Synergistic Antinociceptive Effect of Amitriptyline and Morphine in the Rat Orofacial Formalin Test

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Background: Combination therapy is often used to increase the clinical utility of analgesic agents. The coadministration of two compounds may achieve analgesia at doses lower than those required for either compound alone, leading to enhanced pain relief and reduction of adverse effects. Herein, the authors describe the effect of coadministration of morphine and amitriptyline on cutaneous orofacial inflammatory pain in rats.

Methods: Amitriptyline, morphine, or the combination of amitriptyline and morphine was administered systemically to rats, and antinociceptive effects were determined by means of the rat orofacial formalin test. Isobolographic analysis was used to define the nature of the interactions between morphine and amitriptyline.

Results: Amitriptyline as well as morphine produced a dose-related inhibition in the first phase and the second phase of rubbing activity. ED_{50} values against rubbing behavior were 14.6 mg/kg (95% confidence interval, 10.2–33.5 mg/kg) and 1.3 mg/kg (95% confidence interval, 1.0–1.7 mg/kg) for amitriptyline and morphine, respectively. Combinations of increasing fractional increments of amitriptyline and morphine ED_{50} doses produced a synergistic effect against rubbing behavior, as revealed by isobolographic analysis.

Conclusions: The current study suggests that systemic amitriptyline and morphine synergistically inhibit cutaneous orofacial inflammatory pain in rats.

THE orofacial region is a frequent site of acute and chronic pain. However, the mechanisms underlying these types of pain are still poorly understood. This is partly because of the relative scarcity of investigations devoted to the study of nociception mechanisms in the trigeminal region. As a consequence, only a few analgesic trials have been undergone in trigeminal region. Therefore, it seems necessary to study new analgesic strategies that could be particularly efficacious in the treatment of orofacial pain using appropriate animals models.

Multimodal analgesia is currently recommended for effective pain control.[1,2] It is achieved by combining different analgesics that act by different mechanisms, resulting in additive or synergistic analgesia.[2] The main goals of such combinations would be to improve analgesia and/or to reduce the adverse effects induced by each of the drugs administered separately. The opioid analgesic drugs remain the most effective therapy available for the treatment of moderate to severe pain. However, the side effect profile of opioids, which includes nausea/vomiting, sedation, constipation, and respiratory depression, should be considered when using large doses of these drugs. Using combinations of medications that elicit analgesic synergism should allow for reduction in the required dosage and decrease the incidence of adverse effects.[1,2] Multimodal analgesia is often achieved by combining opioids with nonopioid analgesics. For example, animal studies have shown additive and/or synergistic effects between opioids and nonsteroidal antiinflammatory drugs[3] as well as with other drugs, such as gabapentin[4] or clonidine.[5]

The tricyclic antidepressant amitriptyline is widely used to treat various chronic inflammatory and neuropathic pain conditions.[6] These include diabetic neuropathy, postherpetic neuralgia, headache, arthritis, and chronic back pain.[6] In animal studies, antidepressants produce pain relief in acute nociceptive and neuropathic pain tests[6] as well as in models of inflammation.[7,8] Basic studies have also shown an enhancement of antinociception from systemic opioids by intrathecal injection of amitriptyline.[9,10] However, none was designed to determine the nature of the interaction between amitriptyline and morphine by using isobolographic analysis, which constitutes the most rigorous methodology for investigating drug interactions.[11] Lack of a precise knowledge of drug interaction might explain why in clinical studies, interactions between opioids and amitriptyline or other tricyclic antidepressants were not consistent. Different authors have reported that systemically administered antidepressants potentiated,[12–14] had no effect on,[13,15–17] or antagonized opioid-induced antinociception.[18]

The aims of the current study were to determine the analgesic effects of systemic amitriptyline on cutaneous orofacial inflammatory pain, to compare these effects with those produced by morphine, and to examine the effects of combinations of amitriptyline with morphine to determine whether these two drugs could produce additive or synergistic effects. The orofacial formalin test was used because (1) no animal study has yet examined the activity of amitriptyline in models of phasic or persistent orofacial pain; (2) the orofacial formalin test mimics some features of inflammatory pain in humans[19]; and (3) it is considered a more satisfactory model of clinical

