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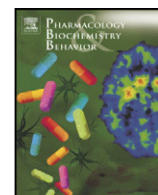

Contributions of peripheral and central opioid receptors to antinociception in rat muscle pain models

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ABSTRACT

Administration of hypertonic saline (HS) is an accepted model to study muscular pain. HS-induced nociceptive responses were tested in masseter, already described, and in two new pain models of spinally innervated muscles (gastrocnemius and triceps) developed in rats at our laboratory. HS administration in the masseter induced vigorous hindpaw shaking and in the gastrocnemius or triceps, paw withdrawal or flexing. Participation of the central and peripheral opioid receptors in HS-induced pain is compared in these muscles: masseter, innervated by trigeminal nerve, and gastrocnemius and triceps by spinal nerves. Morphine and loperamide were used to reveal peripheral and central components of opioid analgesia. Both agonists reduced HS-induced nociceptive behaviours in the masseter and were antagonised by the opioid antagonist naloxone and by naloxone methiodide, an opioid receptor antagonist that poorly penetrates the blood–brain barrier. Unexpectedly, in the gastrocnemius and triceps, morphine, but not loperamide, decreased the nociceptive behaviour and this effect was only reversed by naloxone. So, peripheral opioid receptors seem to participate in HS-induced masseter pain, whereas only central opioid receptors reduced the nociception in gastrocnemius and triceps. Our results suggest that the use of peripheral opioids can be more advantageous than central opioids for treatment of orofacial muscular pain.

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1. Introduction

Muscle pain is the major presenting symptom of many clinically defined conditions. The economic and emotional impact of chronic musculoskeletal pain disorders may be measured in terms of lost productivity and human suffering.

Since a muscle pain model consisting in an intramuscular (i.m.) injection of hypertonic saline (HS) (Kelgren, 1938) was introduced, this and similar models have been used to study human and animal experimental muscle pain (Graven-Nielsen, 2006; Capra and Ro, 2004; Mense, 2009). Muscle injection of exogenous agents activates nociceptive pathways producing reliable nocifensive behaviours (Sluka et al., 2002; Nielsen et al., 2004; Yokoyama et al., 2007).

There are studies related to muscular pain models based on the assessment of behaviours induced by the i.m. administration of algesic agents into masseter, a craniofacial muscle (Shinoda et al., 2008; Ro et al., 2009) or into gastrocnemius or triceps, spinally innervated muscles (Kehl et al., 2000; Capra and Ro, 2004; Fujii et al., 2008). However, there are no data comparing muscle pain induced in the orofacial region with that induced in spinally innervated muscles.

It is accepted that pain in the craniofacial territory, mainly innervated by the trigeminal nerve, presents some specific characteristics, such as a very complex anatomical and physiological organization, and different nociceptive pathways. Some differences described between trigeminal and spinal innervations (Dood and Kelly, 1991; Sessle, 2005; Takemura et al., 2006; Burgos et al., 2010) are:

- The infraorbital and maxillary branches of the trigeminal nerve are actually purely sensory nerves whereas the sciatic nerve is a mixed nerve and contains a significant motor component.
- There is practically no functional overlap between the territory of the three branches of the trigeminal nerve, and they innervate a well defined and restricted region of the face which is very different from the spinal nociceptive innervation.
- Differences in the analgesic effect of several drugs have been described.

Moreover, to our knowledge, there is no algesic agent that has been used in both locations, in the same study, to permit an easy and reliable comparison of the results.

Therefore, our first aim was to establish a model of experimental pain using HS that could be useful in the evaluation of acute muscular pain in the orofacial region (masseter), as well as in spinally innervated muscles (gastrocnemius and triceps). For this, we tested the HS effect in a masseter model already described (Ro et al., 2003)

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and in two new muscular models developed at our laboratory in gastrocnemius and triceps of rat. Models of acute muscle pain have been used as a first experimental approach.

Moreover, it is well known that opioid antinociception is mediated by the activation of central and peripheral receptors (Machelska et al., 2003; Ossipov et al., 2004). The antinociceptive effects of centrally administered opiates in models of acute pain (hot plate and tail-flick tests) are well established (Yaksh, 1999), however, peripheral opioid effects are emerging as interesting therapeutic alternatives and are now challenging old paradigms of target research.

Previous studies investigating the role of peripheral opioid receptors have focused on the local application of opiates at small, systemically inactive doses (Stein et al., 2001; Truong et al., 2003; Kabli and Cahill, 2007) and on the use of opioid ligands with limited access to the central nervous system (DeHaven-Hudkins and Dolle, 2004; Obara et al., 2007; Guan et al., 2008). These alternatives tend to minimise adverse central actions. A different approach may be the identification of areas where peripheral opioid analgesia can be strong enough to become therapeutically relevant.

Our second aim was to compare the role of central and peripheral opioid receptors in HS-induced pain in masseter, gastrocnemius and triceps muscles; this topic is of interest because peripheral opioid antinociception may open new and specific therapeutic approaches. The classic tail-flick test was also used to study centrally mediated opioid effects.

To reveal if central or peripheral opioid receptors are involved, morphine and loperamide, a peripherally acting opioid agonist (DeHaven-Hudkins et al., 1999), were examined; naloxone and naloxone methiodide, an opioid that poorly penetrates into the brain (Russell et al., 1982), were used as opioid antagonists.

