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RESEARCH PAPER

Antinociceptive effects of NCX-701 (nitro-paracetamol) in neuropathic rats: enhancement of antinociception by co-administration with gabapentin

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Background and purpose: Neuropathic pain is characterized by a poor response to classic analgesics. In the present study, we have assessed the antinociceptive activity of NCX-701 (nitro-paracetamol) in neuropathic rats, after systemic and intrathecal (i.t.) administration. In addition, we analysed the possible benefit of the combination of NCX-701 and gabapentin, a well-known potent analgesic, in the treatment of neuropathic pain

Experimental approach: The antinociceptive effects of i.v. and i.t. NCX-701 and paracetamol were studied in spinal cord neuronal responses from neuropathic adult male Wistar rats, using the recording of single motor units technique. The effect of i.v. and i.t. NCX-701 in combination with i.v. gabapentin was studied by isobolographic analysis.

Key results: The experiments showed that NCX-701, but not paracetamol, dose-dependently reduced the nociceptive responses evoked by noxious mechanical and electrical stimulation, after i.v. (ID_{50} 542 ± 5 µmol·kg^{-1} for noxious mechanical stimulation) or i.t. (ID_{50} 932 ± 16 nmol·kg^{-1}) administration. The combined administration of i.v. or i.t. NCX-701 and i.v. gabapentin induced a more intense antinociceptive effect than any of the two drugs given alone. The isobolographic analysis showed a synergistic effect.

Conclusions and implications: NCX-701 is an effective antinociceptive compound in situations of neuropathy-induced sensitization, with an action mainly located in the spinal cord. The combination of NCX-701 and gabapentin induces a synergistic enhancement of the depression of nociceptive responses evoked by natural noxious stimulation. The use of NCX-701 alone or in combination with gabapentin might open up new and promising perspectives in the treatment of neuropathic pain.

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Abbreviations: COX, cyclooxygenase; CPA, N6-cyclopentyladenosine; NO, nitric oxide; NSAID, non-steroidal anti-inflammatory drug; SMU, single motor unit

Introduction

Neuropathic pain is produced by damage or injury to the peripheral or central nervous system and is characterized by a poor response to classic analgesics such as non-steroidal anti-inflammatory drugs (NSAIDs) and opiates. The treatment of neuropathic pain is complicated and not very effective in numerous occasions. It includes the use of tricyclic antidepressants, anticonvulsants like gabapentin and pregabalin, 5-hydroxytryptamine and noradrenaline reuptake inhibitors and topical anaesthetics like lidocaine, but many other drugs are utilized in an attempt to alleviate the intensity of pain in chronic or difficult treatment situations (Moulin *et al.*, 2007). Most of these drugs are, in addition, not devoid of unwanted side-effects, which, for many patients, is an additional problem with a demanding solution. The lack of an effective and safe treatment for neuropathic pain has led to the search for new molecules, or combinations of drugs, which may improve the quality of life for this type of patient.

Experiments carried out in our laboratory have shown that some nitro-NSAIDs (nitric oxide; NO; non-steroidal anti-inflammatory drugs), such as NO-paracetamol (NCX-701, nitro-acetaminophen), are more effective and potent

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analgesics than their parent compounds, either in the absence or in the presence of inflammation (Romero-Sandoval *et al.*, 2001; 2002; 2003; 2007). Nitro-NSAIDs were first developed just to take advantage of the cytoprotective properties of NO in the gastric mucosa. The hypothesis involved a counterbalance of the gastrointestinal damage induced by the action of the NSAIDs (Wallace *et al.*, 1999). However, for a not well-explained reason (Romero-Sandoval *et al.*, 2007), initial experiments showed that the new NSAID derivatives were more effective anti-inflammatory drugs (Fiorucci, 2001) and more potent analgesics than their parent compounds (Al-Swayeh *et al.*, 2000; Romero-Sandoval *et al.*, 2007).

In the present study we investigated whether NCX-701, which has been shown to be markedly effective in nociceptive and inflammatory pain (Romero-Sandoval *et al.*, 2007), exhibits any antinociceptive activity in neuropathy. To test this hypothesis we studied the antinociceptive activity of NCX-701 in responses to noxious natural and electrical stimulation, evoked in neuropathic rats, by means of the recording of spinal cord neuronal responses using the single motor unit (SMU) technique. We also determined whether its effect is mainly located in the spinal cord by comparing its actions after systemic and intrathecal (i.t.) administration. In addition, we analysed the possible benefit of combining NCX-701 with gabapentin, a well-known effective analgesic in the treatment of neuropathic pain (Tremont-Lukats *et al.*, 2000; Jensen, 2002), for the inhibition of nociceptive responses. Preliminary data have been previously published in abstract form (Herrero and Curros-Criado, 2008).

We conclude that NCX-701 is an effective antinociceptive compound in situations of neuropathy-induced sensitization, with an action mainly located in the spinal cord. The combination of NCX-701 and gabapentin induces a synergistic enhancement of the antinociceptive activity. The use of NCX-701 alone or in combination with gabapentin might open new and promising perspectives in the treatment of neuropathic pain.

Methods

Animals, preparatory surgery and groups of experiments

The experiments were performed in 52 male Wistar rats weighing 235–350 g. The animals were housed individually in cages, maintained on a 12 h light/dark cycle and with free access to food and water at all times. In the first part of the study we evaluated the possible antinociceptive activity of NCX-701 after i.v. and i.t. administration, comparing the results with its parent compound, paracetamol. The experiments were divided into four groups: animals treated with paracetamol i.v. (n = 7), animals treated with NCX-701 i.v. (n = 7), animals treated with paracetamol i.t. (n = 6) and animals treated with NCX-701 i.t. (n = 9). The second part of the study consisted of the evaluation of the antinociceptive activity of gabapentin alone i.v. (n = 9) and in combination with NCX-701 i.v. (n = 7) or i.t. (n = 7).

