

Effects of 14-methoxymetopon, a potent opioid agonist, on the responses to the tail electric stimulation test and plus-maze activity in male rats: Neuroendocrine correlates

Leyre Urigüen,¹ Beatriz Fernández,¹ Eva María Romero,¹ Nuria De Pedro,¹ María Jesús Delgado,¹ Carmen Guaza,² Helmut Schmidhammer³ and María Paz Viveros^{1*}

¹*Departamento de Biología Animal II, Facultad de Biología, Universidad Complutense, Madrid, Spain;*
²*Instituto Cajal, Madrid, Spain; and* ³*Institute of Pharmacy, Department of Pharmaceutical Chemistry, University of Innsbruck, Innsbruck, Austria*

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ABSTRACT: We have studied the effects of 14-methoxymetopon (HS 198), a potent opioid agonist, on the responses to the tail electric stimulation test and plus-maze activity of adult male rats. The prototype μ agonist morphine was used as the drug of reference. Besides we addressed the effects of HS 198 on the serum corticosterone levels and on serotonergic systems of discrete brain regions. Both drugs were administered subcutaneously. Morphine (5 mg/kg) and HS 198 (30 μ g/kg) induced a similar effect on the nociceptive test, with both drugs significantly increasing the threshold for the vocalization afterdischarge, which is related to the emotional component of pain. In the plus-maze, morphine (5 mg/kg) and HS 198 (20 and 30 μ g/kg) induced similar increases in the percentages of entries and time in the open arms, two parameters related to the anxiety state of the animals. The results indicate that HS 198 is far more potent than morphine in reducing the emotive/affective component of pain and in inducing an anxiolytic effect. HS 198 (30 μ g/kg) also induced parallel increases in the serum corticosterone levels and the hypothalamic serotonin content. A possible correlation between the anxiolytic action of the drug and its effect on the hypothalamic serotonergic system is suggested. © 2002 Elsevier Science Inc.

KEY WORDS: 14-methoxymetopon, Opioid, Anxiety, Affective pain response, Serotonin, Corticosterone.

INTRODUCTION

Opioid agonists acting at the μ -receptor type, in particular morphine and fentanyl are potent analgesics that are employed in the management of severe pain. However, these agents induce a considerable number of adverse side effects, such as respiratory depression, constipation, tolerance, and addiction [19], which has led to an active search of novel opioid compounds exhibiting a favourable dissociation between analgesic activity and the development of dependence/tolerance and other side effects. A derivative of the

14-alkoxymorphinan series of opioids, 14-methoxymetopon (HS 198), appears to be a potent and selective μ -agonist [15,37], which exhibited an analgesic action far more potent than morphine in diverse nociceptive tests (tail flick, hot plate, acetic acid test) in rats and mice [15,31,37]. The development of physical dependence has been tested in different models in rodents. The dependence liability of 14-methoxymetopon was less pronounced than that of morphine, similar to tolerance to its analgesic action [15]. Moreover, it has been recently reported that, compared with sufentanil, 14-methoxymetopon does not induce hypoxia and hypercarbia, produces less hypotension and bradycardia, and induces less sedative effects, in the dog [13]. Thus, 14-methoxymetopon appears to be a very promising compound that has, besides the high analgesic potency, the advantage of considerably less pronounced adverse side effects than other opioid compounds used in therapy at the moment. However, the nociceptive tests used so far to assess the antinociceptive potency of HS 198 provide little information about the emotional/affective aspects of pain. Other measures like vocalization responses to electrical stimulation of the tail are more informative in this respect. By using this test, we have recently found that the vocalization afterdischarge (a response integrated at neural structures related to the emotional component of pain) is the most sensitive to the effects of morphine [9]. With respect to other possible behavioural effects of HS 198, there is no data available in the literature about its influence on the level of anxiety. The plus-maze is one of the most widely used ecological models in neurobiological research on anxiety [6,10,11,27,36] and has been previously used to assess the anxiolytic properties of morphine [2,18,26]. The aim of this work was to provide some new relevant information about the behavioural effects of HS 198 which can be interesting for a possible future clinical use of the compound, i. e., its effects on the affective pain response and its possible anxiolytic properties. For this purpose, we studied the effects of HS 198 on the behavioural responses of adult male rats in the tail electric

* Address for correspondence: Dr. María Paz Viveros, Departamento de Biología Animal II, Facultad de Biología, Universidad Complutense, 28040 Madrid, Spain. Fax: +34-91-3944935; E-mail: pazviver@bio.ucm.es

stimulation test and in the plus-maze. In both cases we used morphine as the drug of reference. In order to further address other neuroendocrine effects of HS 198, we also studied its effects on adrenocortical activity as well as on brain serotonergic systems that are involved in the modulation of anxiety.