2. Methods

2.1. Animals

Male Wistar rats (250–300 g) obtained from the Veterinary Unit of Rey Juan Carlos University were used for all experiments. Animals were supplied with food and water "ad libitum" and were housed in a temperature-controlled room at 23 ± 1 °C under a standard 12/12-h light/dark cycle (08:00–20:00 h). The animals were housed in the test room for at least two days before experimentation. Throughout the experimental procedure, the international ethics standards for pain-inducing experiments in laboratory animals (Zimmermann, 1983) and the European Communities Council Directive of 24 November 1986 (86/609 EEC, Nov 24, 1986) were followed. All animal procedures were reviewed and approved by the Animal Care and Use Committee of Rey Juan Carlos University.

2.2. Drugs

Morphine (sulphate salt) was obtained from Alcaliber (Madrid, Spain). Loperamide hydrochloride (a peripherally restricted opioid), naloxone hydrochloride (a non-selective opioid receptor antagonist that reaches the central nervous system and shows peripheral and central effects) and naloxone methiodide (an opioid receptor antagonist that does not cross the blood–brain barrier) were purchased from Sigma-Aldrich Química, Madrid, Spain. Loperamide was dissolved in 20% Cremophor EL (Sigma) (DeHaven-Hudkins et al., 1999), and all other drugs were dissolved in saline solution (0.9%). All solutions were made fresh before each experiment.

2.3. General procedures

Test sessions were carried out between 09:00 and 15:00 h. Each group of rats contained 6–8 animals that were used only for one type of experiment, and separate groups of animals were used for each treatment. All experiments and manipulations were carried out by one researcher who was unaware of the treatments.

All drugs were administered intraperitoneally (i.p.) in a volume of 1.5 ml/kg. The effects of the opioid agonists, morphine and loperamide, were studied 30 min after their administration. The antagonists, naloxone and naloxone methiodide, were always administered 15 min prior to the agonists. The effects of the antagonists alone were studied 20 min after their administration. Groups treated with morphine and loperamide were compared to those treated with their vehicles, saline solution and cremophor, respectively.

2.4. Masseter muscle pain procedures

The behavioural assessment of craniofacial muscle nociception was performed in lightly anaesthetised rats as previously described (Ro et al., 2003; Ro, 2003). Animals were anaesthetised with Equitesin (3 ml/kg i.p., chloral hydrate 2.1 g, sodium pentobarbital 0.46 g, $MgSO_4$ 1.06 g, propylene glycol 21.4 ml, ethanol (90%) 5.7 ml, H_2O 23 ml) and the skin over the masseter muscle was carefully shaved. The level of "light" anaesthesia was determined by providing a noxious pinch to the tail or the hindpaw with serrated forceps as previously described (Ro et al., 2003). Animals typically responded to the noxious pinch of the tail with an abdominal constriction and to the noxious pinch of a hindpaw with a withdrawal reflex within 30 min after the initial anaesthesia. Experiments were continued only after the animals showed reliable reflex responses to every noxious pinch as previously described (Ro et al., 2003). A constant level of anaesthesia was maintained by the administration of approximately 0.2 ml every 30 min; to confirm that the level of anaesthesia was satisfactory the withdrawal reflex was tested before every experimental procedure.

The ipsilateral hindpaw shaking behaviour produced by hypertonic saline (HS) stimulation of the masseter muscle is accepted as an index of muscle nociception. 100 µl of HS (5% NaCl) were administered into the mid-region of the right masseter muscle via a 30-gauge cannula. To minimise the effects of insertion of the cannula into the muscle on the hindpaw shaking behaviour, the cannula was inserted into the masseter muscle 10 min prior to the injection of HS. The injection cannula consisted of a 30-gauge needle connected to a PE10 tube and an insulin syringe. The HS was manually infused through the injection cannula for 10 s. Intramuscular injection of HS produced an ipsilateral hindpaw shaking behaviour that was quantified by counting the total number of shakes in a 2-min period after the intramuscular injection of HS. Counting was performed by only one experimenter to maintain consistency. To count the number of shakes, the experiments were recorded on video and then played back in slow motion.

The first set of experiments was done to confirm if the injection of HS into the masseter induced the hindpaw shaking behaviour and to test the reproducibility of the response by performing two repeated i.m. injections with HS, which is known to be short acting and non-sensitising. After the initial injection of HS, the needle was left in place for the subsequent HS administration separated by a 30-min interval (Ro et al., 2003).

The second set of experiments was designed to investigate the antinociception induced by morphine and loperamide (0.6, 1.25 and 2.5 mg/kg, i.p.). These opioid agonists were administered as soon as animals reached a stable state of anaesthesia (between 5 and 10 min after equitesin injection). HS was injected in the masseter 30 min after opioid administration.

Following the same pattern, and to discriminate between participation of the central and/or peripheral opioid receptors, the opioid antagonists naloxone or naloxone methiodide (0.5–2 mg/kg) were i.p. injected 15 min before the agonists, and the hindpaw shaking behaviour induced by HS was evaluated in another group of animals.

2.5. Gastrocnemius and triceps muscles pain procedures

There are no previous reports about the effect of HS injected in spinally innervated muscles. Therefore, the first set of experiments tested whether the HS stimulation of these muscles can cause reliable nociceptive behaviour in rats. Separate groups of animals were used to study gastrocnemius or triceps pain and the nociceptive response was tested in:

- Sham groups (animals only received a puncture with an insulin syringe, in the gastrocnemius or the triceps)
- Saline groups (animals received 0.5 ml i.m. of saline solution in the gastrocnemius or the triceps)
- HS groups (animals received 0.1, 0.25 and 0.5 ml i.m. of HS in the gastrocnemius or the triceps).

