation of voltage sensitive calcium channels (VSCC) in cultured cerebellar neurons (Novelli et al., 2005).

The finding that nefopam blocks sodium influx via VSSCs (Fernández-Sánchez et al., 2001; Verleye et al., 2004) is consistent with the therapeutic properties of this drug, and suggests that nefopam may also have a potential anticonvulsant, local anesthetic and antiarrithmic activity (Catterall, 1987). In fact, nefopam was recently reported to produce dose-dependent protection against maximal electroshock-induced seizures in mice, at lower concentrations than lidocaine (Verleye et al., 2004). In the present study we compared neuroprotection by either nefopam or carbamazepine against neurodegeneration induced by activation of VSSC by veratridine in cultured cerebellar neurons, and we tested nefopam in two experimental models of seizures in mice.

2. Materials and methods

2.1 Materials

Animals for behavioural experiments

Male CD1 mice weighing 25–30 g and 10 day old male Sprague Dawley rats, were purchased from Charles River (Como, Italy).

Animals were housed in groups of ten and kept in a temperature ($21 \pm 1\,^{\circ}C$) and relative humidity (60%) controlled room on a 12 h light/dark cycle (lights on between 06.00 and 18.00 h) with standard diet and water ad libitum. All experiments were performed at the same time of the day to minimize circadian influences. Control and drug experiments were done on the same day to avoid day-to-day variations in convulsive susceptibility. Animals were used on only one occasion. Animals in the control groups received equivalent volumes of the vehicle and were always tested together with the respective experimental groups. This study adhered to the ethical guidelines of the European Communities Council Directive of 24 November 1986 (86/609 EEC).

Drugs and chemicals

Nefopam, isoniazid, and (+)-10,11-dihydro-5-methyl-5H-dibenzo-[a,d]-cyclohepten-5,10-imine hydrogen maleate (MK-801) were obtained from Sigma (St. Louis, MO) and dissolved in distilled water. Diazepam (Valium, Hoffmann-La Roche) was used as commercial solution further diluted with saline. Carbamazepine was obtained from Sigma and dissolved in alcohol. Fluo-3 AM (F1241) was from Molecular Probes. (±)-methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine (BayK8644) was from Research Biochemicals International. The drug solutions were prepared immediately before use.

2.2 Methods

Cell cultures

Primary cultures of rat cerebellar neurons were prepared as previously described (Novelli et al., 1988). 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 μg/ml gentamycin and 10% fetal calf serum. Cells were seeded in poly-L-Lysine coated (5 μg/ml) 35 mm dishes at 2.5×10^5 cells/cm^2 and incubated at 37 °C in a 5% CO_2, 95% humidity, atmosphere. Cytosine arabinoside (10 μM) was added after 20–24 h of culture to inhibit the replication of non-neuronal cells. 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 (Fernández et al., 1991).

Neurotoxicology

Neurons were used between 14 and 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 randomly selected culture fields were taken and live and dead neurons were counted. Results were expressed as the number of live neurons/field. Total number of neurons per dish was calculated considering the ratio between the area of the dish and the area of the field (∼12000).

Confocal microscopy

For determination of intracellular calcium concentrations, neuronal cultures were loaded for 20–30 min with 5 μM Fluo-3-AM ester in a incubation buffer containing (in mM): 154 NaCl, 5.6 KCl, 5.6 glucose, 8.6 HEPES, 1 $MgCl_2$, 2.3 $CaCl_2$, pH 7.4. At the moment of the record the dye was removed and the indicated drugs were added. Fluo-3 emission (>515 nm) was recorded in a Bio-Rad confocal microscope with a krypton-argon laser excitation source (488 nm). Signals were digitized using Bio-Rad interface and analyzed by NIH Image (version 1.61). Concentrations of intracellular calcium ($[Ca^{2+}]_i$) could be estimated as a function of Fluo-3 intensity (F) using the calibration procedure described previously (Segal and Manor, 1992), and according to the following equation:

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$$

where the dissociation constant K_d has been estimated at 400 nM for Fluo-3 at vertebrate ionic strength (Kao et al., 1989), and the mean values

obtained for the camera signal F_{min} and for the maximum fluorescence F_{max} were 6 and 230, respectively.

Maximal electroshock seizure (MES) test

Seizures were induced in groups of 10 mice by delivery of a 50 mA intensity current for 0.2 sec with a pulse train of 60 Hz having a pulse duration of 0.4 msec through ear-clip electrodes (ETC unit model 7801, Ugo Basile, Comerio, Italy) (Swinyard et al., 1952). In mice, MES includes a latency period lasting 1.6 sec followed by a short flexion period, the tonic extension of hindlimbs (THE) lasting about 13.2 sec, and terminal clonus (TC) lasting for about 7.6 sec. Total duration of seizures is 22.3 sec (Swinyard et al., 1963). After MES, mice were observed for both, THE and TC for 30 sec and were then immediately killed by cervical dislocation. Several doses of nefopam (10, 15, 20, 25 mg/kg), diazepam (2 mg/kg) carbamazepine (10 mg/kg) or vehicle were administered (i.p.) 30 min before testing. Abolition of hindlimb tonic extension was considered as protection (Swinyard et al., 1952).

