Nociceptive behaviour upon modulation of mu-opioid receptors in the ventrobasal complex of the thalamus of rats

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Abstract

The role of mu-opioid receptors (MORs) in the inflammatory pain processing mechanisms within the ventrobasal complex of the thalamus (VB) is not well understood. This study investigated the effect of modulating MOR activity upon nociception, by stereotactically injecting specific ligands in the VB. Nociceptive behaviour was evaluated in two established animal models of inflammatory pain, by using the formalin (acute and tonic pain) and the ankle-bend (chronic monoarthritic pain) tests. Control (saline intra-VB injection) formalin-injected rats showed acute and tonic pain-related behaviours. In contrast, intrathalamic administration of [D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin acetate (DAMGO), a MOR-specific agonist, induced a statistically significant decrease of all tonic phase pain-related behaviours assessed until 30–35 min after formalin hind paw injection. In the acute phase only the number of paw-jerks was affected. In monoarthritic rats, there was a noticeable antinociceptive effect with approximately 40 min of duration, as denoted by the reduced ankle-bend scores observed after DAMGO injection. Intra-VB injection of D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP), a specific MOR antagonist, or of CTOP

followed, 10 min after, by DAMGO had no effects in either formalin or ankle-bend tests. Data show that DAMGO-induced MOR activation in the VB has an antinociceptive effect in the formalin test as well as in chronic pain observed in MA rats, suggesting an important and specific role for MORs in the VB processing of inflammatory pain.

Keywords: Inflammatory pain; DAMGO; Pain thalamic processing; Opioid receptors; Ankle-bend test; Formalin test

1. Introduction

G-protein-coupled mu-opioid receptors (MORs) are required for the action of the most potent analgesics such as morphine [19]. The role of MORs in the persistence of inflammatory hyperalgesia has been highlighted by studies in knock-out mice [31] and [61]. Additionally, studies in arthritic rats with long-lasting pain have shown increased MOR synthesis in the spinal cord, dorsal root ganglia and peripheral nerve terminals [56], [60] and [68]. The correlation of MOR with nociceptive mechanisms has been mostly evaluated at the spinal cord level and brainstem circuits [24], [26], [29], [54], [55], [63], [66], [70] and [82]. In contrast, in the thalamus only a few studies have been reported [3] and [16].

The thalamus is especially important for sensory discrimination and transmission/modulation of painful stimuli. The rat ventrobasal (VB) thalamic complex, which comprises the ventroposterolateral (VPL) and the ventroposteromedial (VPM) nuclei, is the main relay thalamic region involved in tactile sensation and medial lemniscal information to the primary somatosensory cortex [59]. The VB is also implicated in pain processing and contains nociceptive-specific neurons, as demonstrated by electrophysiology studies [22], [28], [76] and [80], and observed in our previous studies in formalin-induced inflammation [58] as well as in monoarthritis [47], [49] and [57]. MORs are expressed in the VB, especially within the VPL [37], [38], [39] and [40] but their role in thalamic pain-processing mechanisms is not well understood.

These mechanisms can be partly clarified by evaluating the effects on nociceptive behaviour induced by microinjection of specific MOR ligands into target regions within the central nervous system [19] and [53]. The most commonly used MOR agonist is [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin acetate (DAMGO) because of its potency and high selectivity [12]. On the other hand, CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂) that presents good receptor selectivity, is frequently used as an antagonist of MOR [5] and [12]. DAMGO allows rapid endocytosis of MORs and presents less tolerance and

dependence effects than other agonists that do not promote endocytosis, such as morphine [23], [41] and [78].

The effect of DAMGO or other MOR ligands on nociception has already been studied in supraspinal nuclei implicated in the modulation of painful input, especially at the level of the brainstem such as that in the periaqueductal grey matter (PAG) or in the rostroventromedial medulla (RVM) [24], [26], [29], [63], [66], [70] and [72], as well as in the dorsal reticular (DRt) nucleus [54]. The hypo- or hyperalgesic effects observed were, in some cases, dose-dependent, particularly under chronic pain conditions, as in rats bearing monoarthritis [54]. However, to our knowledge, its effect on pain behaviour when administered into the VB has not been studied so far. Thus the objective of the present research was to evaluate the effect of activating or blocking MORs in the VB upon the nociceptive behavioural responses in acute, tonic and chronic pain in rats.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (Charles River Laboratories, France) weighing between 270 and 300 g were housed in pairs in cages with water and food ad libitum. The animal room was maintained at a constant temperature of 22 °C with controlled lighting (12 h light/dark cycles). All experiments followed the regulations of local authorities in handling laboratory animals, the ethical guidelines for the study of experimental pain in conscious animals [84] and the European Communities Council Directive 86/609/EEC. Moreover all efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Guide-cannula implantation