A volume of 0.5 ml of saline solution and several volumes of HS (0.1, 0.25 and 0.5 ml) were injected directly into the right gastrocnemius or triceps with an insulin syringe. No response was registered in anaesthetised rats and thus conscious animals were used. Every animal was kept in a Plexiglas box and two mirrors were positioned underneath and behind it to permit unobstructed viewing of the paw. The HS injection in gastrocnemius or triceps produced similar responses in both groups consisting in withdrawal or flexing of the injected paw. The time (s) that animals remained with these behaviours was measured for up to 5 min.

Next, we tested if this paw behaviour was reproducible with three 0.5 ml injections of HS into the gastrocnemius or triceps at 30 min intervals and then, we checked if the nociceptive response produced by 0.5 ml of HS i.m. was modified by the i.p. administration of the vehicles, saline and cremophor.

Finally, the modification of this nociceptive behaviour after i.p. administration of morphine (2.5–10 mg/kg) and loperamide (5–10 mg/kg) was assayed. The effect of these opioids was also tested after treatment with the antagonists, naloxone and naloxone methiodide (1 mg/kg).

2.6. Tail-flick procedures

The tail-flick test is widely used for determining the antinociceptive effect of pharmacological agents (D'Amour and Smith, 1941). It is a test of acute nociception in which a high-intensity thermal stimulus is directed to the tail of a rat. The noxious heat stimulation of the tail produces a simple nociceptive reflex response, a flick of the tail away from the heat source, which is a spinally mediated flexion reflex. The time from the onset of the stimulation until the rapid flick/withdrawal of the tail from the heat source was recorded. This test was carried out in conscious and lightly anaesthetised rats.

Conscious rats had to be lightly restrained in a Plexiglas tube and were acclimated to the tube two days before the experiment. Other groups of rats were lightly anaesthetised with Equitesin as described for the masseter pain model.

A tail-flick apparatus (Analgesic-Meter LI7106, Letica Scientific Instruments) was used to test the tail-flick response, and the intensity of the heat source was adjusted to provoke tail-flick latencies of 2–4 s. Radiant heat was focused approximately 5 cm from the tip of the tail. To prevent tissue damage, the cutoff time for each measurement was set to 10 s.

Data from the tail-flick test are expressed as the percent maximum possible effect (% M.P.E.). % M.P.E. = (test-baseline) / (cutoff-baseline) × 100, where the test is the latency to respond after treatment, baseline is the latency to respond prior to treatment, and cutoff is the preset time at which the test ends in the absence of a response.

Separate groups of rats undergoing different treatments with opioid agonists and antagonists opioids were used. Morphine (2.5–10 mg/kg) and loperamide (5–10 mg/kg) were i.p. administered. Naloxone (0.5–1 mg/kg) or naloxone methiodide (1–2 mg/kg) was administered i.p. 15 min before the agonist.

2.7. Data analysis and statistics

Statistical analyses of all data were carried out with One-way analysis of variance (ANOVA), followed by Newman–Keuls post hoc analysis using the Prism program (GraphPad software). In all statistical comparisons, $p<0.05$ was used as the criterion for statistical significance. All data are expressed as the means ± SEM.

Groups treated with opioid agonists were compared to their respective vehicle-treated group (morphine–saline solution and loperamide–cremophor). The values obtained from the groups treated with opioid antagonist and agonist, were compared to those of treated with the agonist alone.

3. Results

3.1. Masseter muscle pain

Animals maintained on light anaesthesia showed no significant hindpaw shaking behaviour. As expected, injection of 100 µl of HS in the masseter muscle induced an immediate and intense ipsilateral hindpaw shaking behavioural response that persisted for 2 min, with the peak number of shakes occurring during the initial 30 s after intramuscular injection. When HS was injected again 30 min later into the same masseter muscle, the response was similar to that induced

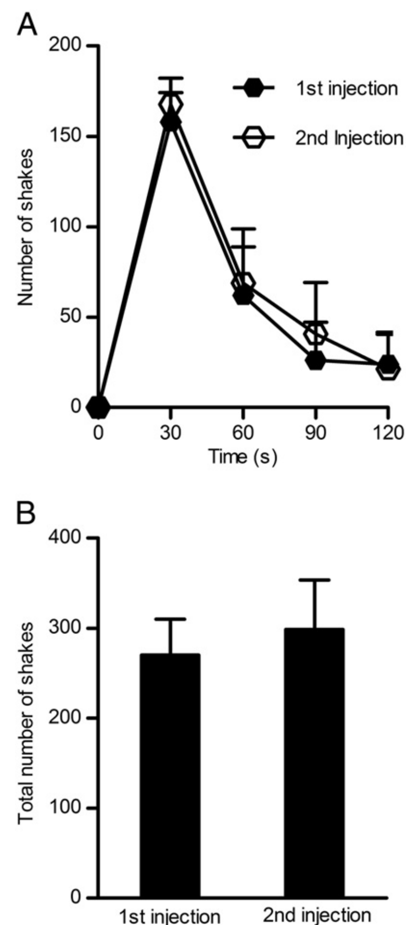

Fig. 1. Time course of the hindpaw shaking behaviour induced by i.m. injection of 100 µl of HS and reproducibility of the nociceptive response (A). HS was injected into the same masseter muscle twice in 30-min interval. The total number of hindpaw shakes was measured (B). Each point shows the mean ± SEM of paw shakes (n ≥ 6).

