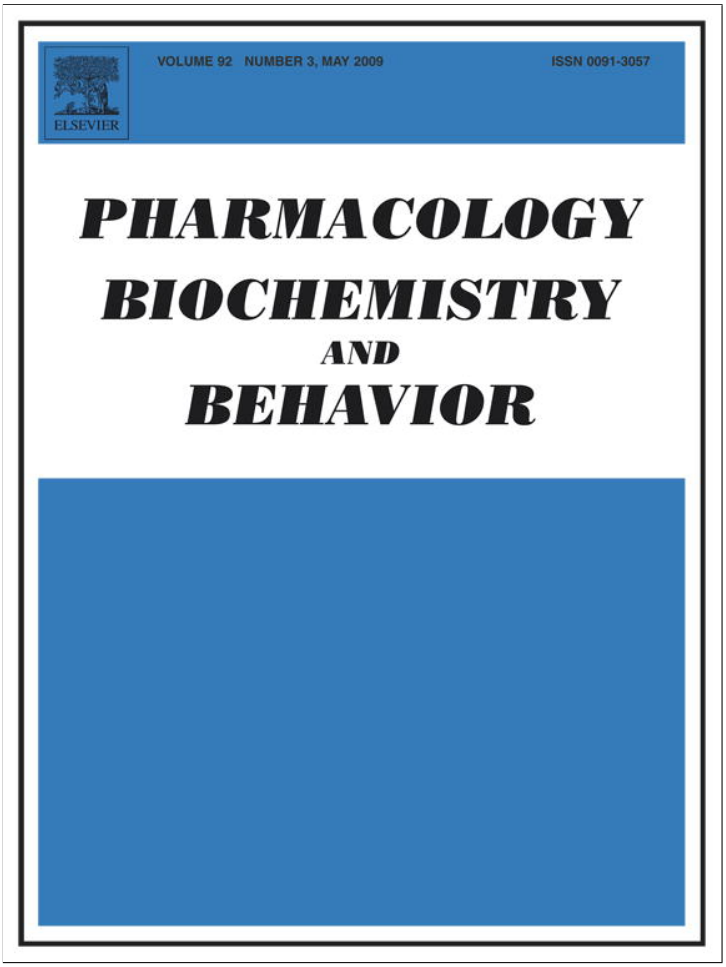

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Anti-nociceptive synergism of morphine and gabapentin in neuropathic pain induced by chronic constriction injury

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ABSTRACT

In order to detect an anti-nociceptive interaction between morphine and gabapentin, the anti-allodynic and anti-hyperalgesic effects of these drugs, administered either separately or in combination, were determined using the von Frey and acetone tests in a rat model of neuropathic pain (Bennett model). Morphine and gabapentin individually induced moderate attenuation of mechanical hyperalgesia, whereas the morphine and gabapentin combination completely decreased hyperalgesia. Morphine showed its maximal effect at 30 min post-injection in the acetone test; however, this effect gradually returned to the baseline value. Gabapentin did not produce an anti-allodynic effect, whereas the morphine and gabapentin combination completely decreased allodynia behavior at 30 min post-injection, an effect that persisted until 120 min. The area under the curve (AUC) of the anti-allodynic or anti-hyperalgesic effects produced by the combinations were significantly greater than the theoretical sum of effects produced by each drug alone or similar to the theoretical sum. The analysis of the effect, expressed as the AUC of the time course, supports the hypothesis that the combination of these drugs is useful in neuropathic pain therapy.

1. Introduction

Neuropathic pain is defined by the International Association for the Study of Pain (IASP) as "pain initiated or caused by a primary lesion or dysfunction in the nervous system" (Merskey and Bogduk, 1994). The spectrum of neuropathic pain includes a variety of diseases and is presented in the clinic by a variety of symptoms. Neuropathic pain is estimated to afflict millions of people worldwide, although precise records are not available, partly because of the diversity of the associated conditions (Hall et al., 2006). Clinically, the presence of neuropathic pain is often characterized by stimulus-independent persistent pain or abnormal sensory perception of pain, such as allodynia (a painful response to a normally innocuous stimulus) and hyperalgesia (exaggerated pain sensations as a result of exposure to a mildly noxious stimulus) (Ueda and Rashid, 2003). The pathophysiological mechanisms underlying neuropathic pain have been reviewed extensively in recent years, and the results reflect both peripheral and central sensitization mechanisms (Moalem and Tracey, 2006). Randomized controlled clinical trials for neuropathic pain treatment have provided an evidence-based approach and specific recommendations for the use of diverse drugs, including topical lidocaine, anticonvulsants, tricyclic anti-depressants, mixed serotonin–norepinephrine reuptake inhibitors, opioids and tramadol. Of these drugs, gabapentin and opioids have been proposed as two of several first-line treatments for neuropathic pain (Dworkin et al., 2007).

Morphine is the most widely used opioid for the treatment of severe pain, but controversies exist regarding its effectiveness in neuropathic pain. In spite of this, it has been proven to be effective in treating patients with neuropathic pain in multiple clinical trials (Dworkin et al., 2007). Gabapentin is an anti-convulsant that has shown analgesic efficacy across a wide spectrum of pain states, including several neuropathic conditions (Levendoglu et al., 2004). Additionally, in preclinical studies, gabapentin has been shown to possess anti-nociceptive properties in animal models of both nociceptive (Feng et al., 2003) and neuropathic pain (Xiao et al., 2007). However, the management of patients with chronic neuropathic pain is complex, and the response to existing treatments is often inadequate. Even with well-established neuropathic pain medications, efficacy is unpredictable, as dosing can be complicated, the onset of the analgesic effect may be delayed, and side effects are common. Consequently, recent experimental and clinical data support the potential benefits of pharmacotherapeutic approaches using a

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combination of drugs for treating neuropathic pain (Gilron and Max, 2005). The therapeutic benefits may include greater efficacy, lower doses and fewer adverse effects (Dworkin et al., 2007). Thus, in the clinical management of neuropathic pain, when pain relief with gabapentin is incomplete, expert panels have recommended adding a second analgesic agent, which may be an opioid. In clinical studies, concomitant morphine with gabapentin administration has been shown to enhance analgesic effects in healthy volunteers and to reduce morphine consumption after mastectomy and after spinal surgery (Turan et al., 2004). In addition, pain intensity was significantly lowered with the co-administration of these drugs in patients with diabetic neuropathy or postherpetic neuralgia, and these drugs also provide greater relief of neuropathic pain in cancer patients compared to opioid drug monotherapy (Keskinbora et al., 2007). Previous preclinical studies have also demonstrated that gabapentin increases the anti-nociceptive effect of morphine in an acute model of nociception (Meymandi et al., 2006) and in a visceral nociception model. An electrophysiological study showed that this combination produced inhibition of evoked dorsal horn neuronal responses in a rat model of neuropathy (Matthews and Dickenson, 2002). However, the anti-allodynic and anti-hyperalgesic effects of different combinations of gabapentin and morphine in a neuropathic pain model have not yet been studied, and no information exists regarding the type of synergistic pharmacological interaction they may generate. Thus, this study was performed to determine the anti-allodynic and anti-hyperalgesic effects and the synergistic anti-nociceptive interaction of morphine and gabapentin, administered either separately or in combination, on chronic constriction injury (CCI) of the sciatic nerve, a rat model of neuropathic pain, using the von Frey and acetone tests.