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pain than tests producing phasic pain, such as hot plate or tail-flick tests.[20]

Materials and Methods

Animals

Adult male Sprague-Dawley rats weighing 190–220 g (Iffa-Credo; Charles River France, Les Oncins, France) were used in the experiments. The experiments were performed according to the Ethical Guidelines of the International Association for the Study of Pain. Rats were housed in plastic cages (four rats per cage) with soft bedding and free access to food and water. They were maintained in climate-controlled (23 ± 1°C) and light-controlled (12:12-h dark:light cycle with light on at 08:00 h) protected units (Iffa-Credo) for at least 1 week before the experiments. Test sessions took place during the light phase between 11:00 and 19:00 h in a quiet room maintained at 23–24°C. The test box had the dimensions of 30 × 30 × 30 cm with three mirrored sides. Each animal was first placed in this box for a 10-min habituation period to minimize stress. The rats did not have access to food or water during the test. Each rat was used only once and was killed at the end of the experiment by the administration of a lethal dose of pentobarbital.

Orofacial Formalin Test

Rats received 50 μl formalin solution, 1.5%, into the vibrissa pad. Formalin was injected subcutaneously through a 27-gauge needle into the center of the right vibrissa pad as quickly as possible, with only minimal animal restraint. After injection, the animals were immediately placed back in the test box for a 45-min observation period. The recording time was divided into 15 blocks of 3 min, and a pain score was determined for each block by measuring the number of seconds that the animals spent rubbing the injected area with the ipsilateral forepaw or hind paw.[19] Movements of the ipsilateral forepaw were sometimes accompanied by movements of the contralateral forepaw. A videocamera was used to record the rubbing response. Analysis of the behavior was made by an investigator who was blinded to the animal's group assignment.

Antinociceptive Effects of Morphine and Amitriptyline

Amitriptyline hydrochloride, morphine chlorohydrate, and formalin were purchased from Sigma Chemical Co. (St. Louis, MO) and were dissolved in saline (0.9% NaCl solution). Amitriptyline and morphine were administered intraperitoneally 20 min before formalin. Control animals received saline at this time. Amitriptyline was administered at doses of 5, 10, 15, and 20 mg/kg, and morphine was administered at doses of 0.5, 1.0, 2.0, and 4.0 mg/kg.

Isobolographic Analysis of Drug Interactions

Isobolographic analysis was used to determine the nature of the drug interaction between morphine and amitriptyline. The method is based on comparison of dose combinations in which the dose combinations are made of doses of each of the two agents that are determined to be equipotent. Thus, from the dose–response curves of two agents alone, the respective ED_{50} values (effective dose resulting in a 50% reduction of control formalin response) are determined. Subsequently, a dose–response curve is obtained by concurrent delivery of the two drugs in a constant dose ratio based on the ED_{50} values of the single agent. Therefore, separate groups received (morphine ED_{50} + amitriptyline ED_{50})/2, (morphine ED_{50} + amitriptyline ED_{50})/4, and (morphine ED_{50} + amitriptyline ED_{50})/8. From the dose–response curves of the combined drugs, the ED_{50} value of the combination was calculated, and these dose combinations were used for plotting the isobologram. In this experiment, the ED_{50} values were determined from the data during phase 2 of the formalin test.

The isobologram was constructed as described previously.[11] The ED_{50} values of the single agents were plotted on the x- and y-axes, respectively. The theoretically additive dose combination was calculated. From the variance of the total dose, individual variances for the agents in the mixture were obtained. Furthermore, to describe the magnitude of the interaction, a total dose fraction value was calculated according to the following formula:

Total Fraction Value

$$= \frac{ED_{50} \text{ of Drug 1 with Drug 2}}{ED_{50} \text{ for Drug 1 Given Alone}}$$

$$+ \frac{ED_{50} \text{ of Drug 2 with Drug 1}}{ED_{50} \text{ for Drug 2 Given Alone}}$$

Total fraction values near 1 indicate additive interaction, values less than 1 indicate synergistic interactions, and values greater than 1 indicate an antagonistic interaction.