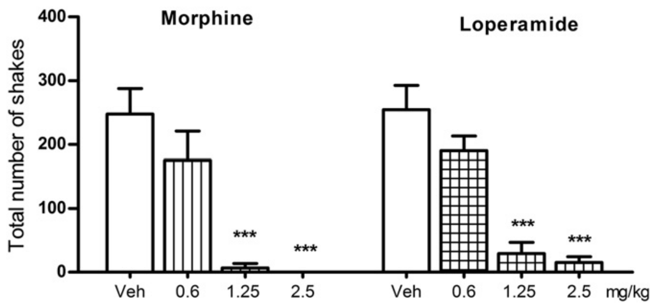

Fig. 2. Effects of the intraperitoneally administered morphine and loperamide on HS-induced nociceptive behaviour after HS injection in masseter muscle. Bars show the total number of shakes (mean ± SEM). ***$p<0.001$ *vs* vehicle (Veh). (One-way ANOVA, n ≥ 6).

by the first injection of HS and no significant differences were observed (Fig. 1).

Intraperitoneal administration of the vehicles (saline solution or cremophor) did not significantly modify the shaking behaviour induced by the i.m. injection of HS.

To investigate the effects of opioid agonists, 0.6, 1.25 and 2.5 mg/kg of morphine or loperamide were i.p. administered 30 min before the injection of HS and changes in the hindpaw shaking behaviour were measured. Morphine and loperamide significantly reduced the hindpaw shaking induced by HS in a dose-dependent manner compared with their respective vehicles (Fig. 2).

To examine the role of central or peripheral opioid receptors in masseter pain, naloxone (0.5–2 mg/kg) or naloxone methiodide (0.5–2 mg/kg) was used. Neither of the antagonists induced significant

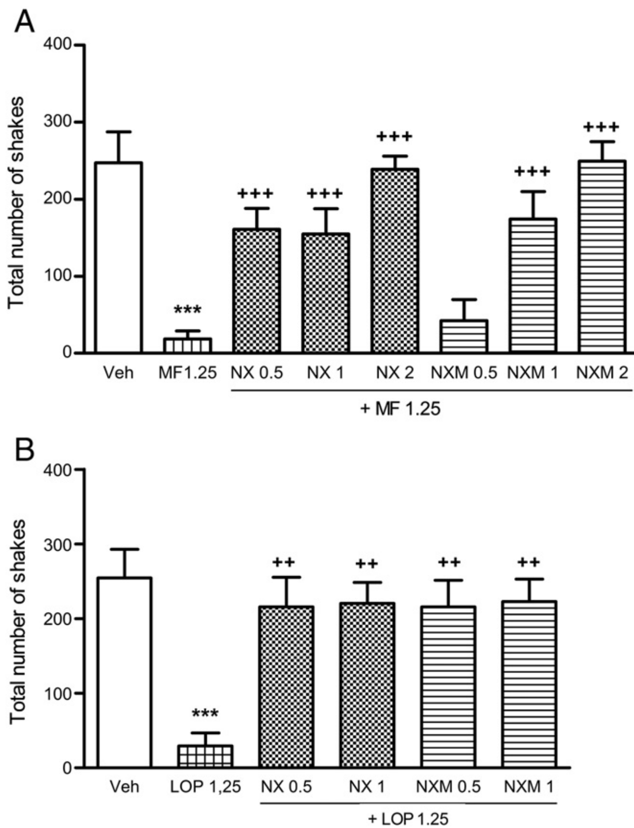

Fig. 3. Blockade of the antinociceptive effect of 1.25 mg/kg morphine (MF) (A) and 1.25 mg/kg loperamide (LOP) (B) by naloxone (NX) and naloxone methiodide (NXM) (0.5–2 mg/kg). Bars represent the total number of shakes (mean ± SEM) induced by HS injection into the masseter. ***$p<0.001$ *vs* vehicle (Veh), +++$p<0.001$, ++$p<0.01$ *vs* 1.25 MF or 1.25 LOP. (One-way ANOVA, n ≥ 6).

changes in the shaking behaviour induced by HS. When they were administered 15 min before 1.25 mg/kg morphine or loperamide, both drugs antagonised, with similar efficacy, the morphine-induced antinociception (Fig. 3A) as well as the loperamide-induced antinociception (Fig. 3B).

3.2. Gastrocnemius and triceps pain

These experiments were carried out on conscious rats because the injection of 0.5 ml of 5% HS into the gastrocnemius muscle of anaesthetised rats did not induce any behavioural responses.

The first set of experiments tested whether an injection of 5% HS in the gastrocnemius or triceps muscle was able to produce a nociceptive behaviour. Neither the pinch of these spinally innervated muscles (sham group) nor injection of 0.5 ml of saline solution induced any significant nociceptive response. On the other hand different volumes of HS (0.1, 0.25 and 0.5 ml) caused withdrawal or flexing of the hindpaw (gastrocnemius) (Fig. 4A) or of the forepaw (triceps) (Fig. 4B). These behaviours were dependent on the volume of injected HS. All further experiments were carried out with 0.5 ml of HS to induce nociception in both muscles.

To demonstrate the reproducibility of this nociceptive behaviour, HS was administered three times, every 30 min, into the gastrocnemius or triceps. No statistically significant differences were observed when the values were compared to the first administration of HS (Table 1).

Next, we determined if the administration of the vehicles (saline solution and cremophor) would alter this nociceptive behaviour 30 min after their i.p. injection; neither of them produced significant changes in the gastrocnemius or the triceps response (Table 2).