The antinociceptive activity of the drugs was assessed by means of the SMU technique, which has been described in detail several times elsewhere (Herrero and Headley, 1991; Solano and Herrero, 1997). Briefly, the preparatory surgery consisted in the cannulation of the trachea, one carotid artery to register the blood pressure, and two superficial jugular veins, and was performed under halothane anaesthesia (5% in oxygen for induction and 2–3% for maintenance). After the surgery, halothane was discontinued, and the anaesthesia maintained with α-chloralose (50 $mg \cdot kg^{-1}$ initial dose and 25 $mg \cdot kg^{-1} \cdot h^{-1}$ by perfusion pump for maintenance in a rate of 1 $mL \cdot h^{-1}$, diluted in saline). The right hind limb was fixed in inframaximal extension in a Perspex block using plaster of Paris. Core temperature was maintained at 37 ± 0.5°C by means of a feedback-controlled heating blanket. Blood pressure was monitored continuously during the experiments and rats with a systolic pressure below 100 mmHg, before the administration of a drug, were rejected from the experiment. In all cases the preparation was left to rest for at least 1 h after the surgery before any drug was tested. Mononeuropathy was induced under the same anaesthetic regime, 7 days before the experiment, using the partial ligation of the sciatic nerve technique (Seltzer *et al.*, 1990). The development of hyperalgesia was assessed by behavioural experiments, studying withdrawal reflex responses evoked by mechanical and thermal stimulation following the technique described previously in detail (Curros-Criado and Herrero, 2005). Briefly, von Frey filaments (60, 80, 100, 200, 300 and 500 mN, applied 10 times for approximately 1 s to the plantar surface of each hind paw) were used to study the frequency of withdrawal reflex responses. Withdrawal of the paw due to the application of the filament was considered as a positive response. Radiant heat generated by an algesimeter (Ugo Basile plantar test; 55°C) was used to test thermal hyperalgesia. Paw withdrawal latencies were measured for each paw using a maximum cut-off time of 17 s to avoid tissue damage.

Intrathecal catheter implantation

Chronic lumbar catheters were implanted in rats under i.p. ketamine/xylazine anaesthesia (2:1; 1×10^{-3} $mL \cdot g^{-1}$: 5×10^{-4} $mL \cdot g^{-1}$) following the technique described by Yaksh and Rudy (1976) at least 7 days before any other procedure. A 7.6 cm long polyethylene catheter (PE-5) was inserted through an incision in the atlanto-occipital membrane and advanced caudally into the i.t. space terminating at the L1–3 spinal segments. The end of the catheter was tunnelled subcutaneously over the front dorsal skull bones and held in this place with dental acrylic. Cefazolin (8×10^{-4} $mL \cdot g^{-1}$) was administered after the surgery to prevent postoperative infection. Rats were housed individually after implantation under the same conditions described above. I.t. catheters were carried for at least 5 days after implantation. Rats showing motor weakness or signs of paralysis upon recovery from anaesthesia were killed immediately. The location of catheters was assessed on completion of experiments by injecting 7 μL of pontamine sky blue (4% in 0.5 $mol \cdot L^{-1}$ sodium acetate).

Stimulus presentation and recording systems

Single motor unit activity was recorded from hind limb muscles by means of a homemade bipolar teflon-coated tungsten electrode (Solano and Herrero, 1997) and a standard electrophysiological set-up. Nociceptive activity was elicited

in 3 min cycles consisting of 10 s of noxious mechanical stimulation and one train of 16 percutaneous electrical stimuli. Noxious mechanical stimulation was applied over an area of 14 mm^2 using a computer-controlled pincher device, and a force of 200 mN over the threshold intensity, threshold being the minimal pressure required to evoke a constant firing rate for at least 10 s of stimulation (Herrero and Headley, 1991; Solano and Herrero, 1997). The electrical stimulation was applied using two 0.2 mm needles inserted percutaneously in the most sensitive area of the cutaneous receptive field, with 16 pulses of 2 ms width, 1 Hz and an intensity of twice the threshold current for long latency responses (C-fibre responses, Herrero and Cervero, 1996a). Only units with a stable firing rate and summation of responses to constant intensity repetitive electrical stimulation (wind-up) were selected for the experiments (Herrero *et al.*, 2000). The drugs were tested only when the responses observed with either stimulus were stable.

Drugs and collection and analysis of data

The drugs studied were prepared fresh everyday, immediately before administration, and were diluted in 0.9% saline. In all cases, i.t. administration was made in a total volume of 7 μL, followed by another 5 μL of saline to flush the catheter. The i.v. injection was made in a total volume of 0.3 mL, followed by another 0.3 mL of saline to flush the catheter. For i.v. administration, paracetamol and NCX-701 were dissolved in DMSO and polyethylene glycol (1:1) in a concentration of 50 $mmol \cdot L^{-1}$, diluted in saline and administered in cumulative log2 regime. The initial dose used was 15 $\mu mol \cdot kg^{-1}$, and the highest dose used was 960 $\mu mol \cdot kg^{-1}$. Each dose was administered every seven cycles of stimulation (21 min; Romero-Sandoval *et al.*, 2001; 2002; 2003). Gabapentin was dissolved in distilled water 0.5 $\mu mol \cdot \mu L^{-1}$ and diluted in saline. The drug was injected i.v. in cumulative log2 regime in a total and constant volume of 0.3 mL in doses of 40–640 $\mu mol \cdot kg^{-1}$. Preliminary experiments showed that the peak effect of gabapentin was observed within the first 7 min after i.v. administration. According to this, the doses studied were administered every three cycles of stimulation (9 min). For i.t. administration, paracetamol and NCX-701 were dissolved in DMSO (20 $nmol \cdot \mu L^{-1}$), diluted in saline and administered in cumulative log2 regime in a dose range of 60 to 960 $nmol \cdot kg^{-1}$.