Testing of Psychomotor Function

Changes in motor performance were assessed using the accelerating rotarod (8500; Ugo Basile; Comerio, Italy), in which rats were required to walk against the motion of a rotating drum, with the speed increasing from 4 to 40 rpm over 5 min. The time taken to fall off the rotarod was recorded as the latency (in seconds). Twenty-four hours before drug testing, rats were trained to stay on an accelerating rotarod for 60 s. The next day, rotarod latencies were measured 20 min and 40 min after systemic administration of amitriptyline (20 mg/kg), morphine (4 mg/kg), association of morphine and amitriptyline (0.66 + 7.31 mg/kg), or saline (n = 5/group).
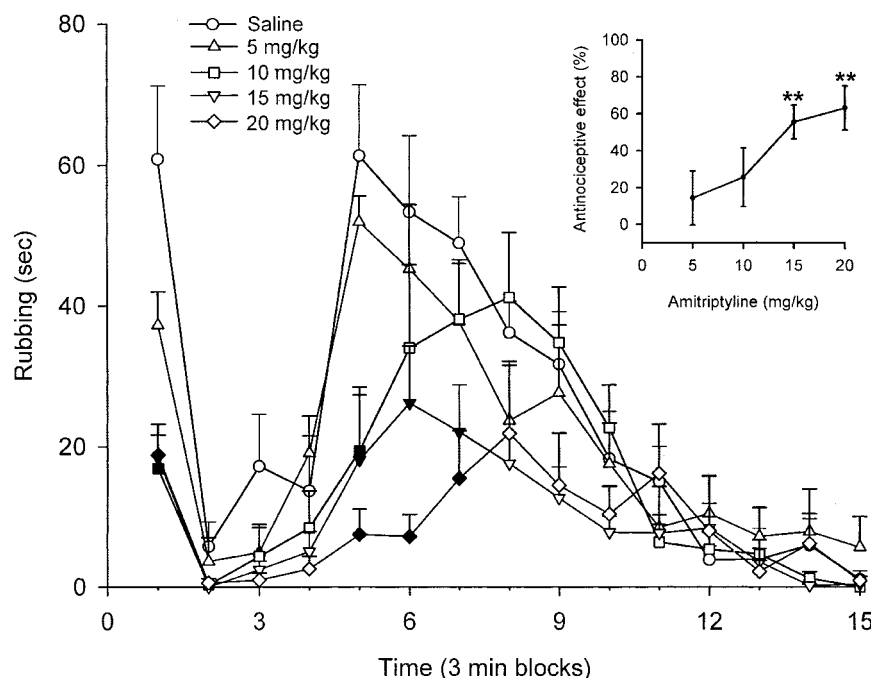
Fig. 1. Time course of the face-rubbing activity evoked by subcutaneous injection of formalin (50 μl, 1.5%) into the vibrissa pad after systemic saline or amitriptyline (5, 10, 15, 20 mg/kg). (*Inset*) Dose–response curve for systemic amitriptyline on the rubbing response during the second phase of the formalin test (n = 10/group). ** P < 0.01. *Solid symbols = significant differences* (P < 0.05) compared with the control group (Dunnett test, subsequent to analysis of variance).

Data Analysis

Results were expressed as mean percentages of antinociceptive effect $\pm$ SEM. The percentage of antinociceptive effect was calculated as 100 − (total time of face rubbing in drug assay/total time of face rubbing in saline assay) × 100. Data were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls test or the Dunnett test for comparisons between groups (n = 8–10 rats/group), and differences were considered significant at P < 0.05. The doses that produced 50% of the antinociceptive effect (ED_{50}) and their 95% confidence intervals were calculated by means of linear analysis of the log dose–response curve. The theoretical and experimental ED_{50} values for the drug combination were compared by means of the Student t test.

