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Nefopam enhances the protective activity of antiepileptics against maximal electroshock-induced convulsions in mice

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Abstract:
Nefopam is a centrally acting non-opioid analgesic with a mechanism of action that is not completely understood. Adverse effects associated with the therapeutic use and overdose of nefopam are mainly associated with the central nervous system, such as hallucinations, cerebral edema and convulsions. The aim of this study was to assess the effect of nefopam on the electrical threshold and its influence on the protective activity of antiepileptic drugs in the maximal electroshock test in mice. A 5 mg/kg dose of nefopam significantly elevated the electric seizure threshold, while a dose of 1 mg/kg failed to protect mice against electroconvulsion. At a subthreshold dose of 1 mg/kg, nefopam significantly enhanced the anticonvulsant activity of valproate against electroconvulsions. The protective activity of phenobarbital and phenytoin was significantly enhanced by co-administration of nefopam at the 5 mg/kg dose, but this same dose of nefopam failed to affect the protective activity of carbamazepine. In conclusion, nefopam exerts an anticonvulsive effect when given alone and significantly enhances the protective activity of certain antiepileptic agents against electroconvulsions induced in mice.

Key words:
nefopam, antiepileptic drugs, maximal electroshock, seizures

Abbreviations: AED – antiepileptic drug, CBZ – carbamazepine, CL – confidence limits, CS_{50} – 50% current strength, ED_{50} – 50% effective anticonvulsant dose, MES – maximal electroshock, NEF – nefopam, NMDA – N-methyl-D-aspartate, PhB – phenobarbital, PHT – phenytoin, SD – standard deviation, SE – standard error, TD_{50} – 50% toxic dose, VPA – magnesium valproate

Introduction

Nefopam (NEF) is a centrally acting non-opioid analgesic used clinically to control acute postoperative pain [1]. Despite its long history of clinical use, the

mechanisms underlying the many pharmacological actions of NEF remain unclear. It is a benzoxazocine that is structurally related to the two drugs orphenadrine and diphenhydramine, which both interact with N-methyl-D-aspartate (NMDA) receptors at the phencyclidine biding site [21]. Additional proposed mechanisms of action for NEF include monoamine reuptake inhibition [13] and interaction with serotoninergic [15] and dopaminergic [10] pathways.

Recently, we reported that orphenadrine significantly elevated the seizure threshold and enhanced the protective activity of valproate against maximal electroshock (MES)-induced convulsions in mice [6]. Based on these results, the aim of this study was to determine the influence of NEF on the electrical seizure threshold and the protective activity of some selected antiepileptic drugs (AEDs) against MES-induced convulsions in mice.

Materials and Methods

Animals

All experimental protocols and procedures were approved by the First Local Ethics Committee of Lublin. Experimentally naive, male Swiss mice between 8 and 10 weeks old, weighing 20–25 g, were used throughout the study. The animals were housed in standard laboratory conditions (12-h light/dark cycle, 21 ± 1°C, relative humidity of 55 ± 5%) with free access to food and water prior to the experiments. After 7 days of acclimation to laboratory conditions, the animals were randomly assigned to experimental groups, each consisting of 10 mice. Each animal was used only once in the experimental procedures. All experiments were carried out between 9 a.m. and 3 p.m.

Drugs

The following drugs were used in this study: NEF (Jelfa, Jelenia Góra, Poland), valproate magnesium (VPA; ICN Polfa, Rzeszów, Poland), carbamazepine (CBZ; Polfa, Starogard, Poland), phenytoin (PHT; Polfa, Warszawa, Poland) and phenobarbital (PhB; Polfa, Kraków, Poland). NEF and VPA were dissolved in sterile saline, and the remaining drugs were suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA). All drugs were administered intraperitoneally (*ip*) at a volume of 10 ml/kg. NEF, VPA and CBZ were administered 30 min prior to experiments, and PhB and PHT were given 60 and 120 min prior to experiments, respectively. The pretreatment times were chosen according to the biological activity of the studied drugs.