Chemically induced seizures

Preliminary studies were conducted to determine the dose of convulsant agent (isoniazid 200 mg/kg, s.c.) producing clonic and tonic seizures in 100% of the animals. Mice ($N = 10$ per group) were injected with nefopam (10, 15, 20, 25 mg/kg, i.p.) or diazepam (0.5 mg/kg i.p.) 30 min before administration of isoniazid. Animals were observed for at least 2 h after administration of isoniazid during which the time of onset of seizure activity, the pattern of seizure and the appearance of TC were recorded.

Assessment of motor coordination

The ability of mice to maintain themselves on a horizontal, turning rod (rotarod) was used to asses the potency of nefopam to interfere with motor coordination. The method used was similar to a previously described procedure (Dunham and Miya, 1957). The apparatus consisted of a rod with a diameter of 3 cm which was suspended horizontally 30 cm above a plain working area. The rod was turned at a speed of 10 revolutions/min by an electric motor. Circular perspex separators were placed at intervals along the rod so that five animals could be tested simultaneously. Before administering any compound, all test animals were placed on the turning rod for 2 min. Any animal which fell from the rod during this time was excluded from the study. Groups of five mice were placed on the turning rod at 15, 30 and 45 min after drug administration. As each mouse fell from the rotarod it was replaced. Performance was assessed by the total number of falls during the test period together with the latency to the first fall.

Assessment of spontaneous locomotor activity

Spontaneous locomotor activity in mice was quantified using an Animex activity meter (LKB, Sweden) set to maximum sensitivity. Mice were placed on the top of the Animex activity meter and each movement produced a signal due to variation in inductance and capacity of the

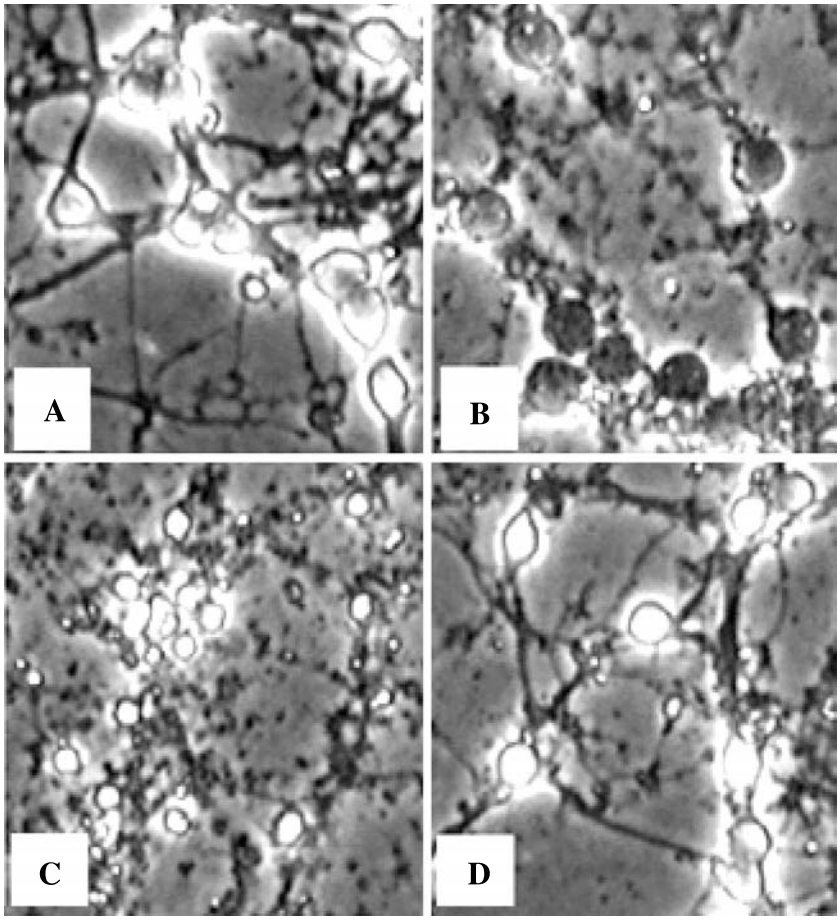

Fig. 1. Phase-contrast microscopy of cerebellar cultures exposed to veratridine. Neuronal cultures were untreated (**A**) or exposed to 5 μM veratridine (VTD) either in the absence (**B**, **C**) or in the presence of either 100 μM nefopam (NEF) or 500 μM carbamazepine (CBZ) (**D**). Cultures were observed for neurotoxicity both at 30 min and 24 h and representative images are shown in **B** and **C**, respectively. Cultures that were untreated (**A**) or received VTD in the presence of either NEF or CBZ (**D**) did not change their morphology throughout the experiment. Images are from one experiment that has been repeated more than 3 times with similar results