2. Materials and methods

2.1. Animals and housing conditions

Male Wistar rats [Crl(WI)BR] weighing between 120–140 g at the time of surgery were used in this study. All animals were housed under standardized conditions in a room on a 12 h light/dark cycle with food and water available *ad libitum*. All experimental procedures were approved by the internal Institutional Animal Care and Use Committee and followed the Guidelines on Ethical Standards for Investigations of Experimental Pain in Animals (Zimmermann, 1983). All tests were performed during the light phase. The number of experimental animals was kept to a minimum, and following the end of the study, rats were euthanized by CO_2 overdose.

2.2. Compounds

Morphine sulfate (Mexican Secretariat of Health, Mexico City, Mexico) and gabapentin (Sigma-Aldrich, Steinheim, Germany) were dissolved in saline solution (0.9% NaCl) and injected subcutaneously (s.c.) in an application volume of 2 ml/kg body weight.

2.3. Surgery

The chronic constriction injury (CCI) of the sciatic nerve model was employed according to methods described by Bennett and Xie (Bennett and Xie, 1988; De Vry et al., 2004). Briefly, rats were anaesthetized by an intraperitoneal (i.p.) injection of a ketamine/xylazine mixture (50:7.5 mg/kg), and the right common sciatic nerve was exposed at the mid-thigh by dissection through the biceps femoris. Proximal to the sciatic trifurcation, the nerve was freed of adhering tissue, and four ligatures with 3-0 silk thread were tied loosely around the nerve with a spacing of about 1 mm. After surgery, the muscle and skin were closed in two layers using absorbable chromic catgut 4-0 for the muscle and 3-0 silk thread for the skin. In sham-operated controls, an identical surgical procedure was performed, except that the sciatic nerve was not ligated. All surgical procedures were performed under normal sterile conditions by the same person.

2.4. Behavioral testing

In order to avoid additional stress shortly after the surgical procedures, behavioral testing with mechanical and cooling stimuli was conducted 10 days after surgery. During testing, the rats were placed on an elevated wire mesh floor enclosed in acrylic containers and were not removed until the completion of both mechanical and cold sensory testing. The rats were adapted to the testing situation, and they were allowed to habituate until exploratory behavior diminished for at least 10 min before stimulation was initiated. Baseline values (BL) for each type of stimulation were obtained prior to drug administration to ensure that consistent behavioral responses were present. The number of stimuli applied to the rats was determined as the minimum needed to evoke reproducible and robust behavioral responses.

2.4.1. Von Frey test

The rats were placed in acrylic cages on top of a wire mesh grid that allowed their paws access to the von Frey filaments. Bending forces of 1, 6, 10 and 15 g to the mid-plantar skin of each hind paw were then applied in increasing order from the weakest to the strongest. Beginning with the lowest force, the filament was placed on the skin until it bowed slightly, with each filament presented ten times at a rate of about 1/s. A different region within the testing area was stimulated with each presentation. A response was recorded if the rat withdrew its hind paw from the filament. Responses were converted into a percent frequency (% = number of responses/10 × 100) (Xiao et al., 2007). Sham-operated rats rarely withdrew from 1 g and 6 g stimuli; the increased level of response seen after CCI is thus indicative of mechano-allodynia (tactile allodynia). Sham-operated rats withdrew from 10 g and 15 g stimuli; the increased level of response to these hairs seen after CCI is thus indicative of mechano-hyperalgesia (tactile hyperalgesia).

2.4.2. Acetone test

Five to 10 min after the last von Frey filament test, the acetone test was performed (cold allodynia). With the animals inside acrylic cages on the elevated grid, a drop of acetone was delicately applied to the plantar surface of the hind paw without touching the skin using a blunt plastic needle connected to a syringe. A response was recorded if the rat withdrew its hind paw in response to acetone application. The time spent with the leg withdrawn from the floor during the 60 s following exposure to acetone was recorded. Both hind legs were tested in each animal, beginning with the unoperated left leg, and each stimulus was applied three times at intervals of approximately 5 min. The duration of lifting of the hind paw after acetone stimuli was recorded with a stopwatch (Dowdall et al., 2005).

2.5. Experimental design

The effects of acute administration of drugs on mechanical and cold sensitivity were tested between day 10 and day 12 post-surgery. Baseline mechanical hyperalgesia and cold allodynia were assessed the day before pharmacological testing in order to confirm the behavioral pathology using the von Frey and acetone tests, respectively (De Vry et al., 2004). The experimental protocol consisted of two sets of experimental groups in which the anti-nociceptive effects produced by morphine and gabapentin given either individually or in combination were studied. In the first set of experimental groups, each dose of morphine (1.8, 3.2 or 5.6 mg/kg, s.c.) or gabapentin (10.0, 17.8, 31.6, or 56.2 mg/kg, s.c.) was given in a volume of 2 ml/kg to six

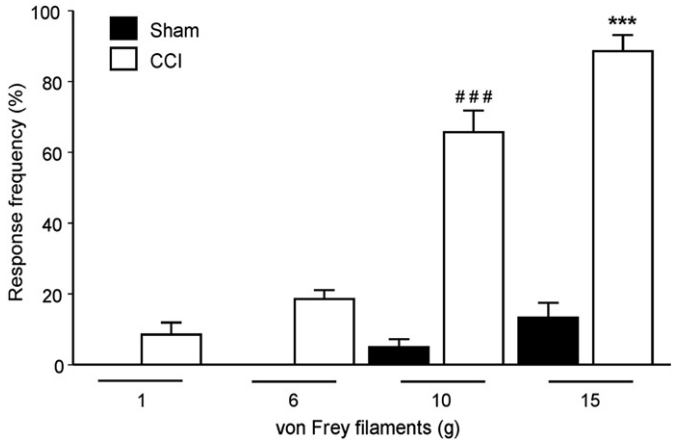

Fig. 1. Response frequency (%) to tactile stimulus with von Frey filaments after unilateral chronic constriction injury of the sciatic nerve (white bars) or sham surgery (black bars) in rats. von Frey filaments (1, 6, 10, 15 g) were applied 10 times to the plantar hind paw, and the number of positive responses was recorded. Data were obtained 10 days after surgery, and the mean ± SEM is shown, $n = 6$/group. *** and ### $P<0.001$ *versus* sham group.