The collection of data and stimulation protocols were performed by computer using commercial software (CED, Cambridge, UK; Spike 2). The effect of the highest cumulative dose was studied for a minimum of 30 min. The number of spikes counted in the last two cycles of stimulation (18–21 min for paracetamol and NCX-701 and 6–9 min for gabapentin) between each dose were averaged and the mean compared with the control response, control being the mean of the three responses obtained before the administration of the first dose (see for further details Herrero and Headley, 1991; Solano and Herrero, 1997). Spikes from mechanical and electrical stimulation were counted and analysed separately. The data from the electrical stimulation were analysed by counting the number of spikes evoked between 150 and 650 ms after each stimulus (C-fibre responses, Herrero and Cervero, 1996a,b). Wind-up responses are presented as actual mean number of spikes or as percentage of control, in order to facilitate comparisons. The effect of the drugs (raw data) and the comparison of regression curves were assessed with the one-way analysis of variance (ANOVA) with *post hoc* Dunnett's multiple comparison test, whereas the comparison of ID_{50} values was made with the two-tail unpaired *t*-test.

Drug interactions were determined by means of isobolographic analysis, described in detail previously (Tallarida *et al.*, 1989; Tallarida, 2001). Supra-additivity or synergistic effect is defined as the effect of a drug combination that is significantly higher than the theoretically calculated equieffect of a drug combination with the same proportions. The isobologram was constructed using the theoretical and experimental ID_{50} doses calculated from the experimental results when the drugs were given alone or combined. The drugs were administered in combination as fixed ratios of the equieffective ID_{50} dose for each drug (1:1). The ID_{50} values (±s.e.mean) for NCX-701 and gabapentin alone were plotted on the *x*- and *y*-axes, respectively, and the theoretical additive point was calculated according to Tallarida *et al.* (1989) and Tallarida (2001). The experimental values were analysed using linear regression and plotted on the isobologram for the comparison with the theoretical value. Student's *t*-test was used to determine significance of the difference between the theoretical additive point and experimental value. A synergistic effect was considered when the *P*-value was less than 0.05. In addition, the interaction index was calculated as experimental ID_{50}/theoretical ID_{50}. If the index value is close to 1, the interaction is additive, whereas values lower than 1 are an indication of the magnitude of supra-additive or synergistic interactions (Tallarida, 2001).

All the statistical tests were made by means of commercial software (GraphPad Prism and GraphPad InStat). Data are presented as mean ± s.e.mean. At the end of the experiments the animals were killed with an overdose of sodium pentobarbital. All experiments in this study were undertaken in accordance with Spanish and European Union legislation regarding the uses of animals for experimental protocols, and all efforts were made to reduce the number of animals used. The methods used in the present study were approved by the Committee of ethics in research of the University of Alcala.

Drugs and materials

α-Chloralose, pontamine sky blue and DMSO were all obtained from Sigma (Sigma-Aldrich, Madrid, Spain); the computer-controlled pincher device, model Estimec, was built by Cibertec (Madrid, Spain); polyethylene glycol, was acquired from Panreac Quimica (Barcebna, Spain); gabapentin from Medichem (Barcelona, Spain) and sodium pentobarbital (Dolethal) from Vetoquinol S.A. (Madrid, Spain). Dolethal (Vetoquinol S.A.). NCX-701 was kindly supplied by NicOx S.A. (Milan, Italy).

Results

Antinociceptive effects of paracetamol and NCX-701 after i.v. administration

The study of the antinociceptive effects of paracetamol and NCX-701 after i.v. administration in response to noxious

