



Contents lists available at ScienceDirect

European Journal of Pain

journal homepage: www.EuropeanJournalPain.com

Antinociceptive effect of the cannabinoid agonist, WIN 55,212-2, in the orofacial and temporomandibular formalin tests

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ARTICLE INFO

Article history:

Received 28 July 2008

Received in revised form 3 February 2009

Accepted 17 February 2009

Available online xxx

Keywords:

Orofacial pain

Cannabinoids

Formalin test

WIN 55,212-2

Ketamine

ABSTRACT

Orofacial pain disorders are frequent in the general population and their pharmacological treatment is not always adequately resolved. Cannabinoids have demonstrated their analgesic effect in several pain conditions, both in animal models and in clinical situations. The aim of the present study was to evaluate the cannabinoid-mediated antinociception in two inflammatory models of orofacial pain (orofacial and temporomandibular joint (TMJ) formalin test) and to compare it with a spinal inflammatory model (paw formalin test). WIN 55,212-2 (0.5, 1 mg/kg), a synthetic cannabinoid agonist, was intraperitoneally (i.p.) administered prior to formalin and significantly reduced the nociceptive behavioural responses in these inflammatory tests. To elucidate which subtype of receptor could be involved in such effect, two selective cannabinoid antagonists were administered prior to WIN. SR141716A (1 mg/kg i.p.), the CB1 receptor-selective antagonist, was able to prevent the cannabinoid-induced analgesia in all three models, whereas SR144528 (1 mg/kg i.p.), the CB2 receptor-selective antagonist, only prevented it in the paw formalin test. A comparison with the antinociceptive effects of morphine (2.5, 5, 10 mg/kg, i.p.), indomethacin (2.5, 5 mg/kg, i.p.) and ketamine (25, 50 mg/kg, i.p.) was also performed. Morphine displayed a dose-dependent reduction of acute and inflammatory pain in all three models, whereas indomethacin and ketamine only attenuated inflammatory pain at the highest tested doses. These results indicate that the cannabinoid-induced antinociception in the orofacial region is mediated by activation of CB1 cannabinoid receptor. Moreover WIN was as effective as morphine and more effective than indomethacin and ketamine, in oral inflammatory pain.

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1. Introduction

The use of cannabinoid derivatives as therapeutic agents, in particular in the management of pain, has been of interest since the identification of the endocannabinoid system, in the early 1990s (Pacher et al., 2006). The antinociceptive activity of cannabinoid agonists has been widely demonstrated, suggesting a regulatory role for the endogenous cannabinoid system in mammalian nociceptive pathways (Rice et al., 2002; Cravatt and Lichtman, 2004; Walker and Hohmann, 2005). Toward this purpose, a wide range of animal models has been used, and the ability of cannabinoids to suppress behavioural responses to acute noxious stimuli, as well as in inflammatory and nerve injury-induced pain models, has been verified (Pertwee, 2001).

CB1 receptors are enriched in the Central Nervous System (CNS) and are also present in some peripheral tissues. Indeed, the activation of peripheral, spinal or supraspinal CB1 receptors can independently reduce nociception, providing strong support for CB1

receptor-mediated cannabinoid analgesia (Hohmann, 2002; Walker and Huang, 2002). CB2 receptors are present mainly in cells of the immune system, although not exclusively (Van Sickle et al., 2005) and their involvement in peripheral antinociception has been shown (Quartilho et al., 2003; Ibrahim et al., 2005).

Orofacial pain displays a high prevalence in today's population (LeResche, 2001; Feinmann and Newton-John, 2004), with a special meaning to the patient because of the emotional and psychological aspects related to it (Tenenbaum et al., 2001; Sessle, 2005). However, the pathogenesis, diagnosis and even pharmacological treatment of these pain conditions are still controversial. The craniofacial region presents a very complex anatomical and physiological organization, and several differences between the spinal and the trigeminal nociceptive pathways have been described (Sweet, 1984; Dodd and Kelly, 1991; Takemura et al., 2006). Moreover, there are relatively few animal models devoted to the study of nociception in the trigeminal region (Sessle, 2007), so the search for new animal models, and the improvement of the existing ones, is highly justified.

Although, as already mentioned, it has been shown that cannabinoids are effective in relieving spinal inflammatory pain

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(Tsou et al., 1996; Elmes et al., 2005; Borsani et al., 2007), only recent evidence supports the participation of the cannabinoid system in the modulation of orofacial neurotransmission (Ahn et al., 2007; Akerman et al., 2007). A better understanding of cannabinoid action in the trigeminal system, as well as a re-evaluation of some drugs such as ketamine, an NMDA antagonist recently re-introduced for the treatment of pain (Kozek et al., 2006), could help to find potential therapeutic targets and to develop new analgesics for these pain conditions.

Therefore, this study had three aims:

- To evaluate the systemic effect of the cannabinoid agonist WIN 55,212-2 (WIN) and two antagonists (SR141716A and SR144528) on two different models of inflammatory orofacial pain (Clavelou et al., 1989; Roveroni et al., 2001).
- To compare the effect of WIN on orofacial inflammatory pain with its effect in a model of spinal inflammatory pain (the paw formalin test).
- To compare the antinociceptive effectiveness of WIN with other well-known analgesic drugs such as morphine, indomethacin, and ketamine.

2. Methods

2.1. Animals

Adult male Wistar rats weighing 200–250 g (Harlan Ibérica, Spain) were used in these experiments. Animals were housed in clear plastic cages with soft bedding (four/cage) and with free access to food and water. They were maintained in a temperature-controlled room (23 ± 1 °C) with a 12/12-h light-dark cycle for at least five days prior to the experiments. Spontaneous behaviour was observed in the cages before starting the experimental procedure, and rats showing aggressiveness or alterations in motility were discarded.

This study was conducted following a protocol approved by the ethical committee of our University and in agreement with the guidelines recommended by the International Association for the Study of Pain for experimental pain in conscious animals (Zimmermann, 1983).