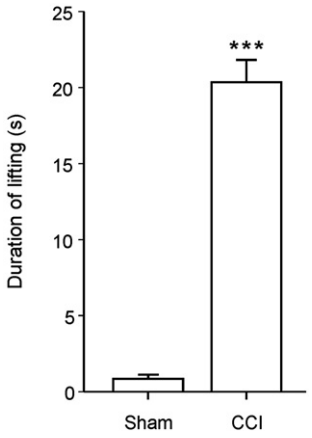

Fig. 2. Duration of lifting (s) in response to cold stimulus with the acetone test 10 days after unilateral CCI of the sciatic nerve or sham surgery in rats. Data show the mean ± SEM, $n = 6$/group. *** $P<0.001$ *versus* sham group.

neuropathic rats to obtain the corresponding dose-response curves. In the second set, morphine and gabapentin were combined to analyze possible synergistic interactions, and were given in six total combinations: 1.8 + 17.8, 1.8 + 31.6, 1.8 + 56.2, 3.2 + 10.0, 3.2 + 17.8, 3.2 + 31.6 (Mor + Gbp in mg/kg, s.c.). Adequate controls were performed with the corresponding vehicles (saline) in CCI and sham rats. In order to determine anti-allodynic and anti-hyperalgesic effects, rats were tested every 30 min for 180 min (3 h) post-injection following s.c. administration.

2.6. Motor coordination test

The motor coordination test was performed to determine side effects of drugs alone or in combination using the rotating rod method (rotarod) (López-Ruvalcaba and Fernández-Guasti, 1994). Briefly, this procedure consisted of placing animals on a rotating cylinder (diameter = 7 cm) at a speed of 11 rpm. Animals were trained to walk on the cylinder for three consecutive sessions prior to pharmacological treatment. For the fourth session, the animals received morphine (3.2 or 5.6 mg/kg, s.c.) or gabapentin (56.2 mg/kg, s.c.) individually or in combination (3.2 + 10.0 mg/kg). The number of falls during a 5 min period was recorded. After a fall, the animal was immediately replaced on the cylinder.

2.7. Data analysis and statistics

Data are expressed as mean ± SEM of $n = 6$ animals/group. The cumulative anti-nociceptive effect during the entire observation period (180 min or 3 h) was determined as the area under the curve (AUC) of the time course. The AUCs for each of the assayed drugs and their combination were calculated by the trapezoidal method (Rowland and Tozer, 1989). Differences between two groups where the data were not normally distributed were evaluated with non-parametric statistics (Mann–Whitney test). An analysis of variance (ANOVA) or Kruskal–Wallis test was used for between-group comparisons, followed by the post-hoc Tukey or Dunn tests, respectively. To determine the synergistic effect from the time course, the anti-hyperalgesic effect as assessed by the AUC produced by the combination was compared with the sum of effects produced by the drugs administered individually using a one-tailed unpaired Student *t*-test. In all statistical analyses $P<0.05$ was considered to be statistically significant. The analyses were performed using SigmaStat 3.0 software (SPSS Inc., Chicago, IL, USA).

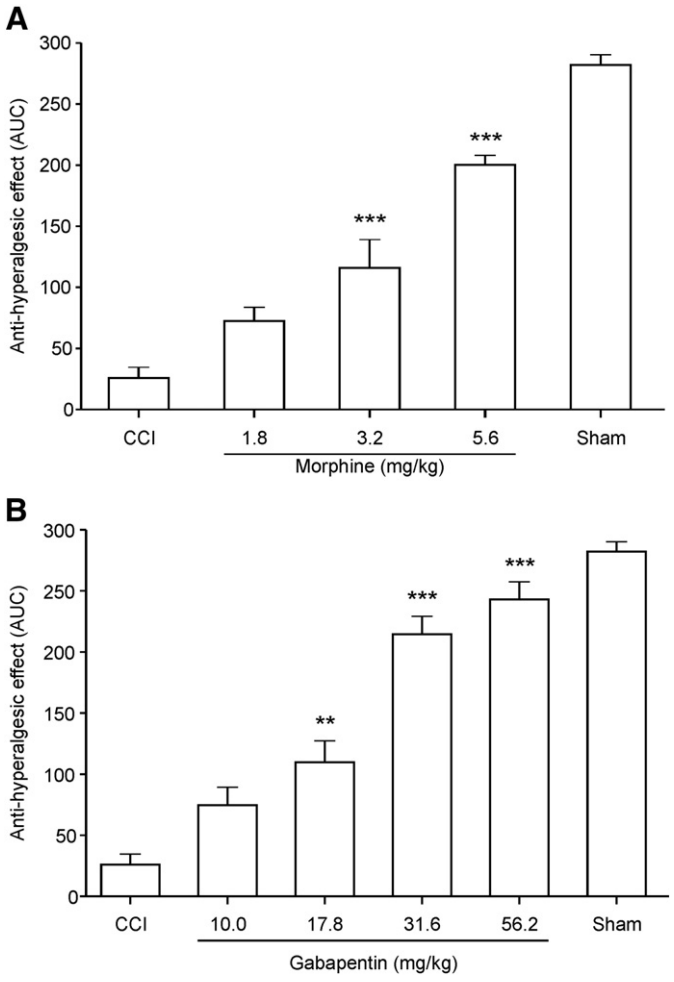

Fig. 3. Dose-response curves expressed as the area under curve (AUC) for the anti-hyperalgesic effects of morphine (Panel A) and gabapentin (Panel B) with a von Frey filament (15 g) after CCI of the sciatic nerve in rats. Rats were treated with vehicle (saline solution) or increasing doses of Mor (1.8, 3.2 or 5.6 mg/kg, s.c.) or Gbp (10.0, 17.8, 31.6, or 56.2 mg/kg, s.c.). Bars are means ± SEM, $n \geq 5$/group. ** $P<0.01$ and *** $P<0.001$ *versus* vehicle (CCI).