apparatus resonance circuit. Signals were automatically converted to numbers. On the day of the experiment mice ($N = 5$ per group) were treated, returned to their home cage, and tested at 5, 15, and 30 min later. For testing mice were introduced individually in a clean box of the same type as the home cage and the box was placed on the Animex apparatus. Activity counts were recorded for 30 minutes. Treatments were matched with saline controls within each test interval to control of inter-trial variability.

Data presentation and analysis

Statistical analysis for data obtained from experiments on both *in vitro* neurotoxicology and chemically induced seizures, was performed by ANOVA, followed by Dunnet test for multiple comparison, while MES test data were evaluated by Fisher's exact test. All experimental results are given as the means ± S.E.M., unless otherwise indicated. Only significant differences relevant to the discussion of the data are indicated in each figure.

3. Results

In order to compare the potency of nefopam and carbamazepine in blocking neurodegeneration following activation of VSSC by veratridine in our experimental system, we first choose to use the same conditions that have we used in a previous paper (Fernández-Sánchez et al., 2001). However, when neurodegeneration was induced by 10 μM veratridine, carbamazepine at concentrations as high as 500 μM failed to show any neuroprotective effect. Higher concentrations of carbamazepine alone were neurotoxic to cultured neurons when present in the growth medium for 24 h (data not shown). In an attempt to obtain a concentration-dependent neuroprotection by carbamazepine we reduced the concentration of veratridine to 5 μM in accord with previously published work (Willow and Catterall, 1982). Activation of VSSC by exposure of cultures to veratridine (5 μM) for 30 min resulted in a toxicity pattern identical to that produced by higher concentrations of veratridine (Fernández-Sánchez et al., 2001), namely an early neuronal swelling and darkening compared to the control (Fig. 1A and B), and a significant reduction in surviving neurons 24 h later

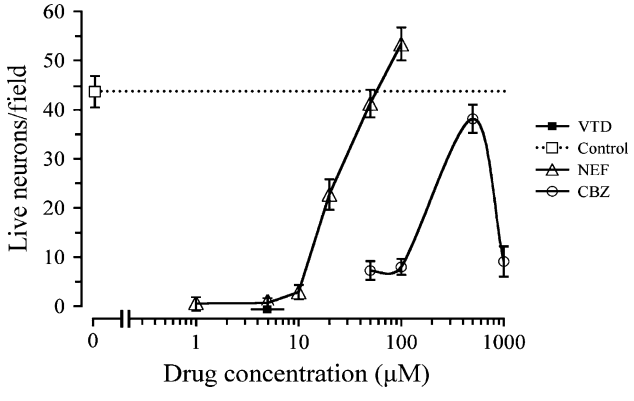

Fig. 2. Concentration-dependent protection by nefopam and carbamazepine against neurodegeneration induced by veratridine. Neuronal cultures were pretreated with the indicated concentrations of either nefopam (NEF) or carbamazepine (CBZ) for 15 min before the addition of 5 μM veratridine (VTD). All drugs were added to the culture growth medium. Neuronal survival was determined 24 h later as indicated in Materials and methods. Data represent the mean ± S.E.M. ($n = 16$)

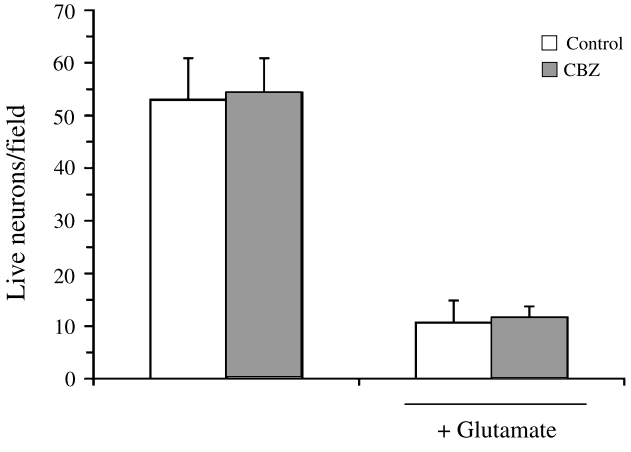

Fig. 3. Carbamazepine does not reduce excitotoxicity. Cerebellar neurons in primary culture were exposed to the indicated drugs. 40 μM glutamate and 500 μM carbamazepine (CBZ) were used. When used together, CBZ was added 5 min before glutamate. Neuronal survival was determined 24 h later as indicated in the text. Values represent the mean ± SD ($n = 2$–6) from one experiment that has been repeated with similar results