2.2. Testing procedure

Test sessions were carried out between 09:00 and 17:00 h in a quiet room maintained at 23 °C. Prior to testing, the rats were acclimated to the test chamber for a 20-min habituation period to minimize stress (Abbott et al., 1986). The test box had the dimensions of $28 \times 28 \times 15$ cm and two mirrors were positioned underneath and behind it to permit unobstructed viewing of the injected area. The test chamber was carefully cleaned for each testing procedure. The rats did not have access to food or water during the test. Number of animals per separate experimental group was at least 8. Each rat was used only once, and was sacrificed at the end of the experiment. An observer who was blind to drug treatment conducted all the behavioural assays.

2.2.1. Orofacial, TMJ and paw formalin test

Formalin 2.5% (Tjølsen et al., 1992; Clavelou et al., 1995) was injected into the upper lip just lateral to the nose (subcutaneous (s.c.)) (Raboisson and Dallel, 2004), into the TMJ region (intraarticular (i.a.)) (Roveroni et al., 2001) or subcutaneously into the ventral side of the rat hindpaw (intraplantar (i.pl.)) (Dubuisson and Dennis, 1977). The volume of injection was 50 μ l in the orofacial and TMJ formalin test (Roveroni et al., 2001; Raboisson and Dallel, 2004) and 20 μ l in the paw formalin test (Tjølsen et al., 1992).

Following formalin injection, rats were returned to the test box for a 51-min observation period. The recording time was divided into 17 blocks of 3 min and corresponding formalin-induced nociceptive behaviour was measured for each block.

The characteristic time course of the behavioural and electrophysiological responses to formalin consists of two distinct periods, the first period (early phase) corresponding to acute pain, and the second period (late phase) with an important inflammatory component (Dubuisson and Dennis, 1977; Dickenson and Sullivan, 1987a,b; Clavelou et al., 1989). Nevertheless, in the first description of the test, behavioural responses in the formalin-induced TMJ pain model did not show this bi-phasic pattern because the first phase was masked by the use of anaesthesia to facilitate the manoeuvre (Roveroni et al., 2001). In contrast with previous works, in our experiments, the i.a. injections into the rat TMJ were carefully performed, without anaesthesia, by two experienced investigators. This procedure avoids possible interactions between the drugs and the anaesthetic agent, and permits unmasking of the early phase of this test. Since it is known that manipulation can produce opioid-mediated stress-induced analgesia in the animals, our procedure could induce an antinociceptive effect that could mask our results. To investigate this possibility, we blocked, in one group of rats, the endogenous opioid activity using naloxone (2 mg/kg i.p.), a μ receptor antagonist, 30 min before formalin administration into the TMJ.

To examine whether nociceptive behavioural responses were directly due to the noxious stimulus of formalin, the same volume of saline solution was injected into the vibrissal pad (s.c.), into the TMJ region (i.a.) or into the hindpaw (i.pl.).

2.2.2. Nociceptive assessment

Animals showed different nociceptive behaviours depending on the region where the formalin was administered. It is accepted that a combination of behaviours provides a better measure of pain intensity than any single behaviour (Tjølsen et al., 1992;Coderre et al., 1993; Abbott et al., 1995; Roveroni et al., 2001). In this work, all the behaviours that showed a direct correlation with the formalin concentration (indicating a relationship with the nociceptive stimulus) were evaluated together.

In the orofacial region, the s.c. injection of formalin solution (2.5%) generates characteristic behavioural responses consisting of recurrent and persistent episodes of paw strokes directed to the perinasal area, called face rubbing, which has been demonstrated as a reliable index to quantify nociceptive sensitivity (Clavelou et al., 1995; Raboisson and Dallel, 2004). Thus, the number of times the animals rubbed the injected area with the ipsilateral fore- or hindpaw was defined as the nociceptive score.

Formalin administration into the TMJ region evokes two behavioural responses which are closely related to the site of injection: rubbing the orofacial region with the ipsilateral fore- or hindpaw and flinching the head in an intermittent and reflexive way characterized by high frequency shakes of the head (Roveroni et al., 2001). The sum of these behaviours (number of times) was considered as the formalin-induced nociceptive response.

In the rat paw formalin test, nociception was assessed by summing the number of times the animals showed two stereotyped behaviours: flinching and licking/biting the formalin-injected paw (Dubuisson and Dennis, 1977; Tjølsen et al., 1992; Abbott et al., 1995).

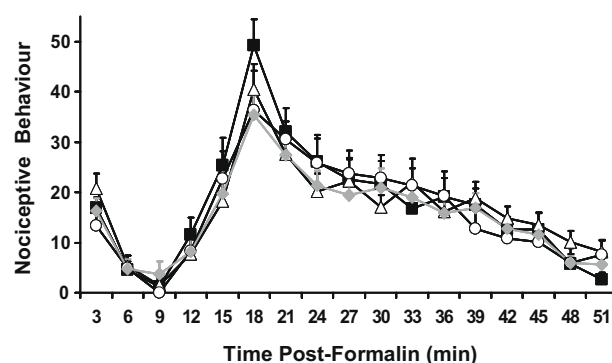
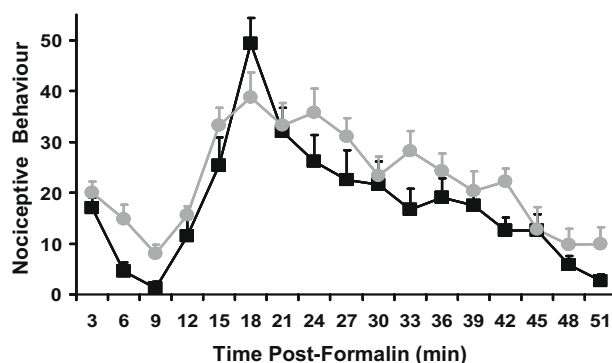
2.3. Drugs

The formalin solution was prepared from commercially available stock formalin (an aqueous solution of 37% formaldehyde) further diluted in isotonic saline to reach a final formaldehyde concentration of 2.5%. Non-selective cannabinoid receptor agonist