The animals were anaesthetized with a mixture of ketamine hydrochloride (Ketalar, 60 mg/kg) and medetomidine hydrochloride (Domitor, 0.25 mg/kg), and the skull of the rat was fixed in a stereotaxic frame. After trichotomy, a linear median incision was performed in the skin of the head and periosteum. Soft tissues were gently dissected exposing the bone of the skull for the osteotomy. A guide cannula (Neolus 23 gauge, Terumo, Belgium) was implanted 3–5 mm dorsal to the injection site in the right VB, by using the procedure previously described [45], and following the stereotaxic parameters of Paxinos and Watson [52] (dorsal–ventral: –6.0 mm; latero-medial: –3.0 mm; rostro-caudal: –3.3 mm, relatively to the interaural line). Fixation to the skull was achieved with three stainless steel screws and dental acrylic cement. During the surgery, the eyes of the rats were kept wet with saline. The animals were allowed to recover from surgery for 7 days before formalin or ankle-bend tests were performed.

2.3. Intrathalamic injections in acute and tonic inflammation and formalin test

For evaluation of the acute and tonic pain behaviours the formalin test [73] and [74] was performed 7 days after cannula implantation. In order to decrease the variability in formalin-evoked behaviours, room temperature was kept between 23 and 25 °C [2], [27] and [62] and the animals were habituated to the experimenter and to the behavioural test room as described elsewhere [58].

Before the formalin test, the right VB of normal rats was stereotaxically injected through a needle (B–D Micro-Fine + 29 gauges, Becton Dickinson, France) inserted into the guide cannula. For intra-VB injections, the animals were randomly selected for the drug administered. Moreover, in order to minimize the variability, the animals for each of the four different experimental groups were processed in parallel in all the experimental series. In the control group 2 μ L of saline (control formalin group, $n = 6$) was administered. For the other experimental groups 0.58 nmol of DAMGO (a specific MOR agonist, DAMGO group, $n = 6$; Sigma, St. Louis, USA), 4.71 nmol of CTOP (a specific MOR antagonist, CTOP group, $n = 6$; Sigma, St. Louis, USA), or CTOP followed by DAMGO after 10 min (CTOP + DAMGO group, $n = 5$) were injected. The injection needle was attached by a polyethylene tube to a 10 μ L Hamilton syringe and the solutions were administered over a 60 s period. In order to apply a standard concentration of DAMGO or CTOP in a volume that remained confined within the VB, but could reach the major extent of this complex, the volume injected in the VB was chosen according to the previous reports [57] and [58]. The selection of DAMGO or CTOP concentrations alone or of DAMGO after CTOP (CTOP + DAMGO group) and the period of time between the antagonist or agonist injection and the start point of the formalin test were selected according to our own preliminary trials, results from parallel investigations (where the 0.58 nmol dose of DAMGO showed best antinociceptive effects; see Section 2.4), and previous reports [5], [6], [26], [29], [54], [63], [67], [70] and [72]. In addition, a higher dose of CTOP, 9.410 nmol/rat was also tried, but the animals exhibited clear deficits in posture control and locomotion, including involuntary spinning movements. The data obtained from these animals were discarded. DAMGO and CTOP were diluted in saline.

Ten minutes after injection into the VB (saline, DAMGO, CTOP, or CTOP + DAMGO), 50 μ L of 5% neutral formalin was injected subcutaneously into the dorsal surface of the hind paw contralateral to the injected VB (left hind paw) in all animals. The rats were returned to the test chamber immediately after and their behaviour was recorded with a video camera connected to a computer during the following 60 min. Subsequently, behaviour scoring was performed by a researcher who was unaware of the drug injected into the VB by using the Etholog 2.25 free software [50].