Table 1. Effect of nefopam and carbamazepine on intracellular calcium fluorescence in cultured cerebellar neurons

	Additions		Δ	% inhibition
	None (f.u.)	BAYK (f.u.)		
None	28 ± 5	125 ± 13	97	–
NEF	25 ± 7	72 ± 8*	47	52
CBZ	30 ± 6	126 ± 15	96	1

Neuronal cultures were loaded for 20–30 min with 5 μM Fluo-3-AM ester and then examined under a laser confocal microscope. MK801 (2 μM) was always present to avoid both calcium influx via NMDA receptor channels and excitotoxicity. Cultures were exposed to 2 μM BayK8644 + 50 mM KCl (BAYK). Either 100 μM nefopam (NEF) or 500 μM carbamazepine (CBZ) were added 5 min before exposure to BAYK. Images were taken before and each second after addition of BAYK. Reported data are from images taken before and 30 sec after the addition of BAYK, when fluorescence is maximal (Díaz-Trelles et al., 2002). Data are the mean ± S.E.M. of the quantification of fluorescence units (f.u.) in about 12 neurons from two independent experiments. *$p < 0.0001$ vs. BAYK

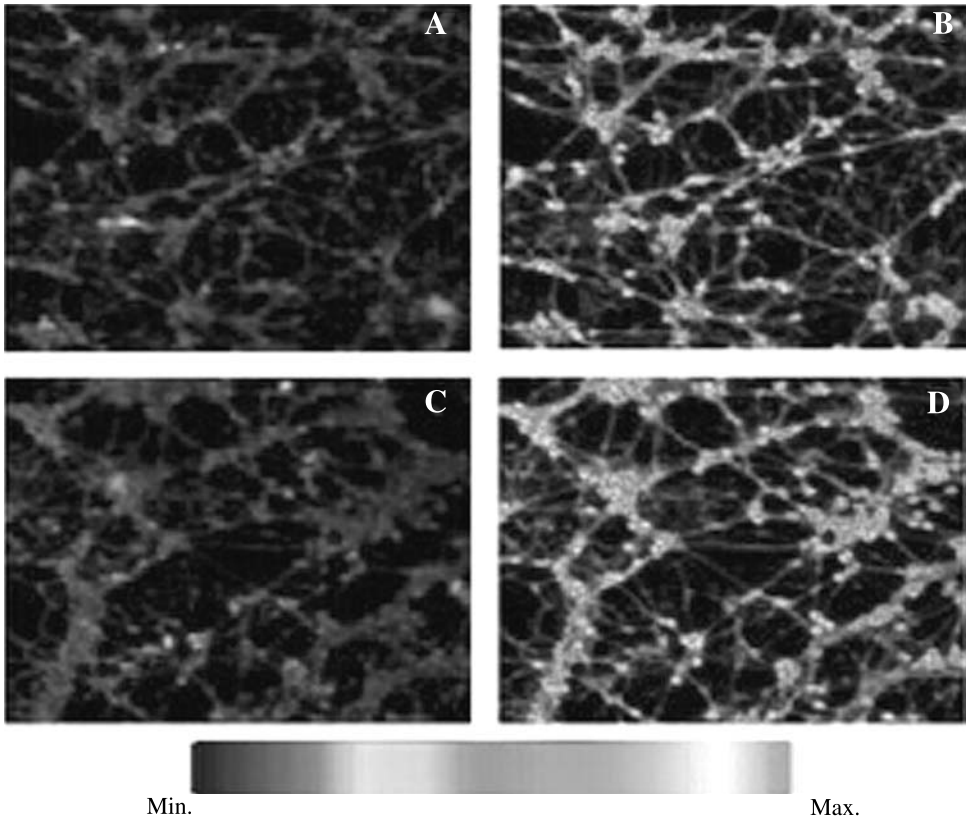

Fig. 4. Effects of carbamazepine on intracellular calcium following specific stimulation of voltage sensitive calcium channels. Neuronal cultures treated as explained in the legend of Table 1. **A** Cultures before and 30 sec after (**B**) exposure to 2 μM BayK8644 + 50 mM KCl (BAYK). (**C**) Cultures exposed to 500 μM carbamazepine (CBZ) alone and 30 sec after BAYK (**D**). Scale color bar spans from black (minimum) to red (maximum) (For an interpretation of the reference to colour in this figure, the reader is referred to the online version of this paper under www.springerlink.com)

(Fig. 1C), and toxicity by veratridine at 30 min, was completely abolished by the specific NMDA receptor antagonist MK-801 (2 μM) (Fernández-Sánchez et al., 2001, and data not shown). Exposure of neurons to increasing concentrations of either nefopam or carbamazepine for 10 min before VSSC stimulation with veratridine, resulted in a progressive, concentration-dependent reduction of both neuronal swelling and darkening, and neuronal death for both drugs. Nefopam and carbamazepine provided 50% protection at 20 and 200 μM, respectively, while full neuroprotection was achieved at 50 and 500 μM of nefopam and carbamazepine, respectively (Figs. 2 and 1D).