Table 1
Interaction between morphine and gabapentin in the von Frey and acetone test in rats.

Treatment (mg/kg, s.c.)	von Frey test AUC (a.u.)	Result of comparing AUC of anti-hyperalgesic effects produced by combination and theoretical sum	Acetone test AUC (a.u.)	Result of comparing AUC of anti-allodynic effects produced by combination and theoretical sum
Mor (1.8)	72.5 ± 11.1		106.2 ± 24.2	
Gbp (17.8)	109.6 ± 17.6		62.6 ± 14.3	
Theoretical sum	182.1 ± 14.7		168.8 ± 28.1	
Mor + Gbp (1.8 + 17.8)	180.0 ± 14.7[a]	Addition	181.8 ± 17.0[a]	Addition
Mor 1.8	72.5 ± 11.1		106.2 ± 24.2	
Gbp (31.6)	214.6 ± 14.6		46.8 ± 16.6	
Theoretical sum	287.1 ± 14.0		153.0 ± 29.3	
Mor + Gbp (1.8 + 31.6)	272.9 ± 2.1[a]	Addition	118.2 ± 30.2[a]	Addition
Mor (1.8)	72.5 ± 11.1		106.2 ± 24.2	
Gbp (56.2)	242.9 ± 14.4		130.7 ± 7.5	
Theoretical sum	315.4 ± 12.9[c]		236.9 ± 25.3	
Mor + Gbp (1.8 + 56.2)	274.2 ± 3.6	–	261.3 ± 9.6[a]	Addition
Mor (3.2)	115.8 ± 23.2		99.8 ± 11.4	
Gbp (10.0)	74.6 ± 14.7		35.3 ± 18.7	
Theoretical sum	190.4 ± 19.4		135.1 ± 21.9	
Mor + Gbp (3.2 + 10)	270.4 ± 5.1[b]	Supra-addition	216.1 ± 7.7[b]	Supra-addition
Mor (3.2)	115.8 ± 23.2		99.8 ± 11.4	
Gbp (17.8)	109.6 ± 17.6		62.6 ± 14.3	
Theoretical sum	225.4 ± 20.6		162.4 ± 18.3	
Mor + Gbp (3.2 + 17.8)	263.3 ± 3.3[b]	Supra-addition	224.5 ± 12.3[b]	Supra-addition
Mor (3.2)	115.8 ± 23.2		99.8 ± 11.4	
Gbp (31.6)	214.6 ± 14.6		46.8 ± 16.6	
Theoretical sum	330.4 ± 19.4[c]		146.5 ± 20.2	
Mor + Gbp (3.2 + 31.6)	275.8 ± 0.5	–	250.2 ± 6.1[b]	Supra-addition

Area under the curve (AUC) of the whole anti-allodynic or anti-hyperalgesic effects displayed by morphine (Mor) or gabapentine (Gbp) during 3 h, either alone or in combination. Results correspond to the mean ± SEM, $n = 6$ rats.

[a] $P \geq 0.05$.

[b] $P < 0.05$ *versus* theoretical sum.

[c] The theoretical sum exceed the maximum value of the AUC (300 a.u.), then it is not correct to determine interaction.

3. Results

3.1. Behavioral characterization

Ten days after surgery, CCI rats developed statistically significant increases in responses to 1, 6, 10 and 15 g von Frey filament stimulation. CCI rats showed 8.5 ± 3.4% and 18.5 ± 2.6% of response frequency to 1 g and 6 g von Frey filament stimuli, respectively, compared with the sham-operated group, which did not show any reaction. Thus, this effect was a mechano-allodynic response. However, the stimuli of 10 g or 15 g von Frey filaments produced mechano-hyperalgesic responses in rats on day ten post-surgery. For example, when a 10 g filament stimuli was used in the sham group, 5.0 ± 2.2% was the observed response, while in CCI rats the percentage of response was 65.7 ± 6.1%. Similarly, when using the stimulus of a 15 g von Frey filament, the responses were 13.3 ± 4.2% and 88.5 ± 4.6% for the sham and CCI groups, respectively (Fig. 1). The most consistent and robust response in neuropathic rats was obtained with the 15 g filament, which evoked significant increases in the hind paw withdraw frequency of CCI rats compared with the sham group ($P < 0.001$). Therefore, this filament was used to evaluate possible drug effects. Pronounced cold allodynia in response to acetone stimulation of the ipsilateral hind paw was observed in the CCI group in the acetone test 10 days after surgery. This test indicated a significant increase in lifting time for CCI rats (20.4 ± 1.5 s) compared with control sham rats (0.9 ± 0.3 s) ($P < 0.001$), as assessed by the Mann–Whitney rank sum test (Fig. 2).

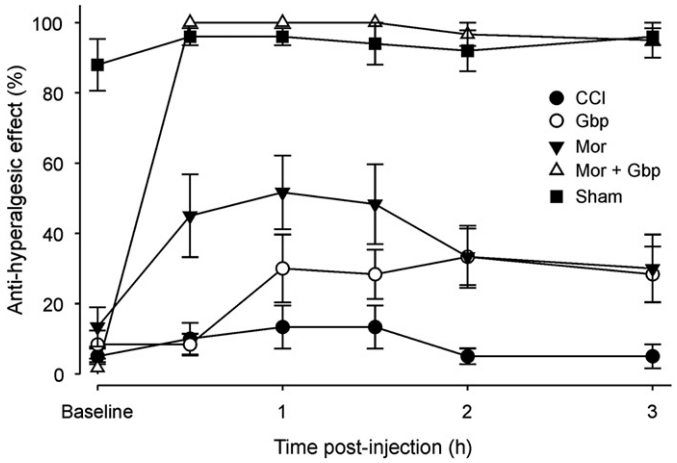

Fig. 4. Time course of anti-hyperalgesic effects of morphine (3.2 mg/kg), gabapentin (10.0 mg/kg), and morphine and gabapentin in combination at the same doses on mechanical hyperalgesia with a 15 g von Frey filament after CCI of the sciatic nerve. Control groups (sham and CCI) were treated with equivalent volumes of vehicle. Data are expressed as mean ± SEM, $n = 5$/group.