For the evaluation of animals' behaviours, the scores were attributed and divided into three different categories [4] and [14]: (1) time spent in focused pain-related motor activity directed towards the injected paw, including biting, licking and shaking of the paw; (2) time spent in non-focused pain-related motor activity not directed towards the injected paw, but modified to protect the paw during movement, such as limping and keeping the paw elevated from the floor ("guarding behaviour"); and (3) number of jerks/flinches (involuntary movements) of the injected paw or hindquarters, named here as paw-jerks. Nociceptive behaviour was measured by the time spent in category 1 (focused pain behaviour) and 1 plus 2 (total pain behaviour) during 12 successive periods of 5 min, and by the number of paw-jerks occurring in the same periods [4]. The behavioural evaluation period lasted for 60 min in accordance with the previous studies indicating that the injection of formalin provides a subcutaneous stimulus inducing pain behaviour with this time duration [74].

2.4. Intrathalamic injections in chronic inflammation and ankle-bend test

Chronic pain was assessed in rats with monoarthritis (MA) at two time points of evolution of the disease, 4 and 14 days of inflammation. To induce MA, the rats were injected intraarticularly into the left tibiotarsal joint with 50 μ L of complete Freund's adjuvant (CFA, [7]). The inflammatory reaction was assessed daily by using a subjective scoring [9] ranging from 0 to 4, where 0 corresponds to no inflammatory signs and 4 to severe inflammatory symptoms affecting the animal's motor activity. The animals displaying any symptom of polyarthritis were discarded from the experiments. In order to minimize fear-motivated behaviours, the animals were habituated to the experimenter for several days before CFA injection and during the evolution of MA until behavioural experiments were performed at 4 (4 d) and 14 days (14 d) after CFA injection. In the 14-days MA group the surgery for implanting the guide cannula was performed 7 days after CFA injection, while the 4-days MA rats were operated 3 days prior to CFA injection to allow recovering from the surgery for 7 days.

In the experimental day the ankle-bend test was performed in the arthritic and contralateral hind paws in order to score the pain intensity from 0 to 20, in reaction to five flexions and five extensions, at time point 0 (before intrathalamic injections). Ankle-bend score was based on the animal's reaction (squeak or struggle) during paw manipulation according to Buttler and Weil-Fugazza [8]. This test was specifically devised for the monoarthritic rat model of chronic pain and has been demonstrated to be an appropriate procedure to assess the antinociceptive effects of a given substance [8].

Then MA rats were randomly selected for intra-VB injections performed through a needle inserted in the guide cannula, as described above. The 4 d and 14 d MA control groups received 2 μ L of saline ($n = 5$ for 4 d and $n = 6$ for 14 d). In order to build a dose-response curve the effect of DAMGO was assessed at

three different concentrations in both the 4 d and 14 d MA rats: 0.39 nmol/2 μ L (n = 5 for 4 d and 14 d), 0.58 nmol/2 μ L (n = 6 for 4 d and n = 5 for 14 d) and 0.78 nmol/2 μ L (n = 5 for 4 d and 14 d). A higher dose (0.97 nmol, n = 3 for 14 d MA) was also tested. However, the rats presented clear motor function alterations such as loss of righting reflex [13], [75] and [77], positional sense reflex [79], withdrawal reflex and toe spread reflex [75] and altered posture and locomotion. Thus this dose was not used in this study. An extra group of 14 d MA rats (n = 5) has also been injected with 2 μ L of the MOR antagonist (CTOP, 4.71 nmol) 10 min before 2 μ L of DAMGO (0.58 nmol). Time between CTOP and DAMGO injection was chosen according to the preliminary experiments where the peak of maximum agonist activity was approximately 10 min. DAMGO and CTOP were diluted in saline. After the injections in the contralateral VB, the ankle-bend test was performed again, both in the same arthritic and in the healthy hind paws, for 15 succeeding time points for 70 min. In the CTOP + DAMGO group of 14 d MA rats ankle-bend scores were also evaluated at a time-point between CTOP and DAMGO injections.

2.5. Histological examination

After formalin or ankle-bend nociceptive behavioural tests all the animals were briefly anaesthetized with isoflurane (5% for induction; 2.5% for maintenance) and 2 μ L of 2% Chicago sky blue 6B dye (Sigma, St. Louis, USA) was injected through the implanted guide cannula. Subsequently the rats were sacrificed by decapitation. The brains were removed and kept at -80 °C for subsequent processing and histological examination of the injection sites in 30 μ m thick coronal sections stained by the Nissl technique. Examination of the injection sites was made with reference to the Rat Brain Atlas [52]. The photos of the brain slices were obtained using a computer-assisted image analyzer (Optimas-Bioscan, USA) equipped with a Leica Axioplan microscope and a Sony Hyper HAD Digital colour video camera.