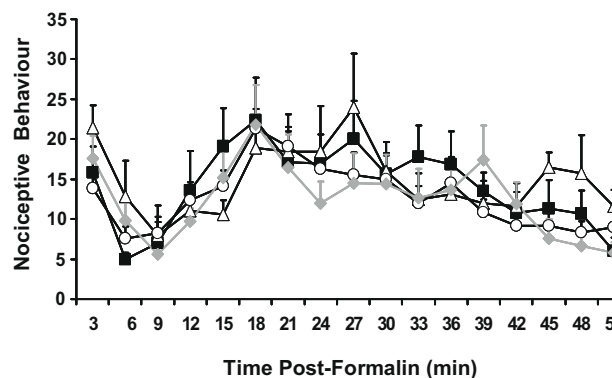
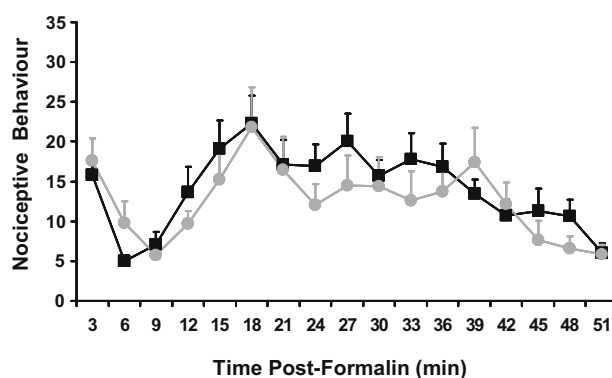
WIN (Tocris, Spain), CB1 receptor-selective antagonist SR1 and CB2 receptor-selective antagonist SR2 (both antagonists kindly supplied by Sanofi-Synthelabo S.A., France) were dissolved in ethanol 1 mg:1 ml and subsequently in ethanol and Tween 80 (1:2), after which the ethanol was evaporated and saline added to reach final concentration (Pertwee et al., 1992).

Whilst morphine sulfate and naloxone hydrochloride were dissolved in 0.9% NaCl, sonication for about 30 min was required for dissolving indomethacin (Sigma, Spain for these three drugs) in 0.9% NaCl. All drugs were administered intraperitoneally in a volume of 1 ml/kg. Ketamine hydrochloride (kindly supplied by Pfizer, Spain) was also administered in the same volume by i.p. injection.

A) OROFACIAL FORMALIN TEST



B) TMJ FORMALIN TEST



C) PAW FORMALIN TEST

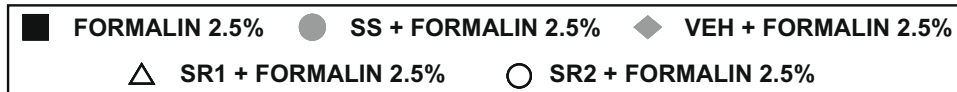
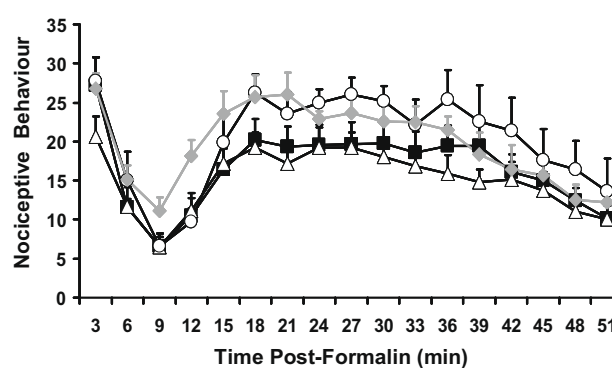
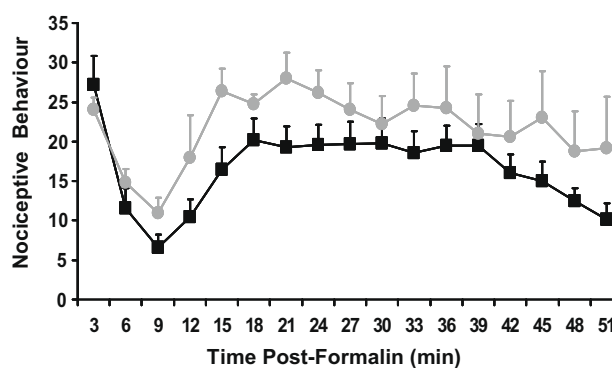


Fig. 1. Time course of the nociceptive behaviours observed after 2.5% formalin injection into the orofacial region (A), into the TMJ (B) and into the hindpaw (C). Data are expressed as the mean (\pm SEM) number of nociceptive behaviours for each 3 min block over the 51 min of post-injection observation period. Animals received an i.p. injection of saline solution (SS), of the vehicle used to solve the cannabinoids (VEH), of CB1 antagonist (SR1; 1 mg/kg), or CB2 antagonist (SR2; 1 mg/kg) ($n \geq 8$ per group, $n = 12$ for saline solution).

2.4. Experimental groups

In order to evaluate the role of cannabinoids in different formalin-induced orofacial pain models, as well as to compare it with other pharmacological treatments, different groups were constructed as follows:

- Control groups: rats were treated with saline solution (i.p.) 30 min before formalin injection (s.c., i.a., i.pl.).
- Additional control groups with cannabinoid vehicle and antagonists were performed: cannabinoid vehicle, SR141716A (SR1, 1 mg/kg, i.p.) and SR144528 (SR2, 1 mg/kg, i.p.) were administered 30 min before formalin administration (s.c., i.a., i.pl.).
- WIN 55,212-2 groups (WIN): rats received WIN (0.5, 1 mg/kg, i.p.) 30 min prior to formalin (s.c., i.a., i.pl.). WIN was chosen because it has been demonstrated that it can act both on CB1 and CB2 receptors, and that its antinociceptive effects can be blocked by CB1 or CB2 antagonists, depending on the receptor implicated. Although high doses of cannabinoid agonists are capable of altering motor activity, previous studies (Liang et al., 2007) have demonstrated that the doses used here do not modify locomotor activity evaluated by rotarod.
- SR1/SR2 groups: rats were pretreated with CB1 or CB2 receptor-selective antagonists, SR1 (1 mg/kg, i.p.) or SR2 (1 mg/kg, i.p.), 30 min before WIN injection (1 mg/kg, i.p.), that is, 60 min before formalin administration (s.c., i.a., i.pl.).
- Morphine groups: these groups were treated with the standard analgesic drug morphine at different doses (2, 5 and 10 mg/kg, i.p.), 30 min before formalin administration (s.c., i.a., i.pl.).
- Naloxone groups: rats received naloxone (1 or 2 mg/kg, i.p.) 30 min before morphine administration (5 or 10 mg/kg, i.p.), that is, 60 min before formalin injection (s.c., i.a., i.pl.).
- Indomethacin groups: animals were treated with two doses of this Non-Steroid Anti-inflammatory Drug (NSAID) (2.5, 5 mg/kg, i.p.), 60 min prior to formalin (s.c., i.a., i.pl.).
- Ketamine groups: these groups were treated with the NMDA receptor antagonist ketamine (25, 50 mg/kg, i.p.), 60 min before formalin administration (s.c., i.a., i.pl.).