Electroconvulsions

Electroconvulsions were produced by an alternating current (0.2 s stimulus duration, 50 Hz) delivered *via* ear-clip electrodes by a Hugo Sachs stimulator (Rodent Shocker, Type 221, Freiburg, Germany).

To evaluate the threshold for electroconvulsions, at least 4 groups of mice were challenged with electroshocks of various intensities to construct a current intensity-effect curve, according to the log-probit method by Litchfield and Wilcoxon [22]. The convulsive threshold was evaluated as CS_{50}, which is defined as the current strength in mA with 95% confidence limits (CL) necessary to produce tonic hind limb extension in 50% of the animals tested. The threshold for electroconvulsions was denoted for 2 different doses of NEF, 1 and 5 mg/kg.

To estimate the anticonvulsant properties of NEF and AEDs (given alone or in combination), mice were pretreated with different doses of the drugs and then challenged with MES (0.2 s stimulus duration, 50 Hz, 25 mA). With these data, we were able to construct a dose-effect curve. Subsequently, the ED_{50} values (50% effective anticonvulsant dose) with 95% CL were calculated.

Chimney test

The chimney test described by Boissier et al. [3] was used to assess potential adverse effects as a result of VPA administration combined with NEF treatment. To estimate the motor coordination impairment produced by VPA treatment (either alone or in combination with a fixed dose of 5 mg/kg NEF), mice were pretreated with different doses of VPA and were then required to climb backwards up a plastic, transparent tube (3-cm inner diameter, 30 cm long). The impairment of motor performance was indicated by the inability of the mice to climb backwards up the tube within 60 s. Subsequently, a dose-response curve was constructed, and TD_{50} values (50% toxic doses) with 95% CL were calculated.

Free plasma concentrations of the tested AEDs

Measurement of free plasma concentrations of the tested AEDs was performed at the doses corresponding to their calculated ED_{50} values against MES-induced convulsions. Mice were pretreated with an AED in combination with either NEF or saline. At the times scheduled for MES analysis, mice were sacrificed by decapitation. Blood samples of approximately 1 ml were collected into heparinized microfuge tubes and subsequently centrifuged at 5,000 × g for 5 min. Plasma samples (350 µl) were transferred to the micropartition system (MPS-1; Amicon, Danvers, MA, USA) to determine the free (non-protein bound) AED concentration and were centrifuged at 5,000 × g for 10 min. Filtrates (50 µl) free of protein-bound microsolutes were pipetted into original Abbott system cartridges, and the free plasma concentration of AED was estimated by immunofluorescence using an Abbott TDx analyzer (Abbott, Irving, TX, USA). Plasma concentrations were expressed in µg/ml as the means ± SD of eight separate blood samples.

Total brain VPA concentration

Pharmacokinetic evaluation of total brain AED concentration was only performed in mice treated with the combination of NEF and VPA at the dose corresponding to the ED_{50} value of VPA calculated from the MES-test. Specifically, mice pretreated with VPA alone or in combination with NEF were decapitated at time points reflecting the peak of the maximum anticonvulsant effect for the drug in the MES-test. The whole brains of the mice were removed from skulls, weighed, harvested and homogenized using Abbott buffer (1:2 w/v; Abbott Laboratories, North Chicago, IL, USA) in an Ultra-Turrax T8 homogenizer (IKA Werke, Staufen, Germany). The homogenates were then centrifuged at 10,000 × g for 10 min. A volume of 100 µl of each sample supernatant was collected and then analyzed for AED content. The total brain VPA concentration was measured with a fluorescence polarization immunoassay using an analyzer (Abbott TDx) and the manufacturer-supplied reagent kits (Abbott Laboratories, North Chicago, IL, USA). Total brain AED concentrations are expressed in µg/ml of brain supernatants as the means ± SD of eight separate brain preparations.