Results

Orofacial Formalin Test

The administration of formalin into the vibrissa pad induced a typically biphasic nociceptive response consisting of a first, short-lasting response followed by a second, prolonged response (fig. 1). These two phases were separated by a period of relative inactivity. The first phase (measured during the first 3 min) started 20–30 s after the formalin injection, and the mean duration of rubbing activity was 61 $\pm$ 10 s. The second phase of intense rubbing activity was identified between blocks 4 and 12, *i.e.*, 12–36 min after the formalin injection, and its total duration was 283 $\pm$ 44 s.

Antinociceptive Effects of Amitriptyline

Systemic administration of amitriptyline produced a dose-dependent depression of the first phase of rubbing behavior (fig. 1). In this experiment, all four used doses had a pronounced antinociceptive effect. The amplitude of the rubbing response was reduced by 38.7 $\pm$ 7.8%, (P = 0.05), 72.2 $\pm$ 7.8% (P < 0.001), 69.8 $\pm$ 5.4% (P < 0.001), and 69.1 $\pm$ 7.2% (P < 0.001) after administration of 5, 10, 15, and 20 mg/kg, respectively.
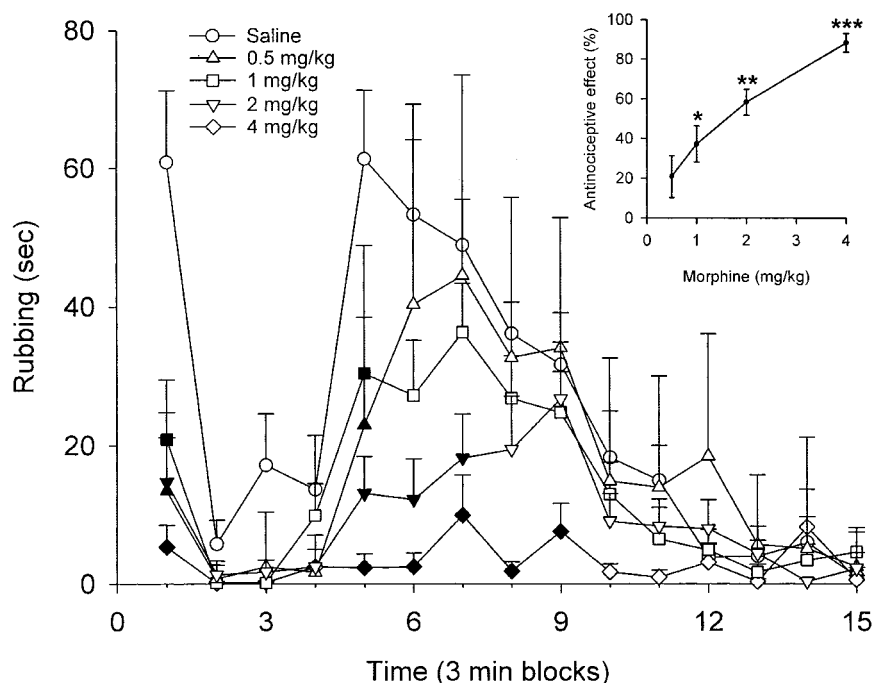
The administration of amitriptyline resulted in a dose-dependent inhibition of the second phase of the rubbing response (fig. 1, *inset*). The amplitude of the rubbing response was reduced by 14.3 $\pm$ 14.8%, 25.5 $\pm$ 15.9%, 55.5 $\pm$ 9.2%, and 63.3 $\pm$ 12.0% after administration of 5, 10, 15, and 20 mg/kg, respectively (fig. 1). The ED_{50} value for suppressing the rubbing response during the second phase was 14.62 mg/kg (95% confidence interval, 10.23–33.54 mg/kg).

Antinociceptive Effects of Morphine

Systemic administration of morphine produced a depression of the first phase of rubbing behavior (fig. 2). The amplitude of the response was reduced by 77.8 $\pm$ 8.3% (P < 0.001), 61.0 $\pm$ 6.4% (P < 0.001), 75.8 $\pm$ 10.6% (P < 0.01), and 91.8 $\pm$ 5.2% (P < 0.001) after administration of 0.5, 1, 2, and 4 mg/kg, respectively.