In order to assess whether carbamazepine may act as an antagonist at excitatory amino acid receptors, neurons treated with carbamazepine were exposed to glutamate. Similarly to nefopam (Fernández-Sánchez et al., 2001), carbamazepine did not reduced excitotoxicity following direct exposure to 40 μM glutamate for 24 h (Fig. 3), as the survival of treated vs. untreated neurons was approximately 20% for both glutamate alone and glutamate in the presence of 500 μM carbamazepine.

Table 2. Effect of nefopam on maximal electroshock-induced seizures (MES) in mice

Treatment (mg/kg)	Convulsions		Terminal clonus	
	No. of animals	%	No. of animals	%
Vehicle	15/15	100	15/15	100
Nefopam (10)	8/10	80	7/10	70
Nefopam (15)	$4/10^{**}$	40	$3/10^{***}$	30
Nefopam (20)	$2/10^{***}$	20	$0/10^{***}$	0
Nefopam (25)	$2/10^{***}$	20	$0/10^{***}$	0
Carbamazepine (10)	$5/10^{**}$	50	$0/10^{***}$	0
Diazepam (2)	$5/10^{**}$	50	$3/10^{***}$	30

Mice were injected with drugs 30 min before MES
$^{**}p < 0.01$, $^{***}p < 0.001$ compared with controls by Fisher's exact test
Values are the mean ± S.E.M.

Nefopam has been also shown to block BayK8644-induced calcium increase in cultured cerebellar neurons (Novelli et al., 2005), and we asked whether carbamazepine could share a similar property. As shown in Table 1, nefopam but not carbamazepine, significantly reduced BayK8644-induced fluorescence increase by 52%.