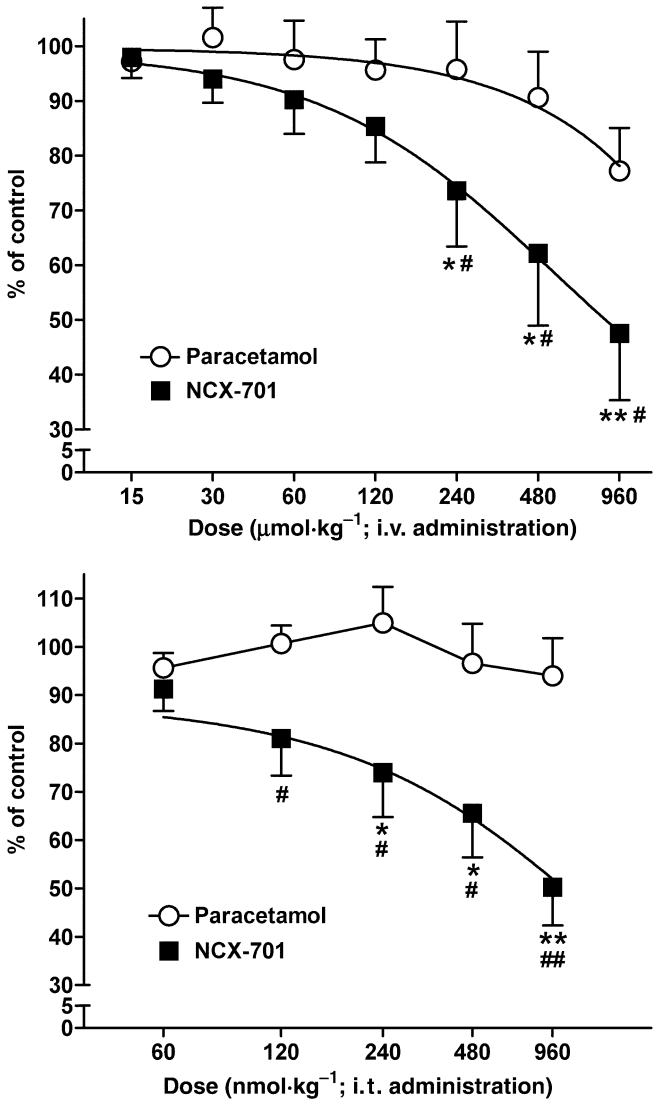

Figure 1 Antinociceptive effect of i.v. (upper panel) and intrathecal (i.t.) (lower panel) NCX-701 and paracetamol on responses to noxious mechanical stimulation. The i.v. or i.t. administration of paracetamol did not reduce significantly the responses to noxious mechanical stimulation in neuropathic rats. However, the administration of NCX-701 reduced dose-dependently the responses with an ID_{50} of 542 ± 5 μmol·kg^{-1} after i.v. administration and an ID_{50} of 932 ± 16 nmol·kg^{-1} after i.t. administration. Statistical analysis was made with the one-way ANOVA, with the *post hoc* Dunnett's test, comparing the effect of NCX-701 versus control response (*P < 0.05, **P < 0.01) and versus the effect of paracetamol (#P < 0.05, ##P < 0.01).

mechanical stimulation showed a clear different action of the drugs (Figure 1). Whereas the administration of paracetamol only induced a slight decrease in the responses to noxious mechanical stimulation with the highest dose studied (77 ± 8% of control response), the administration of NCX-701 produced a more intense and dose-dependent reduction of the nociceptive responses. The ID_{50} was 542 ± 5 μmol·kg^{-1}, the minimal effective dose was 240 μmol·kg^{-1} (P < 0.05; 67.7 mg·kg^{-1}), and the maximal effect observed was 47 ± 12% of the control response (P < 0.01). The effect of NCX-701 lasted for a minimum period of 30 min and was significantly lower than that observed with paracetamol from the dose of 240 μmol·kg^{-1} (P < 0.05, Figure 1).

High intensity electrical stimulation induced a clear wind-up in all the experiments performed (see the inset in Figure 2 as an example), and the responses elicited with the first pulse were similar in all curves. The administration of cumulative doses of paracetamol only induced a significant reduction of wind-up with the highest dose studied (53 ± 15%, P < 0.05, Figure 2). The administration of NCX-701, however, induced a more intense reduction of wind-up. Similar to that seen in responses to noxious mechanical stimulation, the effect was dose-dependent, with a minimal effective dose of 30 μmol·kg^{-1} (P < 0.05, Figure 2). The highest dose studied almost completely inhibited the responses (maximal effect of 18 ± 7% of control response, P < 0.01, Figure 2).

Blood pressure was monitored throughout the experiment, and no significant changes in mean arterial pressure were observed after the administration of paracetamol or NCX-701 (data not shown).

Antinociceptive effects of paracetamol and NCX-701 after i.t. administration

Figure 1 shows the effects of paracetamol and NCX-701 on responses to noxious mechanical stimulation after i.t. administration. The administration of cumulative doses of paracetamol was not followed by any significant change in the nociceptive responses. However, the administration of NCX-701 induced a significant and dose-dependent reduction of the nociceptive activity. In this case, the calculated ID_{50} by regression was 932 ± 16 nmol·kg^{-1}, the minimal effective dose was 240 nmol·kg^{-1} (P < 0.05; 67.7 μg·kg^{-1}), and the maximal effect observed was 50 ± 7% of the control response (P < 0.01). Similar to that seen after i.v. administration, the effect of NCX-701 lasted for a minimum period of 30 min and was significantly lower than that observed with paracetamol from the dose of 120 nmol·kg^{-1} (P < 0.05).

High intensity electrical stimulation induced a clear wind-up in all the experiments performed (see an example in inset of Figure 2), and the responses elicited with the first pulse were similar in all curves. The administration of paracetamol did not induce any significant reduction of wind-up with any of the doses studied (Figure 2). The administration of NCX-701, however, induced a significant and dose-dependent reduction of wind-up. The minimal effective dose was 240 nmol·kg^{-1} (P < 0.01, Figure 2), and the highest dose studied induced a reduction of 41 ± 11% of control response (P < 0.01, Figure 2).