The administration of morphine also resulted in a dose-dependent inhibition of the second phase of the rubbing response (fig. 2, *inset*). The amplitude of the response was reduced by 20.8 $\pm$ 10.5%, 37.2 $\pm$ 9.1% (P < 0.05), 58.3 $\pm$ 6.5% (P < 0.01), and 88.4 $\pm$ 4.7% (P < 0.001) after administration of 0.5, 1, 2, and 4 mg/kg, respectively (fig. 2). The ED_{50} value for suppressing the rubbing response during the second phase was 1.32 mg/kg (95% confidence interval, 1.02–1.72 mg/kg).

Fig. 2. Time course of the face-rubbing activity evoked by subcutaneous injection of formalin (50 μl, 1.5%) into the vibrissa pad after systemic saline or morphine (0.5, 1, 2, 4 mg/kg). (*Inset*) Dose–response for systemic morphine on the rubbing response during the second phase of the formalin test (n = 8/group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. *Solid symbols* = significant differences ($P < 0.05$) compared with the control group (Dunnett test, subsequent to analysis of variance).

Drug Interaction Studies

Systemic administration of morphine and amitriptyline produced a dose-dependent inhibition of the first phase of rubbing behavior (fig. 3). The amplitude of the response was reduced by 16.3 ± 18.5%, 58.3 ± 10.4% ($P < 0.05$), and 78.0 ± 9.9% ($P < 0.001$) after administration of 0.17 + 1.83, 0.33 + 3.65, and 0.66 + 7.31 mg/kg, respectively.

The administration of morphine and amitriptyline also resulted in a dose-dependent inhibition of the second phase of the rubbing response (fig. 3, *inset*). The amplitude of the rubbing response was reduced by 22.8 ± 4.6%, 43.7 ± 2.9% ($P < 0.05$), and 66.5 ± 4.2% ($P < 0.001$) after administration of doses of 0.17 + 1.83, 0.33 + 3.65, and 0.66 + 7.31 mg/kg, respectively (fig. 3). The respective ED_{50} values of morphine and amitriptyline were 1.32 and 14.62 mg/kg. Combinations of fixed increments of ED_{50} values for the two drugs exhibited a synergistic effect against the rubbing behavior (fig. 3). This was verified by isobolographic analysis, with a sig-