In the gastrocnemius model, i.p. administration of morphine (2.5–10 mg/kg) induced a dose-dependent and statistically significant decrease in the time that rats spent with the paw withdrawn or flexed after HS injection (Fig. 5A). In contrast, loperamide (5–10 mg/kg) did not produce significant changes and lacked of antinociceptive effect (Fig. 5B).

In the triceps model similar results were obtained: morphine (2.5–10 mg/kg) also significantly reduced the pain behaviour induced

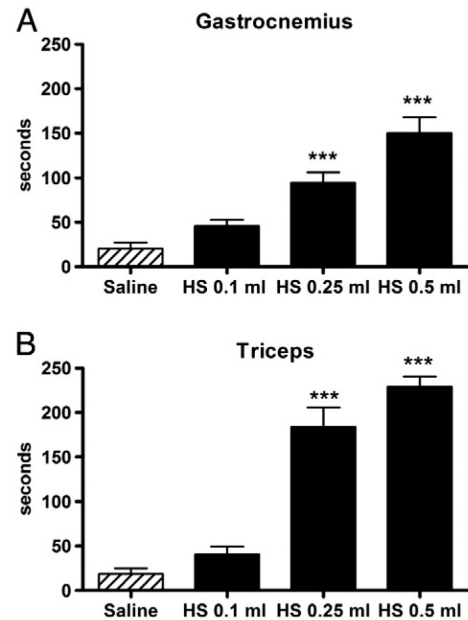

Fig. 4. Nociceptive behaviour induced by different volumes of hypertonic saline (HS) injected in the gastrocnemius (A) or triceps (B). Bars indicate the mean ± SEM of the time (s) that rats spent with the injected paw withdraw or flexed after administration of 0.5 ml of saline solution or different volumes of HS. ***$p<0.001$ *vs* saline (SS). (One-way ANOVA, n ≥ 6).

Table 1
Time (s) that the animals remained with a lifted or retracted paw after the administration of 0.5 ml of HS three consecutive times at 30 min intervals. Data are presented as the means ± SEM, n ≥ 6.

	Gastrocnemius	Triceps
1st injection of HS	149.8 ± 21.3	229 ± 9.9
2nd injection of HS	179.2 ± 20.9	251 ± 3
3rd injection of HS	192.5 ± 11.4	247.3 ± 7.8

Table 2
Time (s) that animals remained with a lifted or retracted paw after the administration of 0.5 ml of HS in animals intraperitoneally treated with 0.5 ml of vehicle (saline or cremophor). Data are presented as the means ± SEM, n ≥ 6.

Treatment	Gastrocnemius	Triceps
HS i.m.	149.8 ± 21.38	229 ± 9.9
Saline i.p. + HS i.m.	174 ± 12.2	216 ± 26
Cremophor i.p. + HS i.m.	142.6 ± 21.5	184 ± 7

by HS (Fig. 6A), whereas loperamide (5–10 mg/kg) did not modify this response (Fig. 6B).

Although 10 mg/kg of morphine may be considered a high dose, its antinociceptive effect cannot be attributed to motor impairment because significant motor behaviour impairments have been previously discarded in our laboratory (Burgos et al., 2010).

To test if the antinociceptive effect of morphine was mediated by central or peripheral opioid receptors, naloxone or naloxone methiodide (1 mg/kg) was injected i.p. 15 min before the administration of 10 mg/kg of morphine. In both muscle pain models naloxone, but not naloxone methiodide, was able to antagonise the morphine effect (Fig. 7A, B).

As expected, neither naloxone nor naloxone methiodide at 1 mg/kg modified the nociceptive response induced by HS injection in the gastrocnemius or triceps.

3.3. Tail-flick test

In the tail-flick test, the i.p. administration of morphine (2.5–10 mg/kg) showed a dose-dependent effect and remarkably increased the nociceptive threshold in both conscious and lightly anaesthetised rats (Fig. 8A, C). In contrast, loperamide (5–10 mg/kg) failed to produce an antinociceptive effect in the two groups of rats (Fig. 8B, D).

When naloxone (0.5–1 mg/kg) or naloxone methiodide (1–2 mg/kg) was i.p. administered 15 min before morphine (10 mg/kg), naloxone significantly and dose-dependently antagonised the antinociceptive effect of morphine, whereas naloxone methiodide was unable to modify the effect of morphine (Fig. 9).

Antagonists alone did not produce any modification on the withdrawal thresholds.

4. Discussion

In the present study, we have tested the response evoked by injection of HS in three different animal muscular pain models: one innervated by trigeminal nerve (masseter muscle) and two spinally innervated ones (gastrocnemius and triceps).

Moreover, considering that there is an increasing interest in the study of peripheral effects of opioids, the role of central and peripheral opioid receptors has been investigated in these muscular pain models.