2.6. Statistical analysis

The formalin test results are expressed as mean $\pm$ standard error of the mean (SEM). It is well known that the pattern of pain behaviour differs in the two phases of the formalin test due to different pathophysiological mechanisms [74]. Thus the behaviours of rats injected with saline (control), DAMGO, CTOP or CTOP + DAMGO, during the first (0–5 min) and second (20–45 min) phases, were independently analysed. Formalin-evoked behaviours during the acute phase (0–5 min) were compared using one-way analysis of variance (ANOVA) followed, when appropriate (whenever the null hypothesis of the F-test was rejected, indicating at least one group with a mean significantly different), by the Dunnett t, 2-sided post hoc test, having as control category the saline-injected group (controls). Pain-related behaviours during the tonic phase were compared using ANOVA-repeated measures' analysis. The effect of group (between-

subjects effect) and the interactions between group and time (within-subjects effect) were tested in the ANOVA-repeated measures' models. In all ANOVA-repeated measures' models the covariance matrices were tested for sphericity and the homogeneity of variances across groups for each within-subject level was tested using the Levene's test. When the sphericity assumption was violated the Huynh-Feldt correction was used to test for the group-time interactions. Whenever group-time interactions were significant, one-way ANOVA was used to test the differences among groups for each of the time points analysed, followed when appropriate by the Dunnett t, 2-sided post hoc test, having as control category the saline-injected group (controls) and corrected for multiple comparisons using the Bonferroni method.

Ankle-bend scores, expressed as mean $\pm$ standard error of the mean (SEM), from 4 d or 14 d MA rats injected with saline (control) or with either dose (0.39, 0.58 or 0.78 nmol) of DAMGO or with CTOP + DAMGO were compared using ANOVA-repeated measures' analysis. Whenever group-time interactions were significant, one-way ANOVA was used to test the differences among groups for each of the time points analysed, followed when appropriate by the Dunnett t, 2-sided post hoc test, having as control category the saline-injected group (controls) and corrected for multiple comparisons using the Bonferroni method.

The statistical analysis was performed using the software programs SPSS 15.0® and Graphpad®, and a level of significance (probability of type I error) of $\alpha = 0.05$ was used.

3. Results

The injection of DAMGO into the VB (Fig. 1A) caused a statistically significant decrease of pain behaviours in both formalin-injected and monoarthritic rats. This effect was specific to DAMGO since rats receiving an injection of the antagonist (CTOP) alone or followed by DAMGO exhibited pain-related behaviours similar to saline-injected animals. Adjacent nuclei do not seem to be contributing to the analgesic effect observed in the VB since injections that, for technical reasons, failed to reach this complex (DAMGO out of VB, Fig. 1B) but reached other areas in the vicinity (e.g. posterior thalamic nucleus, dorsal and/or ventral zona incerta, reticular thalamic nucleus, dorsal and/or ventral medial geniculate nucleus) did not cause any reduction of the pain-related activities. In fact, by using the same statistical methods previously described, no significant difference was found ($p = 0.564$) between the rats injected with saline and the rats injected with DAMGO out of the VB (Fig. 1C).

Fig. 1. Representation of effective and ineffective injection sites. The histological examination of the target nuclei was made by comparison with the Rat Brain Atlas [52]. (A) Composite schematic reconstruction of effective injection sites within the VB (right) where similar behavioural effects were observed and bright field microscopy image of one representative injection site confined to the VB (left). (B) Composite schematic reconstruction of ineffective

injection sites in regions surrounding the VB, such as dorsal part and ventral part of zona incerta, (ZID, ZIV), posterior thalamic nucleus (Po), reticular thalamic nucleus (Rt) and medial geniculate nucleus, dorsal and ventral (MGD, MGV). (C) Graphic demonstrating the specificity of DAMGO injection analgesic effects within the VB (DAMGO in VB, circles) and lack of similar effects when injections were confined to adjacent nuclei (DAMGO out VB, crosses).

3.1. Formalin test

DAMGO-injected rats presented a statistically significant decrease of paw-jerks in the acute phase and of all pain-related behaviours (paw-jerks, total- and focused pain) observed in the tonic phase (Fig. 2 and Table 1) until 30–35 min. Formalin injection into the hind paw of control rats induced pain-related behaviours with the two phases already described in normal (non-operated) formalin-injected animals (Fig. 2) [1], [4], [14], [30], [64], [69] and [74] as well as in the rats that had been subjected to a guide cannula implantation surgery within the VB [58]. This biphasic pattern was also observable in DAMGO-injected rats, even though a shift in the peak of the tonic phase occurred. Thus, in the beginning of the tonic phase (20 min), in contrast to the other groups, DAMGO-injected rats kept presenting less pain. However, after 30–35 min post-formalin injection, their pain activities started to increase until they reached constant levels, around 50 min, similar to the ones observed in saline-injected rats, which remained until the end of the experiment. This wearing off the analgesic effect was especially evident in the paw-jerks and focused pain behaviours.