In order to minimize the impact of environment and manipulation, and their possible effect on the observed behaviours, each drug group had its own control group and in all cases the effects of the different drugs were compared with a group receiving only saline solution.

2.5. Locomotor activity

Separate groups of rats were treated to evaluate the effect of both the noxious stimulus (formalin 2.5%) and the tested drugs (morphine, WIN and ketamine) on locomotor activity. Spontaneous motility was assessed using individual photocell activity chambers (CIBERTEC, Spain). Rats were placed in the recording chambers (55 × 40 cm, spacing between beams 3 cm) after formalin or drug administration (following the same experimental schedule described above) and starting 5 min later. The number of interruptions of photocell beams was recorded over a 30-min period and the mean number of crossings was compared with that obtained from a control group that had received saline solution.

2.6. Statistical analysis

Data are expressed as the mean number of times the rats carried out a nociceptive behaviour ±Standard Error of the Mean (SEM) for each 3 min block over the 51-min of post-injection observation period. Statistical analysis for significant differences

between multiple groups were performed by two-way analysis of variance (ANOVA) followed by Bonferroni test.

In order to facilitate the comparison between the different treatments, data are presented in two separate groups: a first, acute phase (0–3 min) and a tonic phase (12–42 min), for each formalin test.

For all analysis $p < 0.05$ was considered as statistically significant. Comparisons were established using as reference values those obtained from control groups (saline-treated groups).

3. Results

The injection of 2.5% formalin into the upper lip, into the TMJ and into the hindpaw, evoked the classical bi-phasic nociceptive response consisting of two phases: an early, short-lasting phase (0–5 min) followed by a late, prolonged phase (10–51 min). To discard the influence of the manipulation in the responses obtained in animals after TMJ or orofacial administration, the effect of saline solution administered in these areas was evaluated and no significant changes in the animal behaviour were observed (data not shown). No statistically significant differences were found when formalin (2.5%) was injected in i.p. saline-treated rats (control groups) or in naïve animals (Fig. 1).

Fig. 2 illustrates that i.p. administration of naloxone (2 mg/kg) did not modify the nociceptive behavioural responses elicited by i.a. injection of formalin into the rat TMJ, discarding a manipulation-induced stress in those animals.

When WIN was administered i.p. 30 min before formalin, it induced an antinociceptive effect in the three tests (Fig. 3). This effect was statistically significant at the dose of 1 mg/kg, in all models (Fig. 4), and in both phases, except on the acute phase of the TMJ formalin test.

Since WIN is a non-selective cannabinoid agonist, displaying affinity for both CB1 and CB2 receptors (Pertwee, 1999), selective antagonists for each subtype of cannabinoid receptor were administered 30 min before WIN (1 mg/kg i.p.) injection. In the orofacial and in the TMJ formalin test, only the CB1 receptor antagonist SR1 (1 mg/kg i.p.) prevented WIN-induced antinociception (Fig. 3A and B); whereas in the paw formalin test, both SR1 (1 mg/kg i.p.) and the CB2 receptor antagonist SR2 (1 mg/kg i.p.) were able to prevent the antinociceptive effect of WIN (Fig. 3C). Neither the cannabinoid receptor antagonists, SR1 and SR2, nor the cannabinoid vehicle, elicited any significant effect on the formalin-induced nociceptive responses of the three animal models when they were administered alone (Fig. 1).

As expected, morphine (2, 5 and 10 mg/kg, i.p.) also induced a dose-dependent antinociceptive effect in the three tests (Fig. 4).