Fig. 3. Time course of the face-rubbing activity evoked by subcutaneous injection of formalin (50 μl, 1.5%) into the vibrissa pad after systemic saline or fixed increments of ED_{50} values for morphine and amitriptyline (n = 8/group). Dose combinations of morphine plus amitriptyline were (ED_{50} + ED_{50})/2 = 0.66 + 7.31 mg/kg; (ED_{50} + ED_{50})/4 = 0.33 + 3.65 mg/kg; (ED_{50} + ED_{50})/8 = 0.17 + 1.83 mg/kg. *Solid symbols* = significant differences ($P < 0.05$) compared with the control group (Dunnett test, subsequent to analysis of variance). (*Inset*) Isobologram for drug interaction between morphine and amitriptyline on the antinociceptive effect during the second phase of the formalin test. The ED_{50} values for single agents are plotted on the x- and y-axes. The *oblique line* between the *x*- and *y*-axes is the theoretical additive line. The *point* in the middle of this line is the theoretical additive point calculated from separate ED_{50} values. The ED_{50} for combination lies below the additive line, indicating a significant synergy ($P < 0.01$).
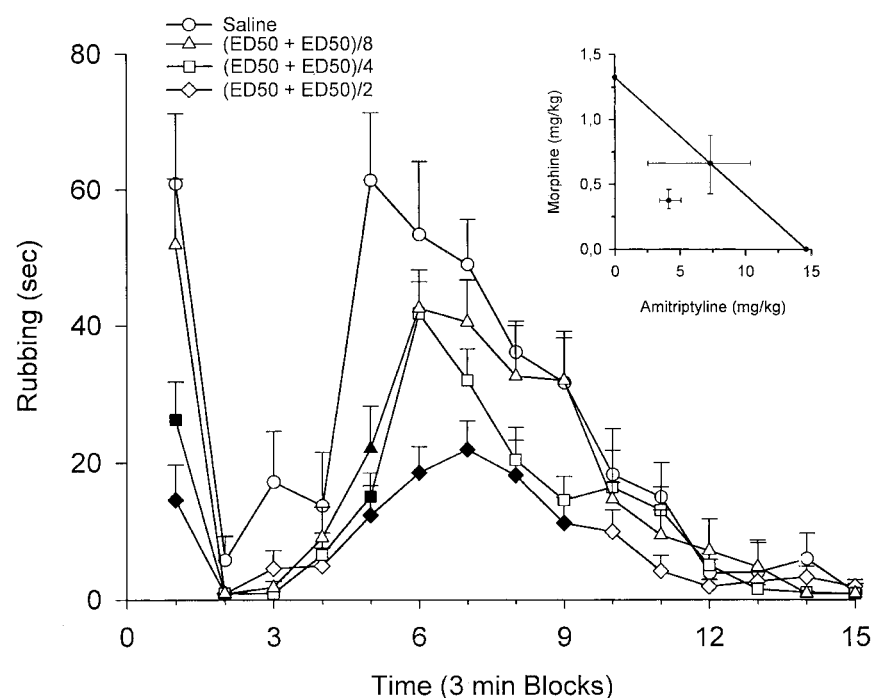
Table 1. Psychomotor Effects Assessed by the Rotatrod Test for Drugs

Treatment	Time after Drug Administration	
	20 min	40 min
Saline	126 ± 40	173 ± 30
Morphine (4 mg/kg)	122 ± 12	133 ± 28
Amitriptyline (20 mg/kg)	152 ± 16	186 ± 26
Amitriptyline + morphine (0.66 + 7.31 mg/kg)	118 ± 13	148 ± 5

Values are presented as mean ± SEM, n = 5/group.

nificant ($P < 0.01$) difference between theoretical (7.97 mg/kg) and experimental (4.49 mg/kg). Corresponding to this synergistic interaction, the dose fraction was 0.56.

Testing of Psychomotor Function

Systemic administration of amitriptyline (20 mg/kg) or morphine (4 mg/kg) did not affect rotarod performance (table 1). Rotarod results also showed no decrease in running time for the combination of amitriptyline and morphine (0.66 + 7.31 mg/kg), a dose that produced a synergistic effect against rubbing behavior.

Discussion

Antinociceptive Effect of Amitriptyline

This study shows that systemic administration of amitriptyline induces an inhibitory effect on the first phase and the second phase of the nociceptive response in the orofacial formalin test. Amitriptyline has also been found to be an effective antinociceptive agent in various animal models of pain.[6] For example, the hyperalgesia evoked by nerve injury[21,22] and inflammation[23,24] was attenuated by amitriptyline. The effects of amitriptyline on the paw formalin test, however, are not consistent. It has been reported that systemic administration of amitriptyline exerted inhibition, facilitation, or no effect, depending on the type of pain measure and the phase of the formalin test. For example, using the rat weighted scores, Acton *et al.*[25] (1992) found that amitriptyline (20 mg/kg) had an inhibitory effect only on the second phase of the formalin test. In contrast, Fuchs *et al.*[26] (1996) reported that systemic administration of amitriptyline (20 mg/kg) produced a significant decrease in weighted scores during both phases of the formalin test. Recently, amitriptyline has been shown to have a differential effect on flinching and licking behaviors during the second phase, enhancing flinching and depressing licking behavior.[8] Other antidepressants also produce a similar suppression of biting/licking behaviors[27,28] but do not affect the flinching behavior.[8] Great controversy exists regarding which of these behaviors or their combination is the best measure of formalin-induced pain in the paw.[29] The current study examined the effect of amitriptyline on face rubbing, which has been shown to

be a reliable index to quantify nociceptive sensitivity because it is evoked by formalin but not by saline,[19,30] positively related to the formalin concentration,[19] and sensitive to both opioid[31,32] and nonopioid analgesic drugs.[33–35] Recently, Cadet *et al.*[36] (1998) revealed a significant correlation between rubbing activity and paw licking, whereas no correlation was found between rubbing activity and paw flinching. Therefore, face rubbing seems more akin to biting/licking behaviors than to flinching.