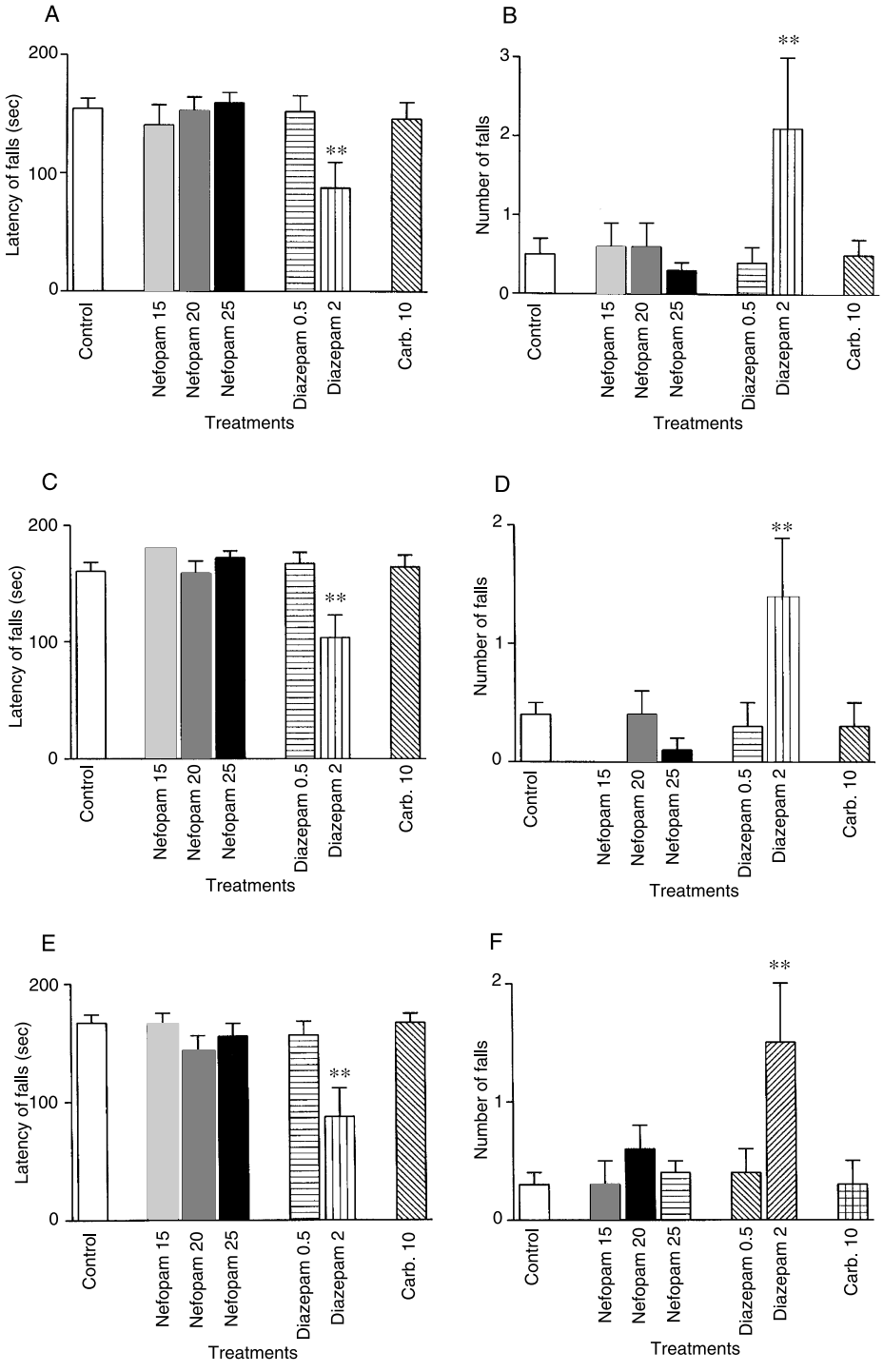

Fig. 5. Assessment of motor coordination after treatment with nefopam. Animals were injected i.p. with three different doses of nefopam that proved to be useful in the MES test. For comparison, two doses of diazepam and one dose of carbamazepine were also used. Both number and latency of falls from the turning rod were measured at the following times after drug treatment: 15 min (**A**, **B**), 30 min (**C**, **D**), and 45 min (**E**, **F**). Five animals/group were used. Data are the mean ± S.E.M. $^{**}p < 0.01$ vs. control treatment

The absence of effect of carbamazepine is also shown in Fig. 4.

Next we tested whether nefopam could have behavioural effects comparable to that of drugs used for the treatment of human seizures. In fact, nefopam has recently been shown to reduce electroshock-induced seizures in mice (Verleye et al., 2004). As shown in Table 2, we also observed that nefopam reduced convulsions in mice in a dose-dependent manner, with a dose of approximately 15 mg/kg i.p. preventing convulsions in 60% of the animals. This dose of nefopam also effectively reduced TC by 70%. Full prevention from TC was achieved with 20 mg/kg of nefopam, a dose that reduced convulsions in 80% of animals. For comparison, carbamazepine at 10 mg/kg reduced convulsions by 50% and fully protected from TC, while diazepam at 2 mg/kg reduced convulsions and TC by 50 and 70%, respectively (Table 2).

In order to evaluate whether the concentrations of nefopam that protect against seizures could also affect either motor coordination or locomotor behaviour, we compared the performance of animals treated with either nefopam, or carbamazepine or diazepam in both the rotarod test and the Animex test of spontaneous locomotion. As shown in Fig. 5, animals treated with nefopam at doses that are effective at preventing both convulsions and TC (15, 20, 25 mg/kg) did not significantly alter either the number or latency of falls from the rotarod at any time after injection. This result was similar to that obtained by using carbamazepine (10 mg/kg), while diazepam at 2 mg/kg significantly increased the number of falls and decreased the latency time of falling, at 15, 30 and 45 min after treatment. The Animex test revealed that nefopam treatment at both 20 and 25 mg/kg reduced significantly the number of animal movements, 15 and 30 min after injection of the drug. A similar result was obtained with diazepam at 2 mg/kg, but not with carbamazepine at 10 mg/kg (Fig. 6).

Finally, we asked whether nefopam could be effective also in reducing seizures elicited by a metabolic imbalance of the excitatory/inhibitory neuronal activity in the brain. For this purpose, we used the animal model of seizures induced by isoniazid. This drug is known to inhibit glutamate decarboxylase activity and to elicit seizures that are blocked by benzodiazepines (Serra et al., 1992; Bernasconi et al., 1992). As shown in Table 3, nefopam significantly reduced convulsions and TC in a dose-dependent manner. Thus, a dose of 20 mg/kg reduced both convulsions and TC by 30 and 40%, respectively, while a dose of 25 mg/kg nefopam reduced convulsions and TC to the same extent (85%). The onset time of both convulsions and TC was also significantly delayed.