3.2. Anti-nociceptive effects of drugs on the von Frey test assayed individually

Fig. 3 shows the dose-response curves expressed as the area under the curve (AUC) of the respective 3 h time courses post-injection for morphine (panel A) and gabapentin (panel B) administered separately. Morphine and gabapentin dose-dependently decreased tactile hyperalgesia in the von Frey test. The maximum value of the AUC obtained in the von Frey test (anti-hyperalgesic effects) under these experimental conditions was 300 area units (a.u.). The maximum value of the AUC obtained in the acetone test (anti-allodynic effect) under these experimental conditions was 300 a.u. Morphine and gabapentin produced reduced anti-allodynic effects, and only the highest doses (5.6 and 56.2 mg/kg, s.c., respectively) decreased cold allodynia ($P < 0.001$), as assessed by the acetone test (data no shown).

3.3. Synergistic interactions between morphine and gabapentin in the von Frey and acetone tests

The synergistic interactions between morphine and gabapentin (Mor + Gbp) in the von Frey and acetone tests in CCI rats are shown in Table 1. When the whole effect, expressed as the area under the curve (AUC), produced by each combination was compared with the theoretical sum of the effects produced by each drug alone, some combinations (1.8 + 17.8 mg/kg and 1.8 + 31.6 mg/kg) did not show statistically significant difference in the von Frey and acetone tests ($P \geq 0.05$ by Student *t*-test). The AUC of the anti-allodynic or anti-hyperalgesic effects produced by some combinations (3.2 + 10.0 mg/kg and 3.2 + 17.8 mg/kg) were significantly greater than the theoretical sum of effects produced by each drug alone ($P < 0.05$ by Student *t*-test). Since, the sum of anti-hyperalgesic effects produced by the combinations

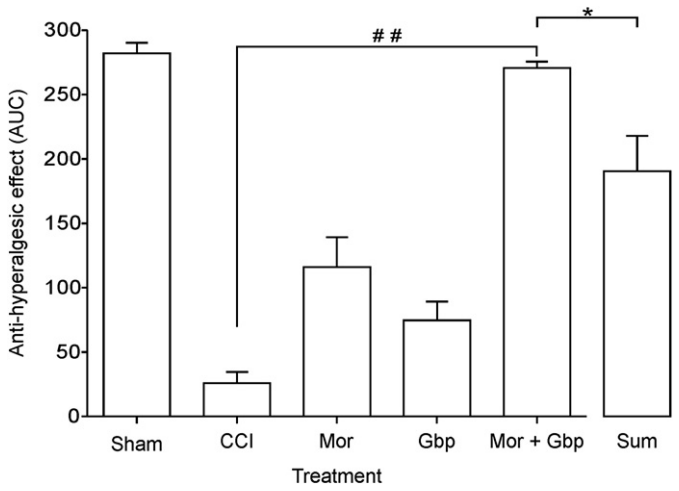

Fig. 5. Area under the curve (AUC) of anti-hyperalgesic effects produced by morphine (3.2 mg/kg) (Mor), gabapentin (10.0 mg/kg) (Gbp), and morphine and gabapentin in combination (Mor + Gbp) at the same doses. Bars are the means ± SEM, $n > 5$/group. *$P < 0.01$ *versus* theoretical sum (Sum), # #$P < 0.01$ *versus* vehicle (CCI).

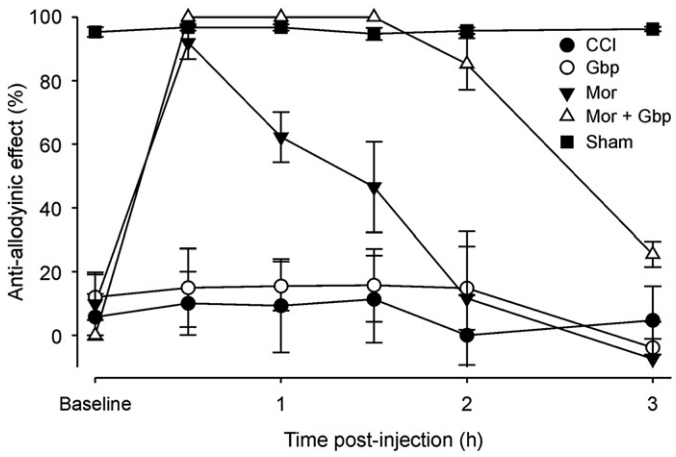

Fig. 6. Time course of anti-allodynic effects of morphine (3.2 mg/kg), gabapentin (10.0 mg/kg) and the combination of morphine and gabapentin at the same doses on cold allodynia after CCI of the sciatic nerve. Control groups (sham and CCI) were treated with equivalent volumes of vehicle. Data are expressed as mean ± SEM, $n = 6$/group.

of (1.8 + 56.2 mg/kg) and (3.2 + 31.6 mg/kg) exceed the maximum value of the AUC, it was not possible to determine the synergism in the von Frey test ($P < 0.05$), while the same combinations led to additive ($P \geq 0.05$) and supra-additive ($P < 0.05$) interactions, respectively, in the acetone test.

3.4. Drug effects on mechanical hyperalgesia and synergistic interaction of morphine + gabapentin (3.2 + 10.0 mg/kg)

One hundred eighty minute time courses of the effects of 3.2 mg/kg morphine (▼), 10 mg/kg gabapentin (○) and the combination of morphine and gabapentin (3.2 + 10.0 mg/kg) (Δ) on mechanical hyperalgesia after a single s.c. administration are shown in Fig. 4. Prior to drug administration, the mean baseline mechano-hyperalgesia of all CCI rats was 5.0 ± 2.2% with a 15 g von Frey filament. CCI rats that were injected with vehicle continued to show the same percentage baseline mechano-hyperalgesia to von Frey stimulation throughout the observation period, which was the highest percentage of hyperalgesia (● CCI), whereas the sham-operated group (■ Sham) presented a minimal response to this mechanical stimulus during the entire 3 h period of observation. Morphine achieved its maximum effect at 30 min post-administration, showing a increase in anti-hyperalgesia values of 45.0 ± 11.8%, and gabapentin produced its maximum anti-hyperalgesic effect 1 h after administration, increasing the percentage of anti-hyperalgesia to 30.0 ± 9.7%. These effects did not increase during the entire time course, whereas the morphine and gabapentin combination increased anti-hyperalgesia completely (100%) 30 min post-injection, suggesting that anti-hyperalgesic effects remained unchanged during the following 150 min of observation.