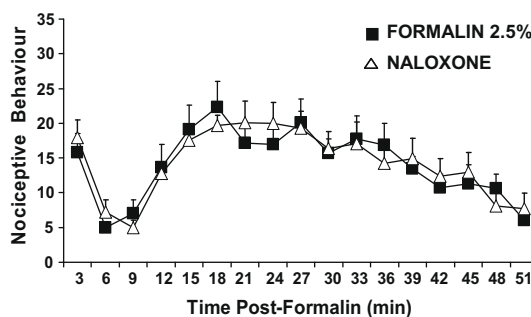
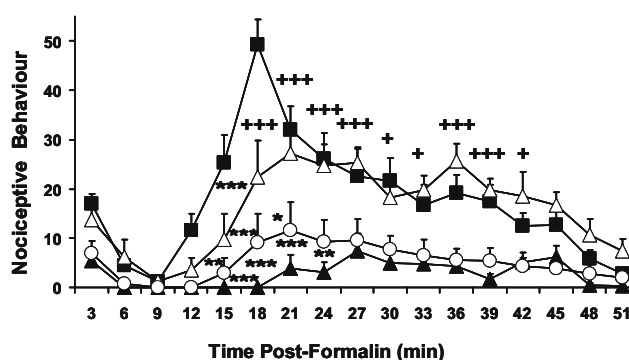
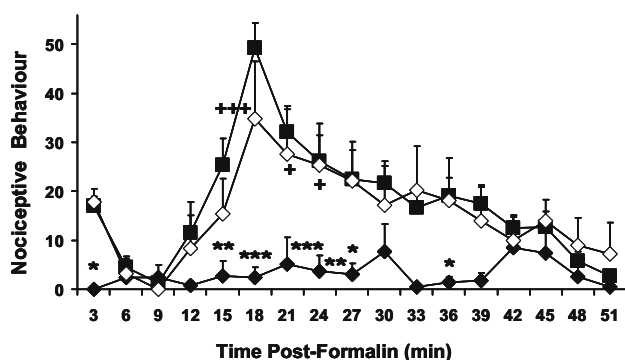
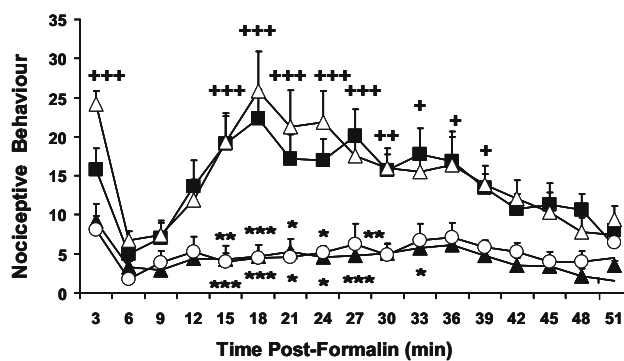
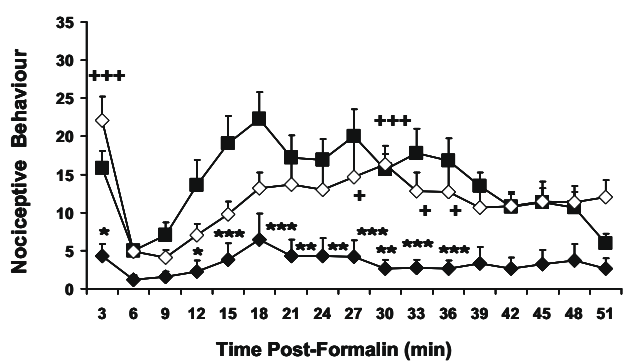


Fig. 2. Time course of nociceptive behaviour observed in rats after 2.5% formalin injection into the TMJ region. Data are expressed as the mean (\pm SEM) number of nociceptive behaviours for each 3 min block over the 51 min post-injection observation period. Animals received an i.p. injection of saline solution (control) ($n = 12$) or naloxone (2 mg/kg) ($n = 10$), before formalin administration.

A) OROFACIAL FORMALIN TEST



B) TMJ FORMALIN TEST



C) PAW FORMALIN TEST

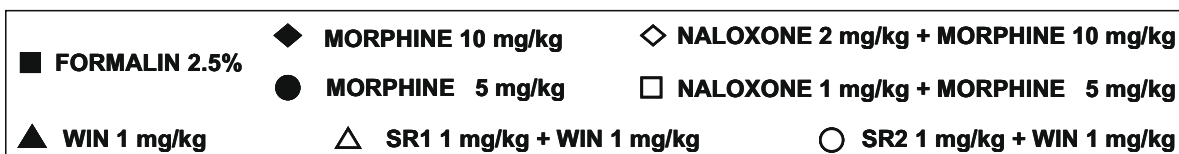
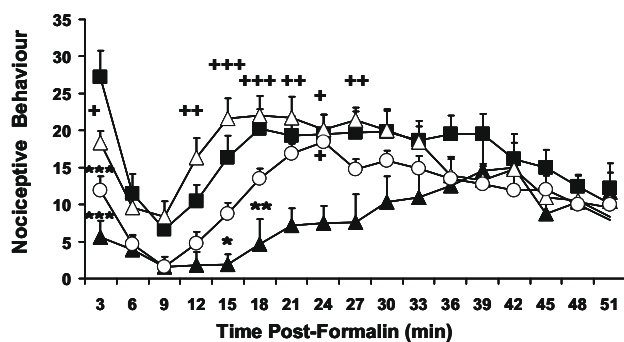
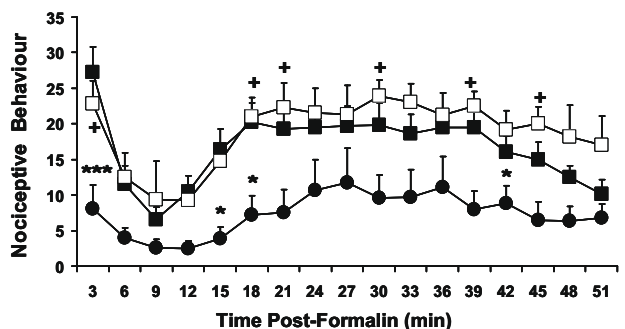


Fig. 3. Time course of the nociceptive behaviours observed after 2.5% formalin injection into the orofacial region (A), into the TMJ (B) and into the hindpaw (C). Data are expressed as the mean (\pm SEM) number of nociceptive behaviours for each 3 min block over the 51 min post-injection observation period. Animals received an i.p. injection of morphine (5 or 10 mg/kg), naloxone (1 or 2 mg/kg) plus morphine (5 or 10 mg/kg), WIN (1 mg/kg), SR1 (1 mg/kg) plus WIN (1 mg/kg) or SR2 (1 mg/kg) plus WIN (1 mg/kg), before formalin administration. *Statistically significant differences vs. control group ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-way ANOVA plus *post-hoc* Bonferroni test). *Statistically significant differences vs. agonist group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-way ANOVA plus *post-hoc* Bonferroni test) ($n \geq 8$ per group, $n = 12$ for saline).