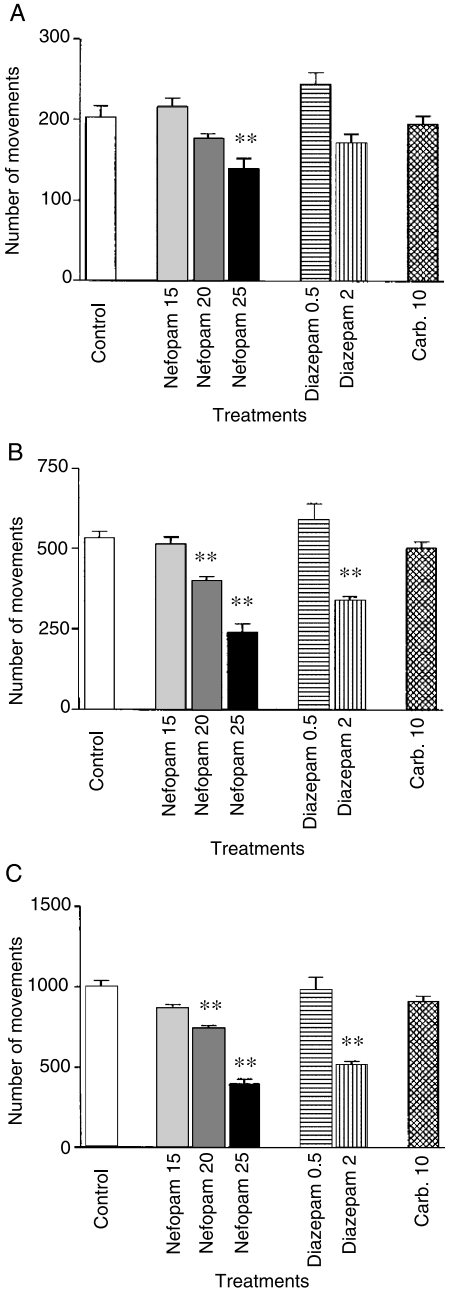

Fig. 6. Assessment of spontaneous locomotor activity after treatment with nefopam. Animals were injected i.p. with three different doses of nefopam that proved to be useful in the MES test. For comparison, two doses of diazepam and one dose of carbamazepine were also used. Number of movements in the Animex activity meter were measured for 30 min. at the following times after drug treatment: 5 min (**A**), 15 min (**B**), and 30 min (**C**). Five animals/group were used. Data are the mean ± S.E.M. $^{**}p < 0.01$ vs. control treatment

Table 3. Effect of nefopam on isoniazid-induced seizures in mice

Treatment + isoniazid (200) (mg/kg)	Convulsions			Terminal clonus		
	Onset (min)	No. of animals	%	Onset (min)	No. of animals	%
Vehicle	45 ± 1	26/26	100	53 ± 1	26/26	100
Nefopam (10)	52 ± 5	14/15	93	64 ± 6	13/15	87
Nefopam (15)	63 ± 5[a]	12/15*	80	77 ± 6[b]	11/15*	73
Nefopam (20)	81 ± 4[b]	14/20*	70	92 ± 4[b]	12/20*	60
Nefopam (25)	97 ± 8[b]	3/20***	15	111 ± 3[b]	3/20***	15
Diazepam (0.5)	72 ± 5[b]	17/20	85	101 ± 4[b]	11/20**	55

Mice were injected with drugs 30 min before isoniazid
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with controls by Fisher's exact test
[a] $p < 0.05$, [b] $p < 0.01$ by ANOVA
Values are the mean ± S.E.M.

4. Discussion

The molecular structure of nefopam resembles that of carbamazepine, a clinically used anticonvulsant drug, that is well known to act on VSSCs (Catterall, 1987), but no comparison of the pharmacological profile of these two drugs has been done to date. Two independent investigations have recently shown that nefopam reduces sodium influx following activation of VSSCs (Fernández-Sánchez et al., 2001; Verleye et al., 2004). Nefopam also reduces calcium influx via VSCCs (Novelli et al., 2005), and provides neuroprotection from excitotoxicity in cultured neurons following activation of either VSSC or VSCC (Fernández-Sánchez et al., 2001, 2002; Novelli et al., 2005), although it does not appear to act as an antagonist at glutamate receptors (Fernández-Sánchez et al., 2002; Verleye et al., 2004). Furthermore, nefopam appears to displace batrachotoxinin from its binding site on VSSC, to a similar extent than veratridine ($IC_{50} = 5.7$ and 4.1 μM, respectively, Verleye et al., 2004). This allows nefopam to protect cultured neurons from neurodegeneration induced by 10 μM veratridine with an EC_{50} of 57 μM (Fernández-Sánchez et al., 2001). In the current study, such concentration of veratridine proved to be too high to demonstrate neuroprotection by carbamazepine at any of the concentrations tested. A lower, but still fully neurotoxic concentration of veratridine (5 μM), allowed us to properly compare the potency of nefopam vs. carbamazepine. Thus, in these experimental conditions the EC50 of nefopam was approx. 10 times lower than that of carbamazepine. This result is of particular interest considering that these two drugs target the same site on VSSCs (Verleye et al., 2004; Willow and Catterall, 1982). Furthermore, it is worth noting that in our experimental system, carbamazepine did lack the capability to act on the same population of VSCC that are blocked by nefopam, similarly to previous observations both in neuroblastoma cells and in epilepsy prone mice (De Sarro et al., 1992; Kito et al., 1994), although carbamazepine may be capable of reducing calcium influx through various types of calcium channels, including the L-type (Schirrmacher et al., 1993; Stefani et al., 1997; Schumacher et al., 1998; Ambrosio et al., 1999; Zhu et al., 2002).