The overall effects expressed as the area under the curve (AUC) of the respective time courses (during the first 180 min or 3 h post-injection) of morphine (3.2 mg/kg, s.c.) and gabapentin (10.0 mg/kg, s.c.), administered either separately or in combination, were analyzed in order to determinate the synergistic antinociceptive interaction (Fig. 5). It should be mentioned that in this case the area values were calculated by using the values of the response percentages in the graph of the time course (Fig. 4) in order to clarify the anti-hyperalgesic effects shown by the corresponding treatments. Morphine and gabapentin administered individually showed 115.8 ± 23.2 a.u. and 74.6 ± 14.7 a.u., respectively, but although having a slight anti-hyperalgesic effect, did not present significant differences compared with the CCI control group without treatment (25.8 ± 8.8 a.u.). However, the AUC obtained with morphine and gabapentin in combination (270.4 ± 5.1 a.u.) was significantly greater ($P < 0.01$) than in the CCI control (Kruskal–Wallis test followed by the Dunn test) and did not present a significant difference compared with the sham control group (282.0 ± 8.2 a.u.). A statistically significant difference ($P < 0.01$) was observed when the theoretical sum of effects produced by each drug alone (190.4 ± 27.5) was compared with the effect obtained from morphine and gabapentin in combination by a Student *t*-test.

3.5. Drug effects on cold allodynia and synergistic interaction of morphine + gabapentin (3.2 + 10.0 mg/kg)

The 3 h time courses of the anti-allodynic effects of 3.2 mg/kg morphine (▼), 10.0 mg/kg gabapentin (○) and the combination of morphine and gabapentin at the same doses (Δ) after a single s.c. administration are shown as the anti-allodynic effect (%) (Fig. 6). Prior to drug administration, the mean allodynia baseline of all CCI rats was 5.8 ± 7.2% using the acetone test. The percentage baseline allodynia in CCI rats that were injected with vehicle (● CCI) remained the same throughout the observation period, whereas the sham operated group (■ Sham) did not present any response to this cold stimulus during the entire 3 h period of observation. Morphine achieved its maximum

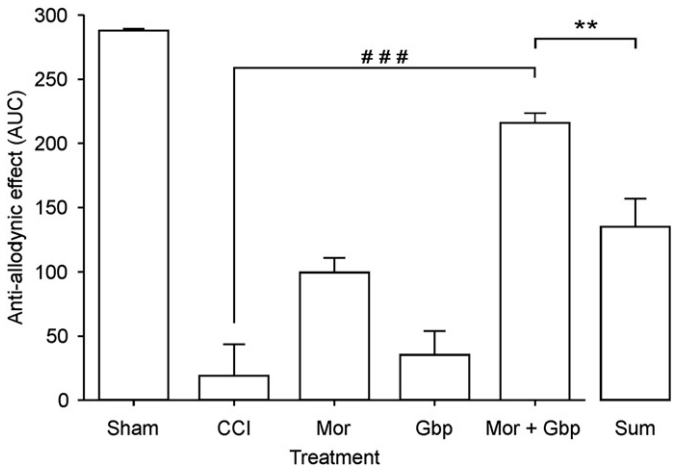

Fig. 7. Area under the curve (AUC) of anti-allodynic effects produced by morphine (Mor 3.2 mg/kg), gabapentin (Gbp, 10.0 mg/kg), and the morphine and gabapentin combination (Mor + Gbp) at the same doses. Bars are the means ± SEM, $n = 6$/group. ** $P < 0.01$ *versus* theoretical sum (Sum). ### $P < 0.001$ *versus* vehicle (CCI), morphine and gabapentin alone.

effect at 30 min post-administration, showing an increase in the anti-allodynic effect of 92.0 ± 5.3%, although this effect diminished over the time course, returning to baseline values at 3 h. Gabapentin alone did not produce any anti-allodynic affect, as a similar response was shown in the CCI vehicle group in the acetone test, and it did not decrease the duration of lifting. However, the combination of morphine and gabapentin completely increased anti-allodynia behavior at 30 min post-injection, such that 100% of the anti-allodynic effect remained, and the effect did not change for 2 h after injection.

The AUCs of the effects during the 3 h post-injection period for morphine (3.2 mg/kg, s.c.) and gabapentin (10.0 mg/kg, s.c.), administered either separately or in combination, were analyzed in order to determine the synergistic anti-allodynic interactions in the acetone test (Fig. 7). As was mentioned earlier, the area values were calculated using the values for the anti-allodynic effects shown in the graph of the time course (Fig. 6). The maximum value of the AUC obtained under these experimental conditions was 300 a.u. An AUC of 287.9 ± 1.5 a.u. was observed in the sham group, which did not show allodynic effects. Morphine and gabapentin administered individually showed values of 99.8 ± 11.4 and 35.3 ± 18.7 a.u., respectively. Morphine showed significant differences ($P<0.05$) compared with the CCI control group without treatment (19.2 ± 24.4 a.u.), whereas gabapentin did not present any significant difference. The AUC obtained with morphine and gabapentin in combination (216.1 ± 7.7 a.u.) was significantly greater ($P=0.001$) than the CCI control or the individual morphine or gabapentin groups, as assessed with a one-way ANOVA followed by a post-hoc Tukey test. There was synergistic interaction by this combination, as a statistically significant difference ($P=0.01$) was observed when the theoretical sum of the effects produced by each drug alone (135.1 ± 21.9 a.u.) was compared with the effect obtained with morphine and gabapentin in combination (216.1 ± 7.7 a.u.) by the Student *t*-test (Table 1).

3.6. Drug effects on motor coordination

The effects of individual drugs and drugs in combination on motor coordination were compared to animals receiving vehicle, and were evaluated by the number of falls from the rotarod apparatus. The vehicle did not affected motor coordination (0.17 ± 0.17 falls). The highest dose of gabapentin (56.2 mg/kg) did not affect motor coordination (0.50 ± 0.17 falls) as morphine (3.2 mg/kg) or combination (morphine 3.2 mg /kg and gabapentin 10 mg/kg) did (3.33 ± 0.88 and 2.50 ± 0.62 falls, respectively). The number of falls resulting from the combination of morphine (3.2 mg/kg) and gabapentin (10 mg/kg) was comparable with morphine 3.2 mg/kg alone, ($P>0.5$). However, a large effect on motor coordination (14.83 ± 3.20 falls) was observed with the highest doses of morphine (5.6 mg/kg) compared with vehicle ($P<0.001$).