Antinociceptive effects of the combined administration of NCX-701 and gabapentin after i.v. administration

In order to assess the degree of antinociception induced by NCX-701 in animals with neuropathy, we compared its activity with that of gabapentin, a drug with a well-known antinociceptive activity in neuropathic pain (see only as examples Tremont-Lukats *et al.*, 2000; Jensen, 2002). The results observed in responses to noxious mechanical stimulation are illustrated in Figure 3. The i.v. administration of gabapentin induced a maximal effect of 43 ± 7% of control response (P < 0.01), with an ID_{50} of 414 ± 27 μmol·kg^{-1}. Although the

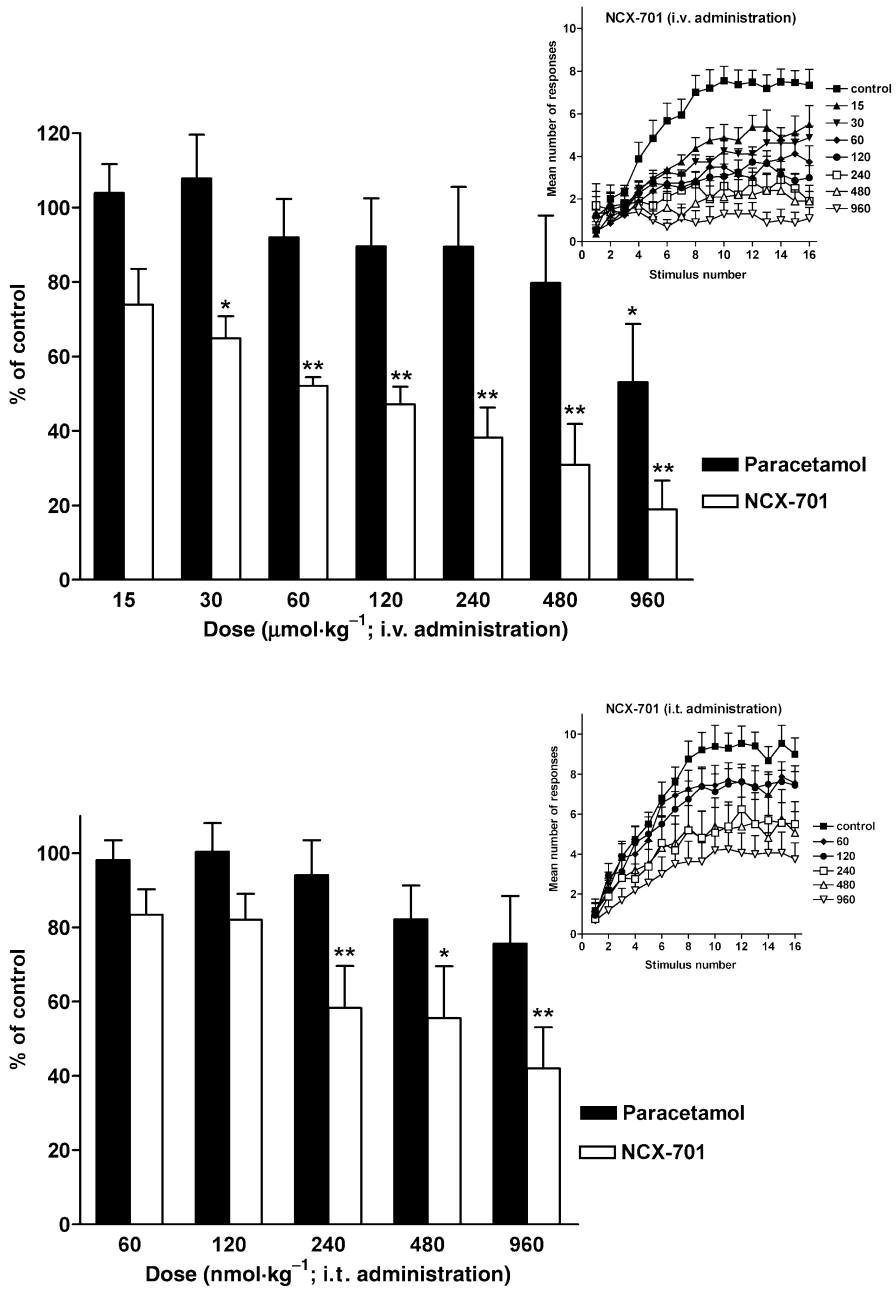

Figure 2 Effect of i.v. (upper panel) and intrathecal (i.t.) (lower panel) NCX-701 and paracetamol on wind-up. The administration of paracetamol only reduced the wind-up phenomenon with the highest dose studied after i.v. administration. No significant effect was observed after i.t. administration. NCX-701, however, induced a dose-dependent and almost complete inhibition of wind-up in neuropathic rats after i.v. administration (maximal reduction of 18 ± 7% of control response). The i.t. administration of NCX-701 also induced a dose-dependent reduction of wind-up in neuropathic rats (maximal reduction of 41 ± 11% of control response). The figure shows the effect of NCX-701 and paracetamol on wind-up as a percentage of control, and the effect of NCX-701 as the actual number of C-fibre-mediated responses (insets). Statistical comparison and layout as for Figure 1.

maximal effect observed was not significantly different from that observed with NCX-701 (47 ± 12% of control response), the ID_{50} values were significantly different (414 ± 27 vs. 542 ± 5 μmol·kg^{-1}, $P < 0.01$). Wind-up was also depressed by gabapentin, with a maximal reduction of 55 ± 20% of control ($P < 0.01$, data not shown in figures). The i.v. administration of NCX-701 combined with gabapentin (Figure 3) induced a more intense antinociceptive action when compared with the effect of NCX-701 alone: minimal effective dose of 60 μmol·kg^{-1} ($P < 0.05$), ID_{50} of 72 ± 18 μmol·kg^{-1} and maximal effect of 30 ± 7% control response. The possible synergistic interaction between the two antinociceptive drugs was studied by the isobolographic method. An isobologram showing the antinociceptive interaction of NCX-701 and gabapentin in responses to noxious mechanical stimulation is shown in Figure 3. The individual ID_{50} values of NCX-701 and gabapentin were plotted on the axes and connected by the theoretical additive line. The point in the middle of the line is