The mechanisms by which amitriptyline induces antinociception have been widely discussed in a previous study.[6] Amitriptyline has a large number of pharmacologic actions, including inhibiting uptake of serotonin and noradrenaline, as well as effects on serotoninergic, adrenergic, cholinergic, and histaminergic receptors.[6] It has been shown that amitriptyline also has antagonistic effects on *N*-methyl-D-aspartate receptors, which are known to play an important role in inflammatory pain states.[7] More recent studies have shown a local peripheral analgesic effect of amitriptyline that is mediated, in part, by an interaction with endogenous adenosine.[37] Therefore, it is possible that combined action on peripheral and central sites may be of importance for the analgesic effect of systemic amitriptyline.

Antinociceptive Effect of Morphine

The current study provides evidence that systemic administration of morphine dose-dependently inhibits formalin-induced rubbing activity. These findings are consistent with previous behavioral studies, which showed that systemic administration of opioids produced antinociception in various trigeminal[31,38] and spinal[39] animal pain models. The effective dose of morphine for the second phase was similar to that found in studies using the paw formalin test[39] or other chemical pain models.[38,40] However, the effective doses of morphine in the formalin test are much lower than those used in the phasic tests, such as tail flicking, hot plate tests, or paw withdrawal from mechanical stimuli.[41] Opioids elicit antinociceptive effects by interacting with peripheral and central opioid receptors.[42]

Antinociceptive Effect of Coadministration of Morphine and Amitriptyline

Although previous studies have shown an enhancement of antinociception from systemic opioids by intrathecal injection of amitriptyline,[9,10] the current study provides the first isobolographic analysis of morphine and amitriptyline interaction on cutaneous orofacial inflammatory pain. The isobolographic approach showed that the interaction between systemic amitriptyline and morphine is synergistic. Other antidepressants, such as fluoxetine[43] and fluvoxamine, were also found to enhance opioid analgesia in other models.[44] Furthermore, Gray *et al.*[24] (1998) showed that pretreatment with en-

kephalinase inhibitors potentiated the amitriptyline antinociception against acid-induced abdominal pain. In clinical studies, interactions between opioids and amitriptyline or other tricyclic antidepressants are not consistent. Therefore, systemically administered antidepressants have been shown to potentiate,[12–14] have no effect on,[13,15–17] or antagonize opioid-induced antinociception[18] depending on the agent, timing of drug administration, and type of pain. In contrast, the current results suggest a clear potentiation of systemic opioid antinociception when a mixed inhibitor of monoamine reuptake is administered by a systemic route. They provide reasons to reexamine the interaction between amitriptyline and morphine in humans.

The mechanisms responsible for synergistic interactions are poorly understood, but a number of hypotheses have been developed to explain these effects. These include pharmacokinetic mechanisms in which one drug increases the active levels of the other by reducing its rate of clearance or altering its metabolism. However, such an interaction is unlikely because administration of amitriptyline did not modify the plasma concentration of morphine.[45] Alternatively, the interaction may also be pharmacodynamic, in which concurrent activation of distinct systems may modulate a common pathway or one compound may enhance the affinity or coupling of the other. Previous studies have suggested that both compounds work *via* peripheral[37,42] and central[6,42] action involving distinct mechanisms; therefore, it is possible that this synergy may occur in the peripheral and central nervous systems. Further work is needed to elucidate the exact mechanism of synergy between amitriptyline and morphine.

In summary, the current study shows that systemic administration of morphine and amitriptyline have antinociceptive effects in the rat orofacial formalin test. Furthermore, a combination of both drugs produces synergistic antinociception.

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