Statistical analysis

The CS_{50}, ED_{50} and TD_{50} values (with 95% CL) were calculated by computer probit analyses, according to Litchfield and Wilcoxon [22]. Subsequently, the respective 95% CL were transformed to SE. Statistical analyses of data collected from the electrical threshold assessment and MES-test were performed with one-way analysis of variance (ANOVA) followed by the *post-hoc* Tukey-Kramer test for multiple comparisons [23]. The TD_{50} values (with 95% CL) were compared using computer probit analyses. Free plasma and total brain concentration data were compared with unpaired Student's *t*-tests.

Results

Effect of NEF on the electroconvulsive threshold

When administered at 5 mg/kg, NEF significantly elevated the electric seizure threshold from 6.0 (5.6–6.4) to 7.4 (6.8–8.1) mA. A dose of 1 mg/kg, however, failed to affect the electric seizure threshold (Tab. 1).

Effect of NEF on the protective activity of AEDs in the MES-test

The administration of 5 mg/kg NEF enhanced the anticonvulsive activity of VPA, PhB and PHT. At a sub-

Tab. 1. Effect of nefopam (NEF) administration on the electrical seizure threshold in mice

Treatment (mg/kg)	CS_{50} (mA)	n
vehicle	6.0 ± 0.15	20
NEF (1)	6.2 ± 0.28	17
NEF (5)	7.4 ± 0.31*	20
F (2,54) = 9.066; p = 0. 0004		

Data are presented as the median current strengths (CS_{50} values in mA ± SE) necessary to induce tonic hind limb extension in 50% of the animals tested. NEF was administered *ip* 10 min prior to electroconvulsions, with the time corresponding to the peak activity of NEF. Statistical evaluation of the data was performed with one-way ANOVA followed by the Tukey-Kramer *post-hoc* test for multiple comparisons; n – number of animals tested at the current strength intensities for which seizure effects ranged between 16 and 84%. F – F-statistics from one-way ANOVA; p – probability from one-way ANOVA, * $p < 0.05$ *vs.* control (vehicle-treated animals)

Tab. 2. Influence of nefopam (NEF) treatment on the anticonvulsant activity of valproate (VPA), carbamazepine (CBZ), phenytoin (PHT) and phenobarbital (PhB), against maximal electroshock-induced convulsions in mice

Treatment (mg/kg)	ED_{50} (mg/kg)	n
VPA + vehicle	330.6 ± 11.65	10
VPA + NEF (0.2)	301.4 ± 15.77	19
VPA + NEF (1)	261.0 ± 17.15*	20
VPA + NEF (5)	210.8 ± 13.35*	10
F (3,55) = 7.236; p = 0.0004		
CBZ + vehicle	9.7 ± 2.26	24
CBZ + NEF (1)	9.2 ± 0.93	15
CBZ + NEF (5)	5.8 ± 0.56	16
F (2,52) = 1.340; p = 0.2707		
PHT + vehicle	5.6 ± 1.39	20
PHT + NEF (1)	5.9 ± 0.48	20
PHT + NEF (5)	3.3 ± 0.42*	20
F (2,57) = 8.585; p = 0.0004		
PhB + vehicle	13.9 ± 1.21	25
PhB + NEF (1)	11.8 ± 0.68	16
PhB + NEF (5)	7.1 ± 0.88*	16
F (2,54) = 12.072; p = 0.0001		

Data are presented as the median effective dose (ED_{50} values in mg/kg ± SE) protecting 50% of the animals challenged with MES-induced seizures. NEF and all AEDs were administered *ip* at time points corresponding to the times of their peak activities: NEF at 10 min, VPA and CBZ at 30 min, PhB at 60 min and PHT at 120 min prior to the MES-test. Statistical evaluation was performed with one-way ANOVA followed by the Tukey-Kramer *post-hoc* test for multiple comparisons; n – number of animals tested at current strength intensities for which seizure effects ranged between 16 and 84%. F – F-statistics from one-way ANOVA; p – probability from one-way ANOVA, * $p < 0.05$ (vehicle-treated animals)

threshold dose of 1 mg/kg, NEF treatment significantly enhanced the anticonvulsant activity of VPA. When administered at 0.2 mg/kg, NEF failed to affect the protective activity of VPA against MES-induced seizures (Tab. 2).