This effect was evident in both the acute and the tonic phase, and the difference from saline-treated groups was statistically significant. Surprisingly, a small, inconsistent and unexpected nociceptive effect of the lower dose of morphine was obtained, only in the paw formalin test and only in its tonic phase (Fig. 4). In order to confirm that the effect of morphine was mediated by the μ opi-

oid receptor, naloxone was also administered i.p., and prevented the antinociceptive effect in each of the three models (Fig. 3). A difference in morphine sensibility in the different tests was obtained, since in the TMJ and orofacial formalin tests, the dose of morphine that produced a statistically significant effect was 10 mg/kg, whereas in the paw formalin test, 5 mg/kg of morphine were

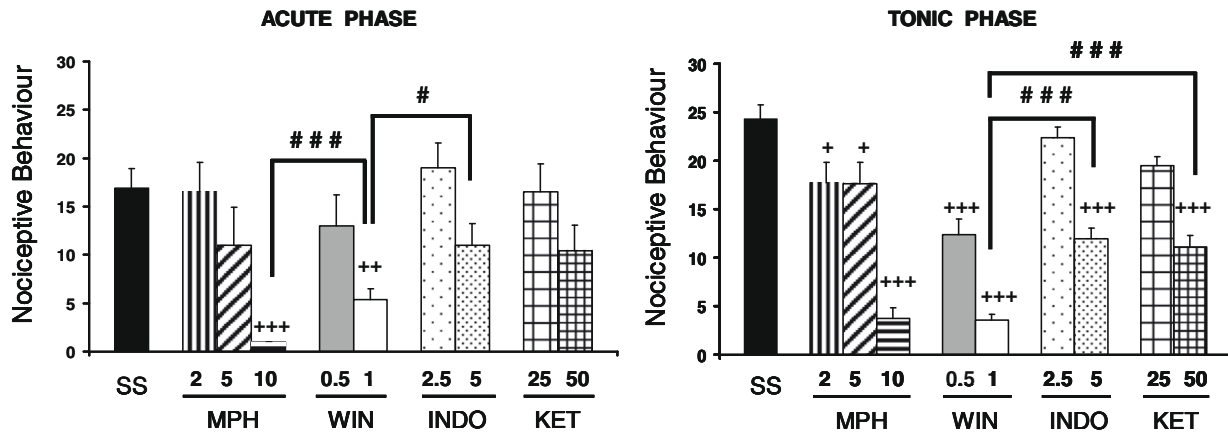
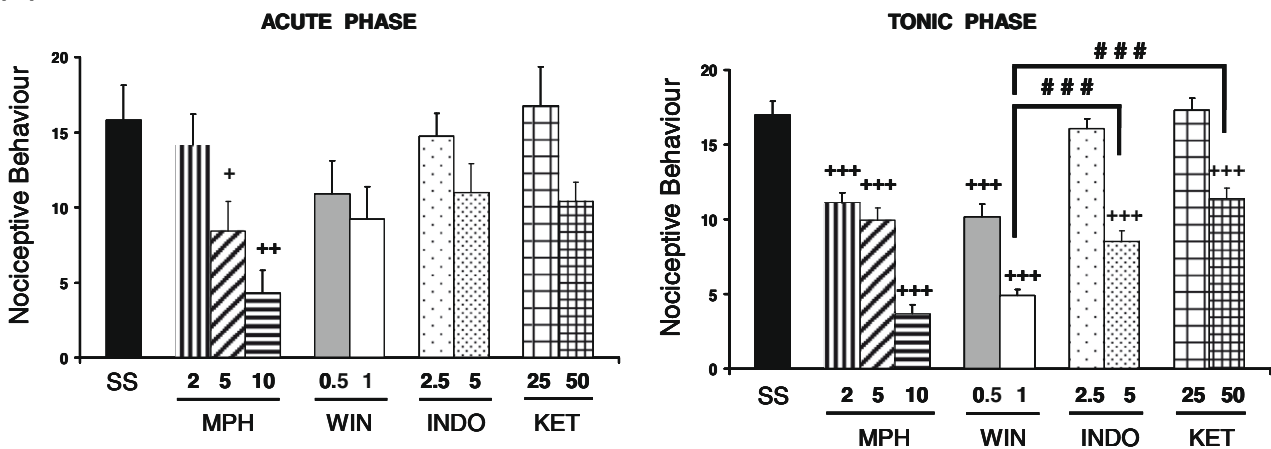
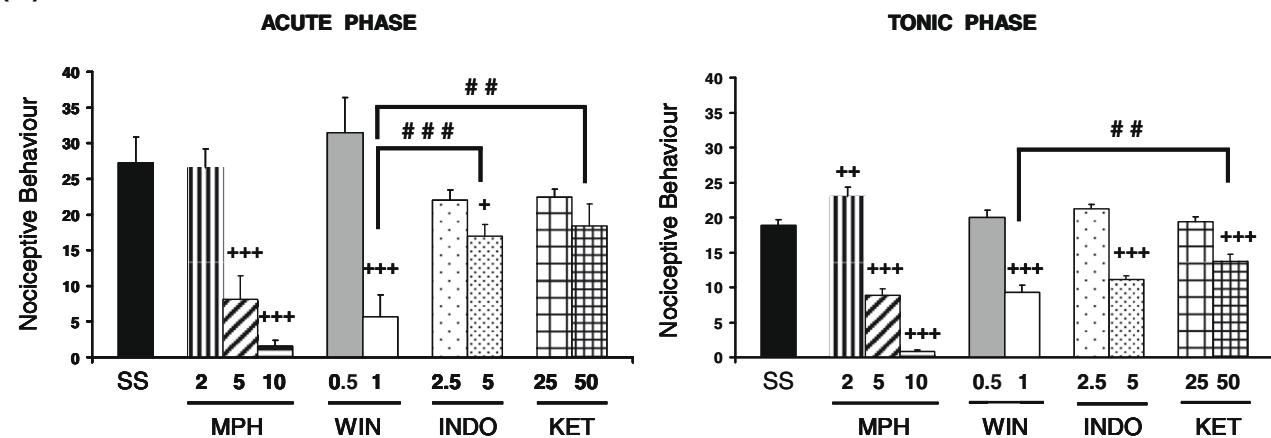
(A) OROFACIAL FORMALIN TEST**(B) TMJ FORMALIN TEST****(C) PAW FORMALIN TEST**

Fig. 4. Effect of intraperitoneal administration of different drugs on the nociceptive behaviours evoked in the orofacial (A), TMJ (B) and paw (C) formalin test. Bars show, in the acute phase (0–3 min) and in the tonic phase (12–42 min) of each formalin test, the antinociceptive effect (mean \pm SEM) of i.p. injection of morphine (2, 5, and 10 mg/kg), WIN (0.5 and 1 mg/kg), indomethacin (2.5 and 5 mg/kg) and ketamine (25 and 50 mg/kg). *Statistically significant differences vs. control group ($p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). #Statistically significant differences between groups ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$). Two-way ANOVA plus *post-hoc* Bonferroni test ($n \geq 8$ per group, $n = 12$ for saline). SS, saline solution; MPH, morphine; WIN, WIN 55,212-2; INDO, indomethacin; KET, ketamine.

sufficient to induce a statistically significant effect. A similar difference was obtained with the antagonist: in the paw formalin test, antinociceptive blockade was obtained with 1 mg/kg of naloxone; in contrast, a higher dose of naloxone (2 mg/kg, i.p.) was needed to prevent the effect of morphine in the TMJ and orofacial formalin

tests, and 1 mg/kg was unable to antagonize the effect of morphine (data not shown).