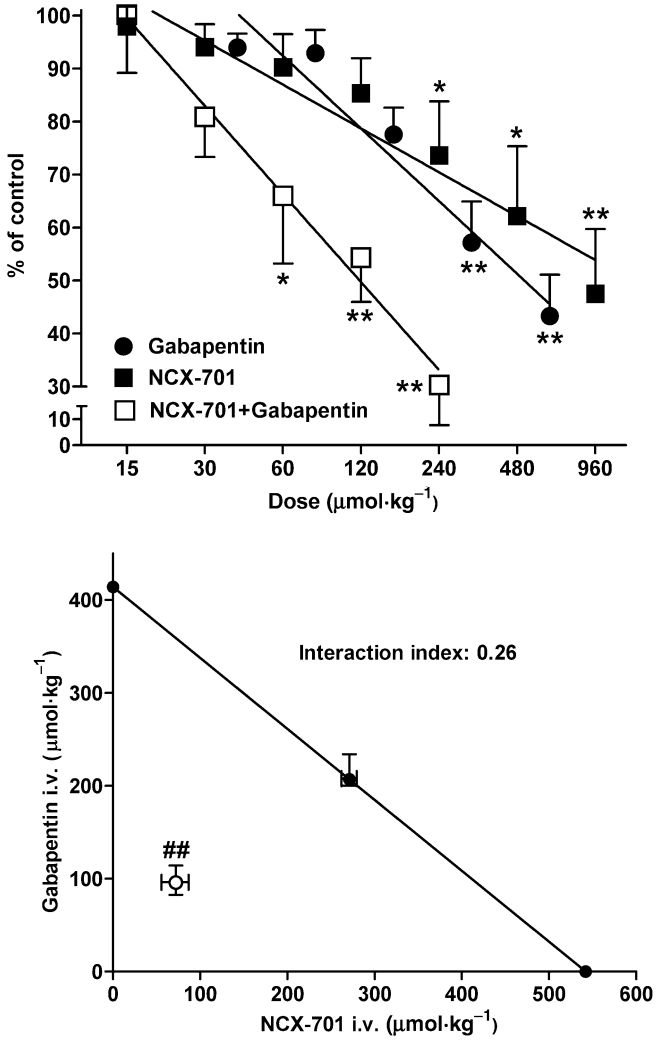

Figure 3 Antinociceptive effect of the combined i.v. administration of NCX-701 and gabapentin on responses to noxious mechanical stimulation. Top panel: the reduction of responses, evoked by noxious mechanical stimulation, observed after i.v. NCX-701 and i.v. gabapentin when given together and separately. The combined i.v. administration of NCX-701 and gabapentin induced a more intense antinociceptive effect than either of the two drugs alone. Lower panel: the isobolographic analysis, which showed that the experimental point lies far below the additive line, indicating a significant synergism. The calculated ID_{50} was 72 ± 18 nmol·kg^{-1}, significantly different from the theoretical ID_{50} (##$P < 0.01$, Student's *t*-test). The figure also shows the interaction index, well below 1. Statistical analysis of the antinociceptive effect was made with the one-way ANOVA, with the *post hoc* Dunnett's test, comparing the effect of the drugs versus their own control responses (*$P < 0.05$, **$P < 0.01$).

the theoretical additive point calculated from the separate ID_{50} values. The experimental result lies far below the additive line, indicating a significant synergism ($P < 0.01$). The interaction index, calculated as experimental ID_{50}/theoretical ID_{50}, was 0.26, which also indicates a synergistic interaction. In wind-up responses, the combined administration of NCX-701 and gabapentin did not induce any increase in the intensity of the effect when compared with the results observed separately (data not shown).

Antinociceptive effects of the combined administration of i.t. NCX-701 and i.v. gabapentin

Because NCX-701 showed a marked antinociceptive effect after i.t. administration, we studied whether the synergistic effect observed with the combined i.v. administration of NCX-701 and gabapentin was mainly located within the spinal cord or in the periphery. However, a possible non-specific chemical interaction between the drugs, if given together by the same route of administration, might interfere with their antinociceptive activity. Therefore, we studied the possible synergistic activity derived of the combined administration of i.t. NCX-701 and i.v. gabapentin. The combined administration of the drugs (Figure 4) induced an intense antinociceptive action on responses to noxious mechanical stimulation: minimal effective dose of NCX-701, 120 nmol·kg^{-1} ($P < 0.05$), ID_{50} of 265 ± 42 nmol·kg^{-1} and maximal effect reduced the response to 29 ± 10% control response. The isobologram showing the antinociceptive interaction of NCX-701 and gabapentin in responses to noxious mechanical stimulation is shown in Figure 4. The experimental result lies far below the additive line, indicating a significant synergism ($P < 0.01$). The interaction index, calculated as experimental ID_{50}/theoretical ID_{50}, was 0.56, which also indicates a synergistic interaction. The inhibitory effect of NCX-701 on wind-up responses was not increased by its combined administration with i.v. gabapentin (data not shown).