Fig. 2. Pain behaviour assessed by the formalin test. Graphics illustrate the time-course of the formalin test performed in rats injected into the VB with either saline ($n = 6$), DAMGO ($n = 6$), CTOP ($n = 6$) or CTOP + DAMGO ($n = 5$). (A) Number of paw-jerks; (B) focused pain-related activity; (C) total pain-related activity. Behavioural data were collected in periods of 5 min after injection of formalin (time 0) and are presented as mean $\pm$ SEM. Data were presented as mean $\pm$ SEM and statistically significant results are shown in bold in Table 1.

Table 1.

One-way ANOVA tests and Dunnett t 2-sided post hoc test with saline as control group, for each time point analysed in the formalin test, for the outcome variable number of paw-jerks (a), focused pain (b) and total pain behaviour (c).

In contrast to saline, DAMGO injection resulted in decreases in the number of paw-jerks as depicted in Fig. 2A. In the ANOVA-repeated measures' models, the p -value for the group-time interaction effect was $p < 0.001$ for the tonic phase, so in both cases one-way ANOVA with post hoc tests corrected for multiple comparisons was used to compare groups at each time point. Significant differences were detected at 20 and 25 min after formalin injection (Table 1a). Interestingly, at 30 min post-formalin injection, a switch was

observed, when DAMGO-injected rats displayed an increased number of paw-jerks, reaching values above those detected in saline-injected rats. This augmented nociception persisted until the end of the experiment being statistically significant at 40, 45, 55 and 60 min post-formalin administration (Table 1a), denoting an hyperalgesic behaviour in the second part of the formalin-elicited tonic pain phase of DAMGO-injected rats.

As observed with paw-jerks, an antinociceptive effect was verified by decreases in the time spent in focused pain-related behaviours (Fig. 2B) detected in the DAMGO-injected group. In the ANOVA-repeated measures' models, the p-value for the group-time interaction effect was $p < 0.001$, for the tonic phase, so that one-way ANOVA with post hoc tests corrected for multiple comparisons, was used to compare groups at each time point. A reduction in nociceptive behaviour was already observed in the beginning of the formalin test, although not statistically significant, with minimum values at 15 min when DAMGO-injected rats presented almost no pain (0.50 ± 0.39 s/5 min). This antinociceptive effect mediated by DAMGO was obvious throughout the tonic pain phase with statistical significance at 20, 25 and 30 min post-formalin injection. At 35 min, DAMGO-injected rats started showing more focused pain-related behaviours, above those observed in control rats, with noticeable significant reversion of the antinociceptive behaviour at 45, 50 and 55 min (Table 1b).

DAMGO injection induced a decrease of all total pain-related activities (Fig. 2C) with a higher magnitude than that observed in focused pain. In the ANOVA-repeated measures' models, the p-value for the group-time interaction effect was $p < 0.001$ for the tonic phase. Thus post hoc tests showed that DAMGO-injected rats exhibited less time spent in total pain-related behaviours which was statistically significant at 20, 25, 30, 35 and 40 min post-formalin injection (Table 1c). After 50 min the animals showed nociceptive behaviours similar to those of saline-injected rats, as previously described.

CTOP injection had no major significant effects upon the nociceptive behaviour of formalin-injected rats in comparison to saline administration. Similarly, the injection of CTOP prior to DAMGO reduced the antinociceptive effect induced by the agonist alone in all behaviours analysed, except for a few time points (Table 1).

3.2. Ankle-bend test

Monoarthritic rats showed severe inflammatory signs restricted to the arthritic paw and avoidance of passive movements as well as "guarding" behaviour. Mean inflammation scores [9] were very constant during the inflammation period and very high until the experimental day (3.97 ± 0.03 at 4 days MA and 3.98 ± 0.02 at 14 days MA), as previously observed [57]. The effects of saline, DAMGO or CTOP + DAMGO administration on the nociceptive behaviour of 4-

or 14-day MA rats, as inferred by the ankle-bend test, are depicted in Fig. 3 and Table 2.