Effect of NEF on motor coordination impairment induced by VPA treatment in the chimney test

The TD_{50} value of the VPA treatment was 669.6 (640.0–699.5) mg/kg. When administered at 5 mg/kg, NEF exposure failed to affect the motor impairment induced by VPA, with the respective TD_{50} value of 644 (582.9–713.0) mg/kg (results not shown).

Tab. 3. Free plasma concentrations of tested antiepileptic drugs (AEDs) administered alone or in combination with nefopam (NEF)

Treatment (mg/kg)	Free plasma concentration (μg/ml)
VPA + vehicle	309.28 ± 28.64
VPA + NEF (1)	292.08 ± 22.72
CBZ + vehicle	1.320 ± 0.14
CBZ + NEF (5)	1.651 ± 0.19*
PhB + vehicle	7.81 ± 0.37
PhB + NEF (5)	7.473 ± 0.88
PHE + vehicle	0.3138 ± 0.02
PHE + NEF (5)	0.3325 ± 0.02

Data are presented as the mean free plasma concentrations (in μg/ml ± SD of eight determinations) of the AEDs. Statistical evaluation of data was performed with unpaired Student's *t*-tests. Blood samples were collected at the times scheduled for MES analysis. * $p < 0.05$ *vs.* control (vehicle-treated animals)

Effect of NEF on free plasma concentrations of tested AEDs

In mice treated with VPA, PhB and PHT, the free plasma levels of these compounds were not affected by co-administration of NEF at 5 mg/kg; however, free plasma levels of CBZ were significantly elevated in animals treated with NEF (Tab. 3).

Effect of NEF on the total brain concentration of VPA

As determined by the fluorescence polarization immunoassay method, the total brain VPA concentration in mice treated with VPA alone was 77.96 ± 7.61 μg/ml. These levels (77.44 ± 8.30 μg/ml) did not differ significantly when co-administered with 1 mg/kg NEF (results not shown).

Discussion

NEF is an analgesic mainly used to control acute post-operative pain. This drug has a unique mode of action distinct from non-steroidal anti-inflammatory drugs and opioids. NEF is pharmacologically unrelated to any other known analgesic agent, and its proposed mechanisms of action include the reuptakes of serotonin, norepinephrine, and dopamine [26]. Recently, NEF treatment was also shown to actively reduce the

shivering threshold in humans, which may be useful if therapeutic hypothermia is necessary [31].

In this study, we demonstrated that treatment with 5 mg/kg of NEF significantly elevated the electric seizure threshold, while a dose of 1 mg/kg failed to protect mice against electroconvulsions. Because adverse effects associated with the therapeutic use and following an overdose of NEF include a variety of pathological symptoms of the central nervous system, such as convulsions, hallucinations and cerebral edema, the anticonvulsant activity observed following NEF treatment may be unexpected [18, 27]. This discrepancy may be explained by the fact that the doses of NEF used in this study were much lower than those required to produce convulsions in our preliminary studies, exceeding 50 mg/kg (unpublished data). The sub-threshold dose of 1 mg/kg enhanced the anticonvulsant activity of VPA against MES, and the protective activity of PhB and PHT was significantly enhanced by the co-administration of 5 mg/kg NEF. Co-administration of NEF did not affect the protective activity of CBZ at the dose range used in this study. This observation was surprising because NEF treatment was shown to elevate the free plasma CBZ levels. This phenomenon may be explained by the fact that when two drugs that share a similar activity profile are combined, a negative interaction is likely to occur [7]. Because NEF and CBZ are known to act as voltage-sensitive sodium channel inhibitors, this assumption seems justified. As NEF had no influence on either free plasma levels of VPA or the total brain concentration of VPA, its combination with VPA seemed to be more pharmacodynamic in nature. It is important to note that, in our study, NEF treatment was effective against electroconvulsions when administered at doses shown to be effective in alleviating pain responses in various animal models of acute nociception. For example, the ED_{50} dose of NEF obtained in the writhing test was approximately 2.15 (0.79–5.85) mg/kg [14].