Discussion

The first observation made in this study is that NCX-701, but not paracetamol, is an effective antinociceptive agent in neuropathic rats. Paracetamol, like other cyclooxygenase (COX)-inhibitors, usually lacks any antinociceptive activity in experiments performed in the normal, non-inflammatory situation, and in neuropathy. Experiments carried out in our laboratory, however, had previously shown that NCX-701 is an effective antinociceptive agent in normal rats and in rats with inflammation (Romero-Sandoval *et al.*, 2001; 2002; 2003; 2007) but, to our knowledge, this is one of the first studies that shows an effective antinociceptive activity of a COX-inhibitor in animals with neuropathy. This observation might open new perspectives for the use of NO-releasing COX-inhibitors in neuropathic-induced sensitization. In addition, the antinociceptive effect of NCX-701 indicates a mechanism of action independent from that of paracetamol, and caused by the combined action of the parent molecule with the release of a low, but maintained, concentration of NO (Del Soldato *et al.*, 1999; Fiorucci, 2001; Kiss and Vizi, 2001; Romero-Sandoval *et al.*, 2002; 2007).

The inhibitory effect of NCX-701 was related not only to nociceptive responses to natural stimulation, but also to those elicited by repetitive electrical stimulation. Repetitive electrical stimulation induces the phenomenon of wind-up (Herrero *et al.*, 2000 for review), a progressive increase of nociceptive responses from spinal cord neurones. Wind-up is mediated by the activation of NMDA (Davies and Lodge, 1987; Dickenson and Sullivan, 1987) and NK_1 receptors (De Felipe *et al.*, 1998) and, therefore, it is the result of the activation of central neuronal circuitry. The depressant effect of NCX-701 on

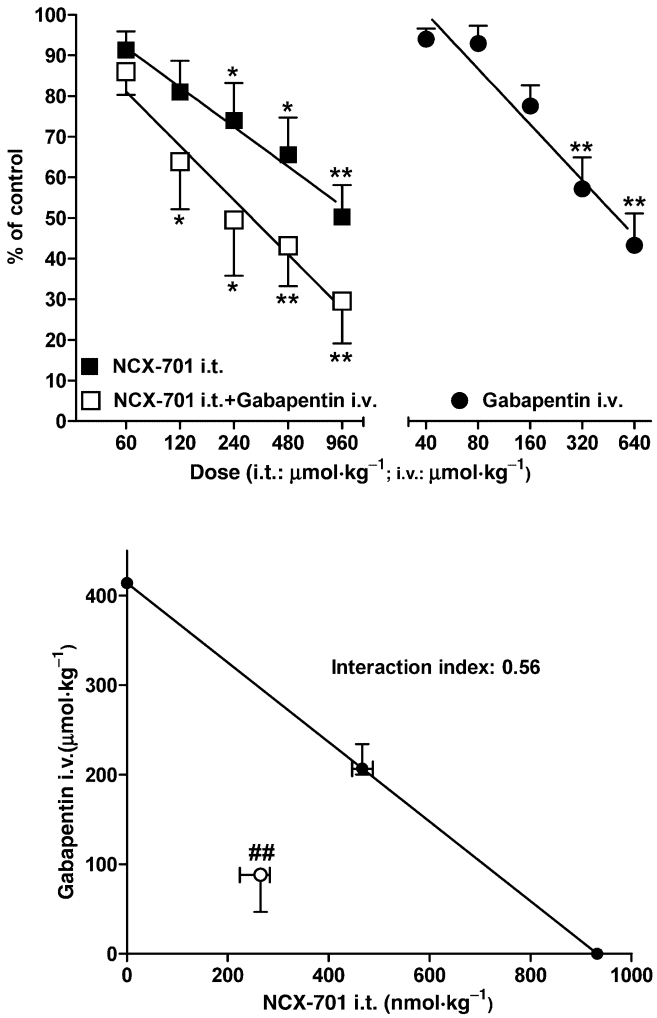

Figure 4 Antinociceptive effect of the combined administration of intrathecal (i.t.) NCX-701 and i.v. gabapentin on responses to noxious mechanical stimulation. Top panel: the reduction of responses, evoked by noxious mechanical stimulation, observed after i.t. NCX-701 and i.v. gabapentin when given together and separately. Similar to that seen in experiments carried out with the systemic administration of the two drugs, the combined administration of i.t. NCX-701 and i.v. gabapentin induced a more intense antinociceptive effect than either of the two drugs given alone. Lower panel: the isobolographic analysis, which showed that the effect observed with the combination of the drugs was the result of a synergistic effect. The calculated ID_{50} was 265 ± 42 nmol·kg^{-1}, significantly different from the theoretical ID_{50} (##$P < 0.01$, Student's *t*-test). The interaction index, well below 1, is also shown. Statistical analysis of the antinociceptive effect was made with the one-way ANOVA, with the *post hoc* Dunnett's test, comparing the effect of the drugs versus their own control responses (*$P < 0.05$, **$P < 0.01$).

wind-up suggests an action located within the spinal cord, similar to the results observed in other experimental situations (Romero-Sandoval *et al.*, 2002; 2003). This is supported by the fact that NCX-701, as well as paracetamol, is able to cross the blood brain barrier and enter the central nervous system easily (Ochs *et al.*, 1985; Bannwarth *et al.*, 1989; Romero-Sandoval *et al.*, 2007 and references within). However, an action located in the periphery cannot be fully rejected from only this observation. It is for this reason that we decided to carry out some experiments studying the effect of NCX-701, in comparison with paracetamol, after i.t. administration. The results of these experiments showed that NCX-701 induced a significant reduction of nociceptive responses evoked by either noxious mechanical stimulation or repetitive electrical stimulation. The i.t. administration of paracetamol, however, did not modify any of the nociceptive responses. The depression of wind-up by NCX-701 confirms an action in the spinal cord circuits involved in the generation of this phenomenon. On the other hand, the intensity of the reduction of nociceptive natural responses was very similar to that observed after i.v. administration, with an ID_{50} roughly 500-fold lower, indicating that most, if not all, of the antinociceptive effect observed after systemic administration of NCX-701 was located in spinal cord nociceptive neurones. These experiments also confirm that paracetamol lacks antinociceptive activity at central sites in neuropathy-induced sensitization. It is, therefore, possible to conclude that NCX-701 is an effective depressant of nociceptive responses evoked in neuropathic rats, and that its activity is mainly located within the spinal cord. On the other hand, it is not possible to deduce the mechanisms involved in this effect from the present experiments. However, previous experiments (Romero-Sandoval *et al.*, 2001; 2002; 2003) have shown that the effect is only observed after the combined administration of paracetamol and the NO-donor molecule, therefore a combination of different mechanisms of action, including those involved in the generation of wind-up (i.e. NMDA and NK_1 receptors) is likely (see Romero-Sandoval *et al.*, 2007 for further discussion).