Fig. 3. Pain behaviour assessed by the ankle-bend test in monoarthritic rats. (A) Four days of MA: scores at 15 different time points performed in rats injected into the VB with either saline ($n = 5$), DAMGO 0.39 nmol/2 μ L ($n = 5$), DAMGO 0.58 nmol/2 μ L ($n = 6$), or DAMGO 0.78 nmol/2 μ L ($n = 5$). (B) Fourteen days of MA: scores at 15 different time points performed in rats injected into the VB with either saline ($n = 6$), DAMGO 0.39 nmol/2 μ L ($n = 5$), DAMGO 0.584 nmol/2 μ L ($n = 5$), or DAMGO 0.78 nmol/2 μ L ($n = 5$) and scores at 17 different time points for CTOP + DAMGO ($n = 5$). Data were presented as mean $\pm$ SEM and statistically significant results are shown in bold in Table 2.

Table 2.

One-way ANOVA tests and Dunnett t 2-sided post hoc test with saline as control group, for each time point analysed in the ankle-bend test. Comparisons of ankle-bend pain scores in monoarthritic rats at 4 days (a) and at 14 days (b).

3.2.1. Four-day MA

Ankle-bend pain scores of saline-injected control animals were high and rather constant throughout the entire period, indicating severe allodynia (15.7 ± 0.2 , mean of 15 time points; Fig. 3A). Likewise, before the agonist administration ($t = 0$ min), the animals receiving DAMGO presented high and constant ankle-bend mean scores with values being comparable to those observed in controls (20.0 ± 0.0 , DAMGO 0.39 nmol; 14.9 ± 1.8 , DAMGO 0.58 nmol; 15.6 ± 1.0 , DAMGO 0.78 nmol). DAMGO injection induced an antinociceptive effect with different response curves according to the dosage (Fig. 3A). In the ANOVA-repeated measures' model, p -values for the group effect and the group-time interaction effect were, respectively, $p = 0.003$ and $p < 0.001$, so one-way ANOVA with post hoc tests corrected for multiple comparisons was used to compare groups at each time point (Table 2a). Thus, when agonist-injected animals were compared, the 0.58 nmol dose of DAMGO was most clearly and

consistently effective in reducing allodynia reaching minimum ankle-bend scores of 4.4 ± 1.3 at about 7 min post-injection. This antinociceptive effect remained significant and relatively stable until 45 min demonstrating that maximum DAMGO effects lasted approximately 40 min (Table 2a). When 0.39 nmol of DAMGO was administered, ankle-bend scores reached a minimum value of 7.0 ± 1.1 at 15 min, with statistically significant decreases only detected from time points 7 to 20 min. The 0.78 nmol dose induced even less consistent antinociceptive effects with a minimum pain value of 9.5 ± 1.8 at 10 min post-DAMGO injection. Statistically significant decreases of ankle-bend scores for this dosage were only observed at 15, 20 and 35 min.

3.2.2. Fourteen-day MA

As verified in the 4-day MA, saline-injected 14-day MA animals presented high scores (19.0 ± 0.2 , mean of 15 time points; Fig. 3B) in ankle-bend test which were constant throughout the entire period, indicating severe allodynia. DAMGO as well as CTOP + DAMGO-injected MA rats displayed also high and constant ankle-bend mean scores before agonist administration ($t = 0$ min; Fig. 3B and Table 2b), with values analogous to controls (15.8 ± 1.1 for DAMGO 0.39 nmol; 18.8 ± 0.4 for DAMGO 0.58 nmol; 18.0 ± 0.7 for DAMGO 0.78 nmol; 19.6 ± 0.4 for CTOP + DAMGO).

In 14 d MA rats, DAMGO was effective in reducing allodynia at the three doses administered (Table 2b), with ankle-bend scores decreasing to minimum values of 9.7 ± 1.4 (0.39 nmol; 25 min), 8.0 ± 2.5 (0.58 nmol; 10 min) and 11.2 ± 0.6 (0.78 nmol; 20 min). In the ANOVA-repeated measures' model, p-values for the group effect and the group-time interaction effect were, respectively, $p < 0.001$ and $p < 0.001$, so one-way ANOVA with post hoc tests corrected for multiple comparisons was used to compare groups at each time point (Table 2b). With all DAMGO dosages used, ankle-bend scores were significantly reduced from time points 5 to 45 min post intrathalamic injection. The lower dose used induced a more prolonged antinociceptive effect until the end of the experiment (Table 2b). As observed with the 4-day MA rats minimum peak values were observed when 0.58 nmol were administered.