4.1. Nociceptive response induced by HS in rat masseter, gastrocnemius and triceps muscles

To correctly study acute muscular nociception and to compare the results obtained in orofacial and limbs regions, it is necessary to use the same kind of stimulation in every tested muscle. It was unexpected to find that none of the muscular models of acute pain used in this study had been previously used to simultaneously investigate nociception in orofacial and in spinal muscles. Thus, our first goal was to establish a simple model to observe a reliable

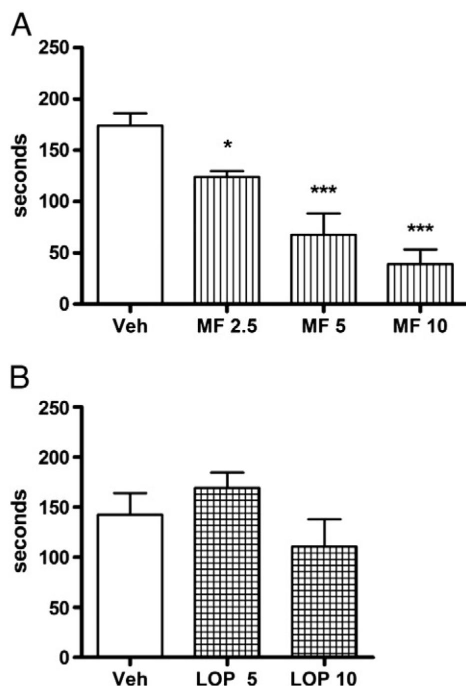

Fig. 5. Antinociceptive effect of morphine (A) and loperamide (B) on gastrocnemius pain induced by 0.5 ml of HS. MF (2.5, 5 and 10 mg/kg), LOP (5 and 10 mg/kg) and their vehicles (Veh), saline and cremophor respectively, were i.p. administered 30 min before HS injection. Bars show the mean time (s) ± SEM spent with the injected paw withdraw or flexed. *$p < 0.05$, ***$p < 0.001$ *vs* Veh. (One-way ANOVA, n ≥ 6).

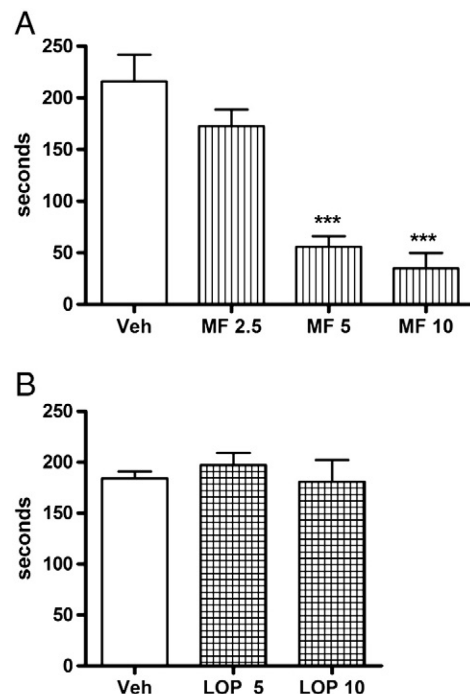

Fig. 6. Antinociceptive effect of morphine (A) and loperamide (B) on triceps pain induced by 0.5 ml of HS. MF (2.5, 5 and 10 mg/kg), LOP (5 and 10 mg/kg) and their vehicles (Veh), saline and cremophor respectively, were i.p. administered 30 min before HS injection. Bars show the mean time (s) ± SEM spent with the injected paw withdraw or flexed. *$p < 0.05$, ***$p < 0.001$ *vs* Veh. (One-way ANOVA, n ≥ 6).

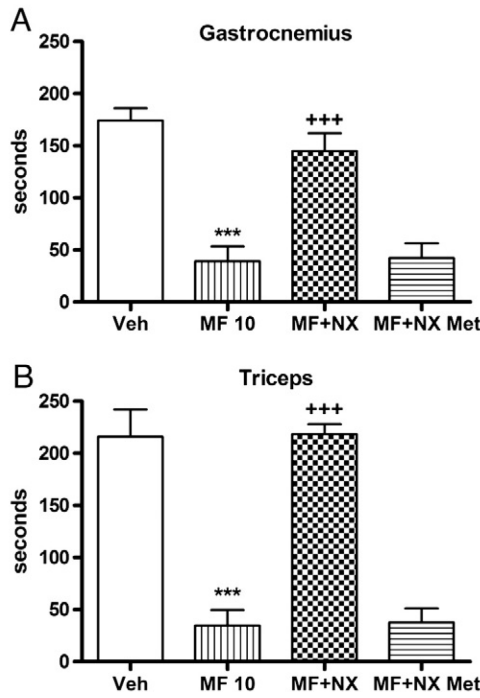

Fig. 7. Blockade of the antinociceptive effect of 10 mg/kg morphine (MF) by 1 mg/kg naloxone (NX) or naloxone methiodide (NX Met), on gastrocnemius (A) or triceps (B) pain induced by HS. Bars show the mean time (s) ± SEM that rats spent with the injected paw withdraw or flexed. $^{***}p<0.001$ *vs* vehicle (Veh), $+++p<0.001$ *vs* 10 MF. (One-way ANOVA, n ≥ 6).