The intensity of the antinociceptive effect of NCX-701 was comparable to that of gabapentin, a compound with well-known antinociceptive activity in neuropathic situations (Tremont-Lukats *et al.*, 2000; Jensen, 2002 and references within). In fact, although gabapentin showed a higher potency after systemic administration, the effectiveness of the reduction of nociceptive responses evoked by noxious mechanical stimulation observed in the present experiments was very similar for the two drugs. This also supports a possible and interesting new perspective in the treatment of neuropathic pain with NO-donors such as NCX-701. Furthermore, apart from gabapentin, adenosine and its derivatives may be considered as one of the very few drugs available with an effective analgesic action in situations of neuropathy (Sawynok and Liu, 2003; De Vry *et al.*, 2004). The adenosine A_1 receptor selective agonist N^6-cyclopentyladenosine (CPA) induces a very potent and intense inhibition of nociceptive responses in experiments similar to those carried out in the present study (Curros-Criado and Herrero, 2005). The antinociception observed in those experiments was certainly more potent and effective than that observed with NCX-701 or with gabapentin in the present study. However, CPA also induced a very intense depression of blood pressure (Curros-Criado and Herrero, 2005). This is not a new observation; it is well known that adenosine is a potent modulator of cardiovascular function and produces hypotension and bradycardia when administered systemically (Barraco *et al.*, 1987; Evoniuk *et al.*, 1987). The side-effects induced by adenosine are an important obstacle in the use of the drug and its derivatives in patients. On the other hand, although gabapentin has a relatively benign side-effect profile, it is not devoid of unwanted

side-effects. In fact, deficits in tests of motor/ambulatory and cognitive functions have been described (Lindner *et al.*, 2006). NCX-701 is a relatively new drug, not available on the market yet, and although further research is needed to ensure its safety, its pharmacological profile appears to be very promising (Romero-Sandoval *et al.*, 2007 for review). It is, therefore, possible to conclude that NCX-701 might represent an alternative treatment of neuropathic pain with a rather safe side-effect profile.

Nevertheless, despite all the above comments, NCX-701 reduced nociceptive responses in the present experiments for just a bit more than 50% of control response. This is an important and significant effect, similar to that seen with gabapentin, but, on the other hand, certainly not as intense as, for example, the antinociception induced by opioids or COX-inhibitors (including NCX-701; Romero-Sandoval *et al.*, 2007) in normal animals or in animals with inflammation-induced sensitization. We, therefore, wondered if the effectiveness, or at least the potency, of the antinociception observed might be enhanced by the combined administration of NCX-701 and gabapentin. In fact, previous experiments carried out in our laboratory had shown that the combination of NCX-701 and the μ-opioid agonist fentanyl induced an important enhancement of the potency, effectiveness and duration of the antinociception, either in normal animals with no inflammation (Gaitan *et al.*, 2003) or in monoarthritic rats (Gaitan *et al.*, 2005). The present experiments showed that the combination of systemic NCX-701 and gabapentin induced an enhancement of the depression of natural nociceptive responses and an intense improvement of the potency. The enhancement was more intense than that expected by the sum of the effects of each drug when given separately, and the results were compatible with a synergistic action. In addition, the synergistic enhancement of the antinociception was also observed after i.t. administration of NCX-701, indicating that this effect of NCX-701 was related to a depression of spinal cord-mediated antinociception. This observation implies also that an intense antinociception is observed with very low doses of NCX-701 and gabapentin. The combination of the two drugs, in consequence, might be very useful in the treatment of neuropathic pain, with a reduction of the risk of unwanted side-effect because of the low doses required to produce an effect. Furthermore, although the elucidation of the mechanisms underlying this synergy requires further studies, it is possible to speculate that a functional link exists between NCX-701 (but not paracetamol) and the mechanism of action of gabapentin, that is, α2-δ1 and α2-δ2 calcium channel subunits (Alexander *et al.*, 2008).

Finally, the enhancement of the inhibition of responses to noxious mechanical stimulation was not accompanied by a similar effect on wind-up responses. The experiments performed in the present study do not provide an explanation for this lack of effect, but it is possible to deduce that the mechanism underlying the synergistic action of NCX-701 and gabapentin is not related to the mechanisms responsible for wind-up (see Herrero *et al.*, 2000 for further discussion on this subject).

In conclusion, NCX-701 is an effective antinociceptive compound in situations of neuropathy-induced sensitization, with an action mainly located in the spinal cord. The combination of NCX-701 and gabapentin induces a synergistic enhancement of the effectiveness and potency of the depression of nociceptive responses evoked by natural noxious stimulation. The use of NCX-701 alone or in combination with gabapentin might open up new and promising perspectives in the treatment of neuropathic pain.

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Conflict of interest

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