The NSAID indomethacin (2.5, 5 mg/kg) and the NMDA receptor antagonist ketamine (25, 50 mg/kg) were administered i.p. 60 min before formalin injection. Indomethacin reduced the nociceptive

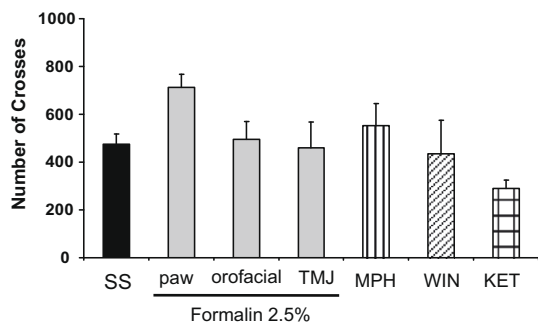


Fig. 5. Effect of formalin and drug administration on spontaneous locomotor activity. Bars show data, expressed as the mean (\pm SEM) number of beam crosses by rats injected with saline (SS), rats treated with formalin (2.5%) into the hindpaw, into the orofacial region or into the TMJ, and rats treated with the highest doses of morphine (MPH, 10 mg/kg i.p.), WIN 55,212-2 (WIN, 1 mg/kg i.p.) and ketamine (KET, 50 mg/kg i.p.) ($n \geq 6$ per group).

responses induced by formalin in all three animal models, at the highest tested dose (5 mg/kg), this effect being statistically significant for the tonic phase (Fig. 4). Additionally, the highest dose of ketamine (50 mg/kg, i.p.) was able to reduce the number of nociceptive behaviours in each of the three models, but these differences were only statistically significant in the tonic phase (Fig. 4).

Comparatively, WIN at 1 mg/kg showed similar efficacy to morphine at 10 mg/kg in the tonic phase of the orofacial test (3.56 ± 0.6 vs. 3.71 ± 1.1) (Fig. 4A), and in the tonic phase of the TMJ test (4.9 ± 0.4 vs. 3.69 ± 0.6) (Fig. 4B). In contrast, the antinociception exhibited by 1 mg/kg of WIN in the paw formalin test was similar to 5 mg/kg of morphine in both phases (5.63 ± 3.1 vs. 8.11 ± 3.3 acute phase, 9.23 ± 1.1 vs. 8.82 ± 1 tonic phase) (Fig. 4C).

Regarding the other drugs evaluated, in all three inflammatory pain models, and for all tested doses, indomethacin and ketamine were less effective than the cannabinoid agonist, being the differences statistically significant for the tonic phase in both orofacial tests (Fig. 4).

When the locomotor activity was evaluated, no differences were observed, neither between the effect of formalin in any of the three models, nor after the different drug treatments (Fig. 5).

4. Discussion

The participation of the endocannabinoid system in the modulation of pain is well documented, in animal models as well as in clinical situations (Martin and Lichtman, 1998; Azad and Rammes, 2005). However, its relationship with orofacial pain has not been analysed until recently (Ahn et al., 2007). Moreover, some chronic pain conditions are manifested in this region (temporomandibular disorders (TMD), post-herpetic and trigeminal neuralgias, burning mouth syndrome, atypical odontalgia, atypical facial pain, etc.) (Sessle, 2007), justifying the development of new behavioural models in animals to replicate inflammatory (Haas et al., 1992; Clavelou et al., 1995; Ren and Dubner, 1996; Roveroni et al., 2001; Pelissier et al., 2002) or neuropathic pain states (Vos et al., 1994; Rodella et al., 2000).

In both peri-orofacial models, WIN induced an antinociceptive effect that was statistically significant for the dose of 1 mg/kg of WIN and equivalent to a dose of 10 mg/kg of morphine ($p < 0.001$ vs. control) although in the acute phase of the TMJ formalin test, the effect of WIN did not reach statistical significance. The classical administration of formalin into the TMJ has been always carried out under anaesthesia (Roveroni et al., 2001) and the first phase of nociception was masked by such condition. Our methodology, using two experimenters to restrain the rat and administer the formalin into the TMJ, avoids the use of halothane and permits us, for

the first time, to obtain the characteristic bi-phasic time course evoked by the injection of formalin, differentiating acute from inflammatory pain in the TMJ region. The reliability of this technique is proven because the i.a. injection of saline solution did not evoke any behavioural response in the animals, and similar results were obtained when it was injected into the orofacial region (s.c.) or into the hindpaw (i.p.). Since the administration of naloxone did not produce any modification of the behaviour in this group of rats, the presence of opioid-related stress-induced analgesia can be discarded.

In contrast, in the paw formalin test, the dose of 5 mg/kg of the opioid agonist was able to reduce the nociceptive behaviour in a statistically significant manner ($p < 0.001$ vs. control), both in the acute and in the tonic phase. Interestingly, the maximum statistically significant effect was obtained when the cannabinoid agonist was administered at the dose of 1 mg/kg, the same as in the peri-orofacial tests.

Morphine was selected for comparative purposes. In each of the formalin tests the opioid agonist was able, at the tested doses, to diminish the number of nociceptive responses. Since no modification was observed in open field activity, this antinociceptive effect does not seem to be related to an impairment of motor activity.