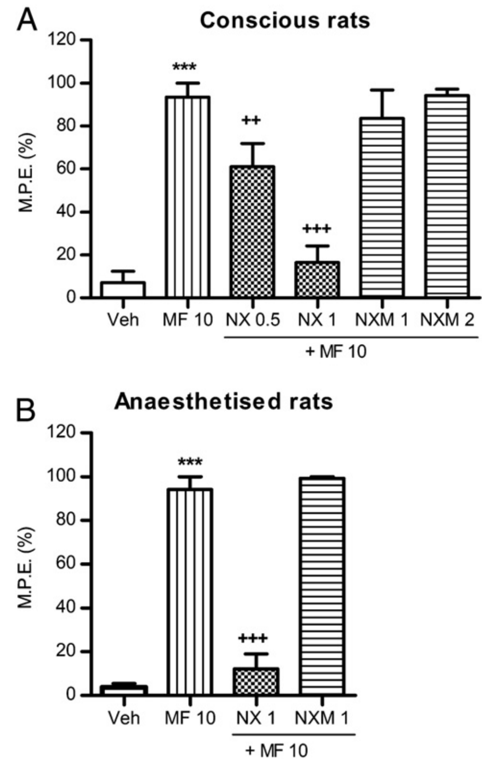

Fig. 9. Blockade of the antinociceptive effect of morphine (10 mg/kg MF, i.p.) by different doses of naloxone (NX) (0.5–1 mg/kg) or naloxone methiodide (NXM) (1–2 mg/kg) in conscious (A) and lightly anaesthetised (B) rats evaluated by the tail-flick test. Bars represent the percentage of the maximum possible effect (M.P.E.) (mean ± SEM). $^{***}p<0.001$ *vs* Vehicle (Veh), $++p<0.01$, $+++p<0.001$ vs 10 MF. (One-way ANOVA, n ≥ 6).

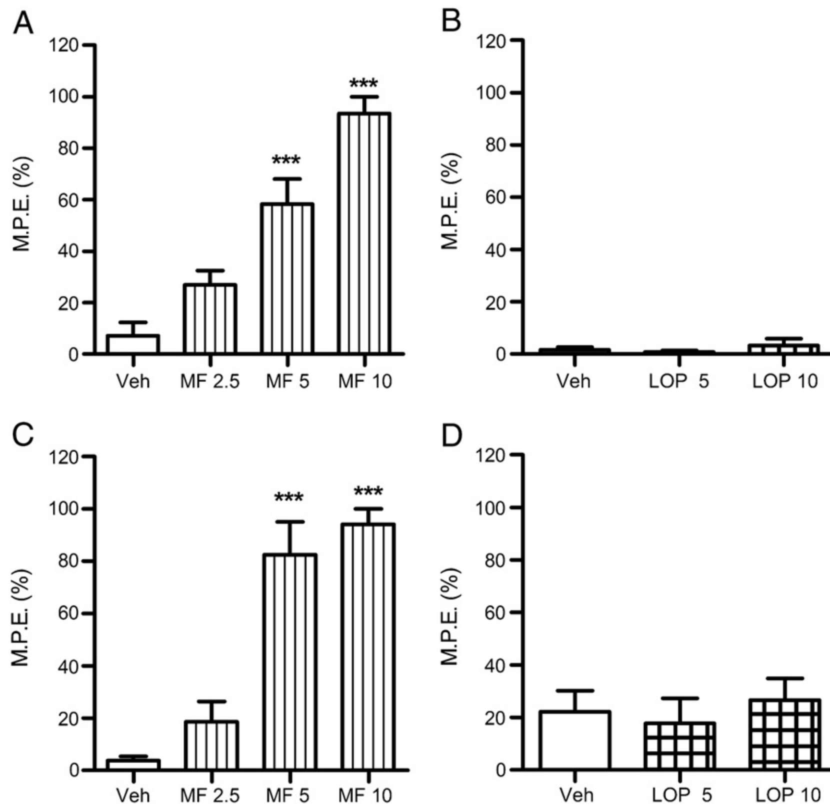

Fig. 8. Antinociceptive effect of morphine (MF 2.5, 5 and 10 mg/kg) and loperamide (LOP 5 and 10 mg/kg) in conscious (A, B) and lightly anaesthetised (C, D) rats evaluated by the tail-flick test. Bars show the mean ± SEM of the percentage of the maximum possible effect (M.P.E.). $^{***}p<0.001$ *vs.* Vehicle (Veh). (One-way ANOVA, n ≥ 6).

nociceptive response after painful stimulation in various anatomical locations.

Recently, a new behavioural assessment system in lightly anaesthetised rats has been introduced to study craniofacial muscle pain by injecting algesic agents (HS, mustard oil, glutamate, formalin) into the masseter and recording the ipsilateral hindpaw shaking behaviour (Ro et al., 2003; Ro, 2003; Han et al., 2008). Among the algesic agents, the use of the HS is advantageous because is used in humans without side effects (Stohler and Lund, 1994) and, furthermore, its effects in the rat masseter muscle are easily reproducible (Ro et al., 2003). In agreement with previous data (Ro et al., 2003), our results show that the intramuscular administration of 5% HS into the masseter muscle of lightly anaesthetised rats produces consistent and vigorous ipsilateral hindpaw shaking behaviour. Nonetheless, there are very few studies that use HS to induce acute pain in spinally innervated muscles in animal models (Martin and Arendt-Nielsen, 2000).

To reproduce this model in spinally innervated muscles, HS was injected into the gastrocnemius or triceps of anaesthetised rats, but no nociceptive behaviour was observed. Conversely, in conscious animals, a reliable volume and time-dependent nociceptive behaviour was recorded. The obtained results are in good agreement with previous results in the masseter muscle (Ro et al., 2003), and taken together they permit the conclusion that the recording of withdrawal or flexing of the paw induced by the injection of HS in gastrocnemius or triceps is a valid method to study acute muscular nociceptive responses. Moreover, considering that the stimulus is the same in limb and orofacial muscles, it could be useful in comparing the characteristics of pain at these locations.

These models of acute pain have been used as a first experimental approach to compare orofacial muscle pain with that of spinally innervated muscles. Nevertheless the use of models of chronic muscle pain, in which inflammation and/or hyperalgesia appear, could be more interesting because of their clinical repercussions.