In animals, our results on the effects of nefopam on MES confirm the original observation of Verleye and collaborators (Verleye et al., 2004), and show that protection from convulsions is obtained using doses of nefopam that also produce a significant increase in animal survival. The doses we found to be effective against MES are approximately 4 times higher than those reported by Verleye et al. (2004). The reasons for this difference may be due to both, the higher electrical intensity of the stimulus used for MES (40 mA in the ear in our study, and 15 mA in the eye in the study of Verleye et al.), and the different route of administration of the drug (i.p. in this study vs. i.v. in the study of Verleye et al., 2004). With respect to this latter point, it should be noted that the i.p dose of nefopam preventing neuronal sensitization in a rat model of neuropathy, is approx. 7 times higher than the i.v. dose used for analgesia (Biella et al., 2003; Mather et al., 2000). Furthermore, the ED50 for nefopam reduction of convulsions is similar to that of carbamazepine. It should be noted that carbamazepine was more effective in reducing TC when considering a similar effect on the reduction of convulsions. The reasons accounting for the difference between the potency of nefopam and carbamazepine *in vitro* and *in vivo* may be various, including metabolism of the drugs and other currently undefined pharmacokinetic and/or pharmacodynamic effects. However, it is worth noting that both nefopam and carbamazepine neuroprotection demonstrated *in vitro*, may contribute to improve animal survival during MES, at doses that do not alter motor coordination. The absence of impaired motor coor-

dination in the rotarod test is particularly important for interpreting properly the results of the Animex test. In fact, the lower number of movements observed in animals treated with nefopam, as compared with untreated animals, cannot be compared with the lower number of movements produced by diazepam, since the latter drug also significantly reduced the performance of animals on the rotarod. It is possible that nefopam may reduce spontaneous locomotion by increasing attention. Such a possibility has been already considered by other authors who did not observe any effect of nefopam on human attention (Belleville et al., 1979). However, it is interesting to note that nefopam has been repeatedly described to affect the biogenic amine system (Vonvoigtlander et al., 1983; Esposito et al., 1986; Jasinski and Preston, 1987; Hunskaar et al., 1987; Rosland and Hole, 1990; Fuller and Snoddy, 1993), and may possess a nootropic effect at the concentrations we used. Further studies will be important to determine whether the effect of nefopam we observed in rats may be related to a psychotropic effect similar to that of carbamazepine in bipolar disorders, considering that the latter drug also appears to involve the biogenic amine system (Ichikawa and Meltzer, 1999; Murakami et al., 2001; Ichikawa et al., 2005; Ahmad et al., 2005).

Finally, the ability of nefopam to reduce convulsions induced by isoniazid may be directly related to the protective effect of nefopam against convulsions induced by i.v. injection of glutamate receptor agonists in mice (Verleye et al., 2004). In fact, isoniazid has been reported to reduce the activity of glutamate decarboxylase, and it may increase endogenous excitatory amino acids neurotransmission, therefore mimicking the effects of direct i.v. injection of excitatory amino acids. Accordingly, the convulsions induced by isoniazid can be effectively antagonized by excitatory amino acid antagonists (Mignani et al., 2002) in addition to anticonvulsant drugs such as diazepam (Serra et al., 1992).

According to the results we obtained, nefopam may possess effective anticonvulsant activity of therapeutical interest. However, two reports in the literature indicate that high concentrations of nefopam may induce convulsions in some patients by possibly lowering seizure threshold (Urwin and Smith, 1999; Pillans and Woods, 1995). In our hands, nefopam at 30 mg/kg elicited convulsions in animals in the absence of other treatments (data not shown). These observations are difficult to explain at a molecular level, and are important for establishing the therapeutic index of nefopam. Side effects that may include convulsions are not unknown with some established antiepileptic drugs such as carbamazepine (Kochen et al., 2002). On the other hand, *in vitro*, the EC100 of nefopam is one order of magnitude lower than the concentration that may cause neurotoxicity in the absence of other treatments, while the EC100 for carbamazepine was only 1/2 of that required to induce neurotoxicity in the absence of other treatments. Thus, the anticonvulsant properties of nefopam may deserve further attention, particularly when considering its actual use as an analgesic, and its potential use against neuropathic pain (Biella et al., 2003).

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