Injection of CTOP prior to DAMGO administration completely abolished the antinociceptive effect observed with the agonist alone (Fig. 3B). Thus in this additional group, 14 d MA rats presented the same high and rather constant severe allodynia throughout the entire period (ankle-bend scores of 19.3 ± 0.3 , mean of 15 time points), as in the saline-injected group (Table 2b). Additionally, in the 10-min period between CTOP and DAMGO administrations ($t = -5$ and $t = -10$ min), when MORs were specifically blocked, ankle-bend scores were also evaluated and remained high, denoting the absence of any antinociceptive effect induced by the antagonist alone (Fig. 3B).

4. Discussion

The importance of the VB in sensory discrimination and transmission/modulation of noxious input has already been reported [22], [59] and [80]. However, although MORs are expressed in the VB of rats [37] and [40], available information on the nociceptive behavioural effects of modulating these receptors within this thalamic region is sparse. In this study, it was observed that specific activation of MOR by DAMGO in the rat's VB induces a decrease of pain-like activities induced by formalin inflammation or chronic monoarthritis. It is known that rodent and human opioid receptors are pharmacologically comparable [19] and that at the brainstem level opioid circuits have similar mechanisms in both species [19] and [20]. However, differences between rodents and primates should be considered so that the findings may not be directly applicable to humans.

The depression of pain behaviour induced by injection of DAMGO at the doses administered was the result of a specific effect on the nociceptive system, and not due to impaired motor function since animals retained their normal ability to walk and to move the affected paw, as well as the entire body, during the evaluated period. Normal motor behaviour was also observed according to standard evaluation tests [13], [75], [77] and [79].

4.1. Formalin test

DAMGO injection into the VB of formalin-inflamed rats significantly decreased the number of paw-jerks monitored within the first 5 min of the test but this antinociceptive effect was not detected in the other pain-related behaviours analysed. The 0.58 nmol dose of DAMGO used in the formalin test induced only a partial antinociceptive effect in this acute phase, which has a strong predominance of peripheral and spinal cord mechanisms [25]. This dose of MOR agonist was previously shown to have the best antinociceptive effects in preliminary trials as well as in studies involving monoarthritic animals at an early period of the disease. Thus it is unlikely that the lack of a complete hypoalgesic effect in the typical responses to formalin injection is due to the dosage of DAMGO employed. A large antinociceptive effect on all pain-related activities evaluated was observed only in the beginning of the second phase, possibly because the maximum analgesic effect of DAMGO in the VB is achieved at around 10–15 min, as observed in the monoarthritis model.

The second or tonic pain phase results from the release of inflammatory mediators or from the hypersensitisation of the spinal cord in the first phase [1], [30], [64], [69] and [71]. At the beginning of the tonic phase, in contrast to the other experimental groups, DAMGO-injected rats kept presenting less pain with a prolonged effect, demonstrating an antinociceptive effect of MOR activation within the VB. Afterwards a switch in nociception occurred, especially marked in the paw-jerks and focused pain behaviours at 35–40 min, when DAMGO-

injected rats started exhibiting even more pain-related activities than animals in other groups. Since the analgesic effect of DAMGO seems to only last about 40–50 min, as observed in experiments in the monoarthritis model, it was only the first part of the second phase of the formalin test that was depressed. When comparing the response curves obtained for saline-injected with those from DAMGO-injected inflamed rats, it looks as if a shift in the peak of the tonic phase has occurred upon DAMGO injection. This shift can be described as a gradual disappearing of the agonist analgesic effect before the responses to chemical irritation induced by formalin in phase two have worn off.

Conversely, the late switch in nociception could also represent a post-DAMGO hyperalgesic effect. Indeed other authors propose that opiates concomitantly activate antinociceptive systems and a NMDA-dependent pronociceptive system which leads to enhanced pain sensitivity (allodynia and/or long-lasting hyperalgesia) after single or repeated opioid administration in rats [10], [11], [33], [34] and [35]. It is possible that analogous events might also be triggered by DAMGO in the VB. Indeed, MOR are expressed in this region [37], [38], [39] and [40], the nociceptive responses of certain VB neurons are largely dependent upon NMDA (and metabotropic glutamate) receptors [15] and [65] and there is evidence that thalamic NMDA receptors are involved in the development and maintenance of inflammation-produced hyperalgesia in rats [32].