4. Discussion

The present study demonstrates the anti-allodynic and anti-hyperalgesic effects of morphine and gabapentin and the synergistic anti-nociceptive interaction of their combination on chronic constriction injury of the sciatic nerve as a model of neuropathic pain. This model is based on a unilateral loose ligation of the sciatic nerve, which is one of the most frequently used models for the study of neuropathic pain and its treatment. This model also shows many of the pathophysiological properties of chronic neuropathic pain in human subjects, such as allodynia and hyperalgesia (Bennett and Xie, 1988; De Vry et al., 2004). Chronic constriction injury in rats simulates the clinical condition of chronic nerve compression such as that occurring in nerve entrapment neuropathy or spinal root irritation by lumbar disk herniation. Animal models of neuropathic pain generally entail injury to a peripheral nerve (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992; Lee et al., 2000; Hofmann et al., 2003) followed by behavior assessment of the animals to make sure that the nerve injury models are related to pain. Behaviors such as allodynia and hyperalgesia are parameters that have been previously used to study the pharmacology and modulation of neuropathic pain (Dowdall et al., 2005). In this study, 10 days after CCI, the rats showed a relatively high degree of similarity with other studies published on neuropathic pain in terms of the degrees of allodynia and hyperalgesia against cold and mechanical stimuli, demonstrated by the increased responsiveness to acetone stimulus and von Frey filaments. The doses used for obtaining the DRCs of morphine and gabapentin alone were selected on an increasing 0.25 logarithmic unit basis. The doses used for analyzing the combinations were selected from the respective DRCs because they produced a weak to moderate anti-nociceptive effect and did not produce adverse effects when were administered alone and because in scientific literature controversies on drug combinations analysis do exist, and it has been presented evidence that evaluation of drug synergism interaction must be done from dose-response curves (Tallarida, 2007).

Since the combination (3.2 + 10.0 mg/kg) of morphine and gabapentin demonstrated supradditive effect on mechanical hyperalgesia and cold allodynia with the lowest dose of gabapentin, this dose was considered the best, and its synergistic interaction on mechanical and cold allodynia has been well described in this report.

Morphine is the most widely used opioid and the standard against which new agents are compared. Morphine mediates its actions by binding and activating receptors in the peripheral nervous system, as well as those found in inhibitory pain circuits that descend from the midbrain to the spinal cord dorsal horn via presynaptic and, to a lesser extent, postsynaptic μ-opioid receptors modulating nociceptive transmission (Nicholson, 2003). This opioid drug has demonstrated antinociceptive efficacy in several models of nociception (López-Muñoz et al., 1993a), including neuropathic pain (De Vry et al., 2004). However, the anti-allodynic and anti-hyperalgesic abilities of morphine in behavioral studies involving neuropathy are somewhat variable and seem to be dependent on the model of neuropathy used, the behavioral assessment, and the nature of the stimuli used, in addition to the route of morphine administration (Matthews and Dickenson, 2002). In the current study, morphine (1.8–5.6 mg/kg) reduced tactile hyperalgesia in a dose-dependent manner in the von Frey test, but only a weak anti-allodynic effect was observed at the highest dose (5.6 mg/kg, s.c.) in the acetone test. These results are consistent with previous findings reported in the same animal model of neuropathic pain (Hama and Borsook, 2005). Gabapentin has been extensively used to treat neuropathic pain. Gabapentin is structurally related to the neurotransmitter GABA; however, it does not interact with GABA receptors or GABA metabolism, and it has no effect on sodium channels like other anti-convulsants (Rose and Kam, 2002). The antinociceptive effects of gabapentin may involve inhibition of the release of excitatory amino acids from presynaptic terminals. Other studies suggest that gabapentin, besides opening K^+ channels, may partially activate the NO-cyclic GMP-PKG spinal pathway in the Chung model of neuropathy (Moalem and Tracey, 2006). The mechanisms of action of gabapentin and its successor, pregabalin, are likely mediated by binding to the α_2-δ subunit of presynaptic voltage-gated calcium channels, which are upregulated in the dorsal root ganglia and spinal cord after surgical trauma. Gabapentin may produce antinociception by inhibiting calcium influx via these channels, and subsequently inhibiting the release of excitatory neurotransmitters (e.g., substance P, calcitonin gene-related peptide, glutamate, and norepinephrine) from the primary afferent nerve fibers in the pain pathway (Taylor et al., 2007). It was also demonstrated that gabapentin acts on supraspinal structures to activate the descending noradrenergic system that terminates in the lumbar spinal cord, where noradrenaline interacts with α_2-adrenoceptors to reduce thermal and mechanical hypersensitivity after partial nerve injury (Takeuchi et al., 2007). Gabapentin is not metabolized and is eliminated unchanged in the

urine with an elimination half-life of 4–6 h. Due to a lack of hepatic metabolism and low protein binding, gabapentin has not shown clinically relevant drug interactions (Rose and Kam, 2002). Gabapentin is well tolerated, with few serious adverse effects. The main dose-limiting side effects are somnolence and dizziness, which are reduced by gradual dosage titration, and peripheral edema. The anti-convulsant gabapentin is becoming widely accepted by clinicians as an alternative treatment for various types of neuropathic pain because it provides reasonable efficacy and is well tolerated (Rose and Kam, 2002; Levendoglu et al., 2004). In neuropathic pain models, the administration of gabapentin in rats reduced tactile allodynia induced by ligation of L5 and L6 nerves in a dose-dependent manner. In the chronic constriction injury model, gabapentin induced a weak to moderate attenuation of thermal hyperalgesia and mechanical allodynia (De Vry et al., 2004). Gabapentin dose-dependently decreased mechanical hypersensitivity in spared nerve injury (SNI) and distal (plantar) nerve injury (DNI) in rats, but only the highest dose of gabapentin (100 mg/kg, s.c.) decreased hypersensitivity in CCI rats, and gabapentin had no statistically significant effect on cold hypersensitivity in either the CCI or SNI models. Similarly, in the current study, gabapentin (10–56.2 mg/kg, s.c.) dose-dependently decreased tactile hyperalgesia in the von Frey test in CCI rats, but only the highest dose of gabapentin (56.2 mg/kg) decreased cold allodynia in the acetone test. Administration of low dose gabapentin (10.0 mg/kg) induced a relatively weak attenuation of mechanical hyperalgesia and had no effect on the duration of the lifting response in the acetone test during the first 3 h post-injection compared with the baseline response and with injection of vehicle. In addition, although gabapentin significantly alleviated mechanical allodynia and heat hyperalgesia in neuropathic animals as well as in patients with neuropathy, the analgesic effect on cold allodynia remains controversial (Levendoglu et al., 2004). While the reason for this disparity is unclear, it is possible that it may reflect differences in the stimulation site (paw versus tail) and stimulus type (paw/tail immersion versus cold spray or data acquisition methods, i.e., withdrawal latency versus response frequency). In fact, some animals exhibited a high withdrawal response frequency to spraying acetone, but a short duration of foot lifting off a cold plate, and *vice versa*. Alternatively, the different modes of nerve damage in these models may be another possible cause (Walczak and Beaulieu, 2006). The acetone spray test incorporates not only an innocuous cold stimulus but, because of the spray technique and the chemical composition of acetone, simultaneous cold, mechanical, and chemical stimulations (Vissers and Meert, 2005).