4.2. Effect of central and peripheral opioids on acute muscular pain

As expected, the i.p. administration of morphine decreased the nociceptive behaviour in the masseter muscle as well as in limb muscles, and i.p. naloxone was able to antagonise the morphine effect at the three locations. Additionally, naloxone methiodide, an antagonist that does not cross the blood–brain barrier (Russell et al., 1982; Lewanowitsch and Irvine, 2002), antagonised the effect of morphine in the masseter muscle but not in the gastrocnemius or triceps muscles. These results suggest the participation of peripheral opioid receptors in the orofacial region, but not in the limb muscles.

To assess this possibility, the effect of a peripheral agonist was tested and, in agreement with the obtained results, loperamide induced antinociception in the masseter, but not in gastrocnemius or triceps. The effect of loperamide was antagonised by naloxone and by naloxone methiodide, confirming the participation of peripheral opioid receptors.

Finally, the effect of the drugs was tested using the classic tail-flick test (Bennet, 2001), where opioids are effective through the stimulation of centrally located opioid receptors. The obtained data were in accordance with those obtained in spinally innervated muscles; i.p. administered morphine induced dose-dependent antinociception that was antagonised only by naloxone, and neither loperamide nor naloxone methiodide were effective. Moreover, these results were similar in conscious and anaesthetised rats, which eliminate the possibility that the anaesthesia was the cause of the different responses to opioids in the different muscles assayed.

The results obtained in the masseter muscle are in line with the results of previous studies which demonstrate that morphine reduces the shaking behaviour induced by mustard oil (MO) injection into the masseter (Ro et al., 2003; Han et al., 2008); topical administration of morphine into the masseter decreases the nociceptive shaking behaviour after injection of MO in lightly anaesthetised rats, suggesting that this effect is mediated by a peripheral μ opioid receptor (Han et al., 2008). Moreover, it has been demonstrated that the intramuscular administration of a selective mu-opioid agonist is able to reduce the nociceptive response induced by HS in the masseter. The effect of this drug was significantly enhanced under inflammatory conditions (Nuñez et al., 2007), during which the expression of mRNA and protein for the mu-opioid receptor were increased in the trigeminal ganglion.

Under our experimental conditions opioids were not topically, but systemically administered. The importance of the peripheral component was pointed out because the effect of morphine was completely antagonised by the administration of naloxone methiodide, which does not reach the central nervous system, and because of the effectiveness of loperamide.

Regarding opioid effects on limb muscles, which are spinally innervated, the involvement of peripheral opioid analgesia does not seem to play an important role, under our experimental conditions. It is well known that peripheral opioid receptors participate in different degrees in the control of the nociceptive information and in the development of secondary allodynia and hyperalgesia in various experimental paradigms, such as the inflammation in the rat´s paw induced by formalin (Sevostianova et al., 2005; Ambriz-Tututi et al., 2009). In this inflammatory model, peripheral analgesia is associated with enhanced axonal transport of opioid receptors toward the periphery, increased mRNA transcription and a higher opioid receptor density in DRG, as well as increased opioid receptor binding and G protein coupling (Obara et al., 2009). The effectiveness of the peripheral opioid analgesia has also been demonstrated in visceral tissues using the acetic acid test in mice (Labuz et al., 2007), as well as in musculoskeletal structures using inflammatory joint pain models (Mecs et al., 2009; Santos et al., 2009).

There are few data on limb muscles; it has been demonstrated that spinal mu and delta opioids but not kappa, are involved in opioid analgesia in a model of chronic pain induced by the repeated administration of acidic saline (Sluka et al., 2002). It has been proposed that central, but not peripheral, mechanisms are involved in the inhibition of the nociceptive reflex, after morphine administration, in the gastrocnemius muscle injected with complete Freund's adjuvant (Li and Zhao, 2003). Our data are consistent with these previous results and also suggest that in cases of acute pain, central mechanisms are involved in opioid analgesia.

In summary, our data provide evidence that morphine and loperamide differentially modulate hypertonic saline-induced nociceptive behaviour after its injection into the masseter, gastrocnemius or triceps muscle. This suggests that differences in underlying mechanisms may exist among these two types of muscle pain. It is important to point out that in the masseter pain model where peripheral opioid receptors are mainly implicated, the doses of morphine necessary to induce antinociception are lower than those at which central opioid receptors become involved (gastrocnemius, triceps and tail-flick test). This is interesting because clinically low doses of opioids imply fewer adverse effects, such as respiratory depression, dependence and tolerance.

In addition, the role of peripheral opioid receptors has also been previously suggested by more authors using other orofacial animal models, such as the face grooming provoked by subcutaneous capsaicin (Pelissier et al., 2002).

The difference between the antinociceptive effect recorded in orofacial muscle and in limb muscles could be also attributed to the experimental model. In the first case animals were lightly anaesthetised whereas in the second they were awake, it could be possible to suggest that central components are reduced in the masseter pain model and this could make more evident the peripheral analgesia. Nevertheless the absence of differences in the results obtained in the

tail-flick tests in anaesthetised and awake rats suggest that the peripheral component does not depend on the administration of the anaesthesia and, in any case, our results demonstrate that peripheral opioid receptors play a role on muscle pain in the masseter.

Although our results are not final because it would be interesting to know if these differences also exist in humans, it can be suggested that peripheral opioid receptors are potential targets for the treatment of muscular orofacial pain, avoiding undesirable central opioid effects.

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