The mechanisms related with the analgesic effect of DAMGO could be possibly explained by supraspinal inhibitory events triggered by MOR overactivation in the VB. Morphine microinjection studies in other thalamic nuclei supported the existence of possible enhanced descending inhibitory mechanisms involving MOR activation [16], [43], [81] and [83]. In fact, there is evidence for the involvement of the submedial nucleus in a GABAergic mechanism of pre-synaptic opioid action leading to indirect activation of an endogenous analgesic system comprising the spinal cord, submedial, ventrolateral orbital cortex and periaqueductal grey [16], [81] and [83]. Also, a recent study in the habenular nucleus suggested a post-synaptic action of morphine on glutamatergic neuronal transmission and activation of the descending antinociceptive pathway in the thalamus [43]. The spinothalamic as well as lemniscal input to the VB is mainly glutamatergic while there are GABAergic terminals from the reticular nucleus neurons projecting into the VB [21]. Also, there is evidence for the expression of glutamate and GABA receptors in this region [42], [46], [48] and [65], so that similar mechanisms might also be elicited by DAMGO in the VB. However, at this point it is hard to speculate whether pre- and/or post-synaptic mechanisms as those proposed in the submedial and habenular nuclei, respectively, will be acting in the VB, since the synaptic localization of MOR within this thalamic region is, to our knowledge, sparsely studied, as well as the

electrophysiological in vitro and in vivo behaviour of VB neurons upon pharmacological manipulations with specific opioid receptors ligands.

4.2. Ankle-bend test in MA: chronic pain

The antinociceptive effect of selective MOR activation by DAMGO was clearly evident in MA animals experiencing chronic inflammatory pain (4 and 14 days). More consistent antinociceptive effects of DAMGO were achieved by all doses at the later time point of MA. This could be explained by the disease progression in time and thus the increased hyperactive state of VB neurons due to spontaneous noxious information arising from the inflamed joint, as suggested by our previous studies [47] and [57]. In this later period of disease, significant neuronal metabolic activity rises were detected in a high number of brain regions, including the VB [47]. Furthermore plastic changes involving the expression of neurotransmitter receptors in VB seem to occur during MA [17], [18] and [49].

The marked antinociceptive effect upon DAMGO administration observed in MA and formalin models reinforces the hypothesis that MORs within the VB are involved in the supraspinal modulation of inflammatory pain noxious inputs. Recent work has suggested that an interaction between MOR and delta opioid receptors (DORs) is crucial for mediation of opioid analgesia [36] and [61], and that activation of DORs might inhibit GABA release in some brainstem sites involved in pain modulation [36] and [51]. It is interesting to note that changes of DORs mRNA expression within the reticular thalamic nucleus, which sends inhibitory GABAergic projections to the VB [59], were found in MA rats [44].

In the present study, activation of MOR by DAMGO administration within the VB probably activated a thalamic inhibitory circuitry, which ultimately led to a reduction in the amount and/or quality of the nociceptive information that was eventually transmitted to the somatosensory cortex by VB neurons, thus leading to less perceived pain, as judged by the behaviour of the animals. In addition or alternatively to activation of descending antinociceptive pathways, as discussed above, is equally conceivable that this thalamic inhibitory circuitry involves the activation of GABAB receptors. In fact, a decrease of pain-related activities in both the acute and tonic phases of the formalin test and in MA rats was also observed upon intra-VB injection of baclofen, a specific GABAB receptor agonist [57] and [58]. In the later time point of MA both MOR (this study) and GABAB receptor activations induced similar antinociceptive effects [57], while in the tonic phase of formalin test baclofen provoked a more sustained effect at the higher dose administered [58]. Thus it is likely to assume a complex mechanism of interaction among receptors in the thalamus, and in the VB in particular, to mediate nociceptive processing of inflammatory pain, suggesting the use of combined drugs to reach better results in analgesic therapy.

In conclusion, the activation of MOR within the rat VB by DAMGO caused the reduction of nociceptive behaviours in both the acute and tonic phases of the formalin test. A similar effect was evidenced in MA rats suggesting an important role of MOR in the pathophysiology of inflammatory pain in rodents. More studies are needed on the synaptic localization and physiology of neurons containing MOR in order to elucidate the thalamic antinociceptive mechanisms involved. Additionally, comparative studies across different animal species, namely non-human primates, are required in order to evaluate the applicability of these findings in humans.

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