The antinociceptive activity of WIN appears more evident in inflammation of trigeminal nerve- than spinal nerve-innervated areas. This difference could be the consequence of the characteristics of the spinal and trigeminal innervations. 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. Moreover, 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 (Dodd and Kelly, 1991; Sessle, 2005; Takemura et al., 2006).

WIN-induced analgesia by specifically acting on CB1 receptors in the orofacial and TMJ regions, since a CB2 antagonist did not modify WIN-induced antinociception. Our results are in agreement with other groups, because it has been recently shown that WIN, intracranially administered, is able to induce antinociception via CB1 receptor activation, in the TMJ formalin test (Ahn et al., 2007). Moreover, electrophysiological recordings have demonstrated that the activity of trigeminovascular neurons can be selectively blocked by activating CB1, but not CB2, receptors (Akerman et al., 2007). Liang et al. (2007), in rat model of trigeminal neuropathic pain, have also described that the WIN-induced antinociceptive effect is selectively blocked by CB1 receptor antagonists. Therefore, this evidence suggests that CB1 receptors play a more important role than CB2 in the antinociceptive effect of cannabinoids in orofacial pain.

The mechanism by which WIN induces this antinociceptive effect is not fully understood. Lee and co-workers (2008) demonstrated the participation of cannabinoid agonists in the antinociceptive effect of mGluR groups II and III agonists in the formalin test, and the group of La Rana recently reported (La Rana et al., 2008) that the enhancement of endocannabinoid activity reduces plasma extravasation in a model of neuropathic pain.

It is unlikely that the inhibition of nociceptive responses by WIN, observed in this work, could be attributed to motor impairment. The ability of cannabinoids to produce side effects, which could mask the antinociceptive effects, by activation of central CB1 receptors (including hypothermia, catalepsy and hypolocomotion) is well established. However, our group (Pascual et al., 2005) showed that a dose of 1 mg/kg (i.p.) of WIN only slightly reduced motor activity, without modifying other psychoactive effects as hypothermia or catalepsy. In the present study, i.p. administration of WIN (1 mg/kg), 30 min prior to the open field assay, did not

modify spontaneous motility when compared with saline- or formalin-treated animals. These data are similar to those previously reported by Fox et al., (2001) and Liang et al., (2007), who did not observe motor impairment at similar time points and doses of WIN.

When the antinociceptive effect of WIN was compared with other analgesic drugs, namely indomethacin and ketamine, the effect of the cannabinoid was always greater. The doses of these two drugs were selected from two previous studies (Qian et al., 1996; Sawynok and Reid, 2002) that determined the highest doses that could be used avoiding toxic effects.

Doses above 50 mg/kg of ketamine produced impaired locomotor activity and other psychotomimetic effects (Qian et al., 1996). However, according to published studies, 45 min after i.p. administration of ketamine (50 mg/kg), no locomotor disturbance was observed (Qian et al., 1996), and a higher dose of 60 mg/kg (i.p.) induced motor uncoordination only for 30 min (Sawynok and Reid, 2002). In our study, the antinociceptive effect of ketamine, at the highest dose (50 mg/kg), was observed 1 h after its administration and no significant reduction of locomotor activity was observed.

In the acute phase, indomethacin was unable to consistently diminish the nociceptive behaviours induced by formalin in any of the three tests. This result was expected since, in this phase, the behavioural response is only related to a direct stimulation of the nociceptors (Dubuisson and Dennis, 1977). However, in the tonic phase, the differences vs. control were statistically significant for the highest tested dose (5 mg/kg), consistent with the well-known anti-inflammatory effect of indomethacin. This result was also expected because nociceptive behaviours of the tonic phase depend on an inflammatory reaction as well as on functional changes in the dorsal horn of the spinal cord (Hunnskaar and Hole, 1987; Tjølsen et al., 1992). It could be hypothesised that higher doses of indomethacin could induce a greater antinociceptive effect; however, since it has been described that doses of indomethacin above 5 mg/kg can induce gastrointestinal lesions (Whiting et al., 1987), we discarded the possibility of evaluating the effect of a higher dose. Interestingly, a link between COX inhibitors and endocannabinoids has been known for several years: blocking COX with indomethacin (Gühring et al., 2002) or flurbiprofen (Ates et al., 2003) induces higher levels of arachidonic acid, facilitating endocannabinoid synthesis. Moreover, it has been proposed that indomethacin inhibits fatty acid amidohydrolase (FAAH), thus blocking endocannabinoid metabolism (Gühring et al., 2002).

Many studies have demonstrated that NMDA receptor activation contributes to the development of noxious stimulus-induced central neuroplasticity suggesting that selective receptor antagonists, such as ketamine, memantine or dextrometorphan, may be useful in pain treatment (Dickenson, 1994; Sawynok and Reid, 2002; Kozek et al., 2006). Currently, ketamine is clinically used for the treatment of chronic pain states which do not respond to classical analgesic drugs. Thus, ketamine was selected for this study as an attempt to approximate the clinical situation.

It is known that NMDA receptors participate in mechanical allodynia in the orofacial area (Ahn et al., 2004; Lee et al., 2004), and previous reports have also demonstrated that ketamine, at lower doses (0.4–12 mg/kg), and subcutaneously administered, is analgesic in the orofacial capsaicin test (Alvarez et al., 2003). In our hands, ketamine only induced an antinociceptive effect in the second phase of the different formalin tests, and only for the highest tested dose. This difference (we used an i.p. administration), could suggest a mostly peripheral effect of ketamine in orofacial pain. Moreover, in this study, the effectiveness of ketamine was always lower than WIN or morphine.

Although more work is needed, the differences shown in the effectiveness of the different pharmacological approaches, related to differences in receptor distribution and in the neurological orga-

nization of the involved areas, permits us to predict there will be differences in the physiology as well as in the pharmacological treatment of orofacial pain.

Acknowledgements

This work was supported by a grant from the Ministry of Education and Science of Spain (SAF2006-13391-CO3-01). E. Burgos is a research fellowship from the Ministry of Education and Science. Thanks are due to Dr. Shannon Shields and Dr. Julian Taylor for English language revision.

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