Since current therapy for pain relief is inadequate for some patients and chronic pain is difficult to treat, the search for new analgesic compounds or therapies must continue. The potential benefits of combining opioid and nonopioid drugs have been described (López-Muñoz et al., 1993b; López-Muñoz, 1994). This is due to enhanced analgesic efficacy, a broader analgesic spectrum, decreased side effects, and prevention of opioid tolerance, although it is important to note that some combinations of analgesic drugs may not have clinical utility in pain therapy due to sub-additive interaction results (García-Hernández et al., 2007).

A recent randomized controlled trial indicated that pain intensity during treatment with a morphine and gabapentin combination was significantly lower than either single agent in patients with diabetic neuropathy or neuralgia postherpetic. However, this study was not designed to test whether combination therapy is synergistic, or even additive. A combination of gabapentin and morphine resulted in enhanced inhibitory effects on the dorsal horn neuronal responses in an electrophysiological study in a rat model of neuropathy (Matthews and Dickenson, 2002). A sub-analgesic dose of gabapentin may enhance the anti-nociceptive effects of both anti-nociceptive and sub-antinociceptive doses of morphine in an acute model of nociception (Meymandi et al., 2006). This group of investigators suggested that future studies are needed to interpret additive or synergistic effects from this combination. In the current study, a synergistic interaction between morphine and gabapentin was confirmed when analyses of the AUCs of the whole anti-hyperalgesic or anti-allodynic effects over a 3 h time course were analyzed post-injection for six different combinations. These results demonstrate that the interaction between morphine and gabapentin is able to produce an additive anti-nociceptive effect when doses of morphine and gabapentin that present weak to moderate anti-hyperalgesic or anti-allodynic effects individually are co-administered, as assessed by the von Frey test (1.8 + 17.8 or 1.8 + 31.6 mg/kg) or the acetone test (1.8 + 17.6, 1.8 + 31.6 or 1.8 + 56.2 mg/kg). A supra-additive effect was observed with the combinations, where either of the individual doses, or at least one of them, do not show any anti-allodynic or anti-hyperalgesic effect with the von Frey (3.2 + 10.0 or 3.2 + 17.8 mg/kg) or acetone tests (3.2 + 10.0, 3.2 + 17.8 or 3.2 + 31.6 mg/kg). Therefore, under these experimental conditions, the synergistic interaction depends on the drug ratio co-administered and the test assessed. Chou (2006), consider: "additive effect is not a single arithmetic sum of two (of more) drugs", and this was clear in the combinations that exceed the maximum value of the AUC, then it was not possible to determine the pharmacological interaction.

In the present study, the synergistic interaction of one combination (3.2 + 10.0 mg/kg) was well described and confirmed, as analysis of the AUC of anti-hyperalgesic effect over a time course of 3 h post-injection produced by this combination indicated a significantly greater AUC value than the theoretical sum of the effects of each drug given alone ($P<0.05$). On the other hand, this combination induced a strong attenuation of cold allodynia when a sub-antinociceptive dose of gabapentin (10 mg/kg), which did not show any anti-cold allodynia, was combined with morphine, and the anti-allodynic effect persisted for 3 h post-administration. Additionally, when the AUC was analyzed, this combination of morphine and gabapentin showed an AUC greater than the AUC of the theoretical sum of each drug alone ($P<0.01$). The present results confirm a positive interaction between morphine and gabapentin, showing enhanced anti-hyperalgesic and anti-allodynic effects.

Since morphine and gabapentin differ in their adverse effect profiles, a potentiation of adverse effects induced by their combination would seem unlikely; additionally, low doses of both drugs were used in this experiment. In this respect, it should be pointed out that during the whole experimental period (3 h) the rats did not show any alteration in their behavior or walking resulting from the administration of gabapentin or morphine in the combination studied. In fact, the effects resulting from the combination (3.2 + 10.0 mg/kg) or morphine alone (3.2 mg/kg) were not different in the motor coordination test; only the highest dose of morphine (5.6 mg/kg) exhibited adverse effect in the motor coordination test. However, future trials are needed to evaluate optimal drug combinations and dose ratios, as well as safety, compliance and cost-effectiveness (Gilron and Max, 2005).

Neuropathic pain and epilepsy share neuronal hyperexcitability as a common underlying mechanism. There are established anti-epileptic drugs that target the generation of neuronal hyperexcitability, and some of these have been proven to be effective in the treatment of various forms of neuropathic pain (Sindrup and Jensen, 2000).

In this study, no attempt was made to deduce the mechanism involved in the interaction of the antinociceptive effects produced by gabapentin and morphine. It is possible that a pharmacodynamic interaction exists between the drugs, although a pharmacokinetic interaction is unlikely since gabapentin is not metabolized and is eliminated unchanged in the urine (Rose and Kam, 2002). Differences in mechanisms of action may explain the potentiation effect obtained with the combinations of morphine and gabapentin used in the current study. Because of their different ionic mechanisms of inhibition, it could be predicted that morphine and gabapentin

would interact positively through a concomitant decrease of excitation and an increase of inhibition, since gabapentin targets the excitatory system and morphine targets the inhibitory system (Matthews and Dickenson, 2002; Gutstein and Akil, 2006).

In summary, the current data support the results obtained by Gilron and Max (2005) and extend previous studies showing that acute treatment with a combination of morphine and gabapentin is effective in improving symptoms associated with different types of pain, including inflammatory, visceral, postoperative, and, in this particular case, neuropathic pain. This is the first study where the anti-allodynic and anti-hyperalgesic effects of several morphine and gabapentin combinations in CCI were assessed, and interaction of these combinations were found, demonstrating that treatment with the combination of morphine and gabapentin greatly reduced cold allodynia and mechanical hyperalgesia with lower doses of each drugs than for either single agent, and showing no increase in side effects on motor coordination.

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