The observed protective activity of NEF against electroconvulsions demonstrated in this study may have resulted from the interaction of NEF with NMDA receptors. Similar to its structural analogue orphenadrine, NEF is suspected to interact with NMDA receptors, but its exact influence on glutaminergic transmission remains to be elucidated [12, 17]. In addition to its chemical structure, there is evidence that suggests a possible influence of NEF on glutamate-mediated neurotransmission. This agent was shown to possess preemptive analgesic activity in an animal model of chronic pain with NMDA receptor involvement [2, 19], and it protected mice against clonic seizures induced by intracerebrally administered agonists of the glutamate receptor [32]. The authors of the latter study also reported a protective activity of NEF against MES with an ED_{50} dose of 3.8 mg/kg when given intravenously. Additionally, Novelli et al. [25] demonstrated that NEF treatment was effective against MES when given *ip* at doses not exceeding 25 mg/kg. It is important to note that the authors of that study also reported that when administered at doses effective against MES, NEF failed to affect either motor coordination or locomotor behavior as assessed by the rotarod test and the Animex test of spontaneous locomotion. These results are in agreement with the results presented in this study. Because NEF treatment produced convulsions when given *ip* at doses exceeding 30 mg/kg, we failed to obtain a dose-response curve for NEF against MES (results not shown). These results are also in agreement with the results obtained by Novelli et al. [25].

Alternatively, NEF seems to inhibit voltage-sensitive sodium channels and does not appear to modulate glutaminergic transmission *via* NMDA receptor antagonism [11]. Verleye et al. [32] hypothesized that NEF treatment reduces the excitability of central neurons without direct interaction with the subtypes of glutamate receptors.

It is widely accepted that NMDA receptor antagonists display anticonvulsant activity and enhance the protective activity of AEDs in various animal models of seizures [28, 29]. Dizocilpine, a potent non-competitive antagonist of the NMDA receptor, significantly increased the electric seizure threshold and strongly enhanced the protective activity of VPA against MES in mice at subthreshold doses [5]. Two competitive NMDA receptor antagonists, D-(–)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonate (D-(–)CPP) and D,L-3-(±)-(2-carboxypiperazin-4-yl)propyl-1-phosphonate ((±)-CPP), were shown to enhance the protective activity of VPA, CBZ and PhB against electroconvulsions [4]. More recently, Makarska-Białek et al. [24] demonstrated that MRZ 2/576, a choline salt of pyridophthalazindione that is a glycine site antagonist of the NMDA receptor, displayed protective activity against electroconvulsions. When co-administered at a sub-protective dose, this antagonist significantly enhanced the anticonvulsant activity of CBZ, OXC, PB, PHT and VPA against MES.

NEF is derived from the histamine H_1 receptor antagonist diphenhydramine. This compound is known to possess proconvulsant activity, which is manifested by producing increased duration of EEG seizures in the MES-test and abolishing the protective activity of histidine and metoprine against amygdala-kindled seizures in rats [16, 17, 20]. Additional histamine H_1 receptor antagonists, such as astemizole and ketotifen, were also shown to diminish the electroconvulsive threshold and to impair the protective activity of various AEDs against the MES-test [30]. The antihistaminic activity of NEF is known to be 90-fold less than that displayed by diphenhydramine, and its analgesic activity was shown to not be mediated by histamine H_1 or H_2 receptors in mouse models of pain [14]. This explanation is also supported by results obtained by Diáz-Trelles et al. [8, 9], who reported that the antihistamine terfenadine blocked voltage-sensitive sodium channels at concentrations much lower than those required to block histamine receptors. Based on these data, any potential antihistaminic activity of NEF does not seem to play a role in its protective activity against electroconvulsions or in the enhancement of the anticonvulsive activity of AEDs against the MES-test in mice.

In conclusion, NEF possesses anticonvulsant activity when administered alone and significantly increases the protective activity of certain AEDs against electroconvulsions. Based on these conclusions, the use of NEF as a drug with potential anticonvulsant activity should be studied further, particularly in patients receiving AEDs.

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