MATERIALS AND METHODS

Animals and Experimental Conditions

Experiments were performed on Wistar albino adult male rats (85–135 days of age) which were served by Harlan Interfauna Ibérica S. A. (Barcelona, Spain). The animals were maintained at a constant temperature of 20°C and in a reverse 12-h dark/light cycle (lights on at 2000 h), with free access to food (commercial diet for rodents A04; Panlab, Barcelona, Spain) and water. Animals were housed in standard laboratory cages, each one containing groups of five to six individuals, and were habituated to the environmental conditions during a 15-day period. We used different animals for the nociceptive test ($n = 36$), the plus-maze ($n = 52$) and the neuroendocrine and neurochemical determinations ($n = 19$). The doses of the drugs, morphine (Alcaliber S. A., Madrid) and HS 198 (provided by Prof. Schmidhammer), and the time points at which nociceptive and behavioural responses were recorded, were chosen on the basis of previous studies [9,15] and pilot experiments performed in our laboratory. Both drugs were dissolved in saline and administered subcutaneously (s.c.) in a volume of 1 ml/kg. Each experimental group contained 8–15 individuals, from a minimum of seven different cages, that were tested on at least 2 different days to minimize interage and interday variability. All experimental procedures were carried out between 0930 and 1430 h. On the day of testing the animals were equilibrated in a quiet laboratory at least 30 min before experimental procedures were begun. Behavioural tests were carried out under the same illumination conditions as those in the animal facilities (red light). All the experiments performed in this study are in compliance with the Royal Decree 223/1988 of 14 March (BOE 18) and the Ministerial Order of 13 October 1989 (BOE 18) about protection of experimental animals, as well as with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Nociceptive Test

Nociception was assessed using the tail electric stimulation test as described previously [9,30]. The animals were placed in horizontal aerated plastic cylinders (Cibertec, Madrid, Spain) and two electrodes were attached at the base of the tail, 2 cm separated from each other, after spreading conductive gel. The electrodes were connected to a stimulator (SH-92 Model; Cibertec) delivering the current [pulse frequency: 60 Hz, train duration: 100 ms, train interval: 5 s, increment of intensity between trains: 0.06 mA, maximal intensity (cut off): 7.62 mA]. The thresholds for the motor response (tail withdrawal), vocalization during stimulus and vocalization after cessation of the stimulus (vocalization afterdischarge) were assessed for each rat. These responses are considered to be integrated at spinal, medulla oblongata and diencephalon-rhinencephalon levels, respectively [4]. Nociceptive responses were recorded immediately before acute administration of morphine (5 mg/kg), HS 198 (20 or 30 µg/kg) or 0.9% saline solution (baseline responses) and 20 min after treatment. The dose of morphine was chosen on the basis of previous studies carried out in our laboratory [9], which indicated that this dose produced submaximal and easily observable responses in this test. To compare the different treatments, antinociception was quantified using the following formula: % MPE (percentage maximum possible

effect) = (post-treatment latency-baseline latency/cut off-baseline latency) × 100.

Plus-Maze

The plus-maze consisted of two open arms (50 × 10 cm) and two enclosed arms of the same size with 40-cm-high walls arranged so that the arms of the same type were opposite each other. The junction of the four arms formed a central square area (10 × 10 cm). The apparatus was made of hard plastic material and elevated to a height of 62 cm. The test was carried out for 5 min. The test was started by placing the animals at the centre of the apparatus facing one of the enclosed arms, as described previously [6]. The measures recorded were frequency and duration of arm visits, separately for open and closed arms. An arm was considered to be visited when the animal entered it with the four limbs. We also estimated the percentage of entries into the open arms to the total number of entries, percentage of time spent in the open arms to the total time in arms, and total number of entries and total time spent in both types of arms. The percentage of entries and time in the open arms of the maze provides the measure of anxiety. The number of closed arms entries and the total number of entries provide a measure of motor activity/exploration in this test [10]. Four experimental groups were formed: a control group, which received saline, two groups receiving a single injection of either 20 µg/kg or 30 µg/kg of HS 198 and a fourth group receiving a single injection of morphine (5 mg/kg). The animals were tested individually in the plus-maze 20 min after receiving the corresponding treatment. The apparatus was thoroughly cleaned at the end of every test.

Endocrine and Neurochemical Determinations

For the endocrine and neurochemical determinations we chose the dose of HS 198, which showed the clearest effects in the behavioural tests, i.e., 30 µg/kg. As in the other experiments the control group received a single injection of saline. The animals were sacrificed by decapitation 20 min after the pharmacological treatment (the same time point at which the behavioural responses had been recorded). Blood samples were collected from the trunk and the brains were quickly removed. Hypothalamus, striatum, midbrain, cortex, and hippocampus were dissected out on an ice-cold glass plate and immediately stored at -80°C until indolamines quantification. Blood samples were centrifuged (3,000 rpm for 15 min), and serum was also stored at -80°C. Corticosterone was measured using a solid phase ¹²⁵I radioimmunoassay (Coat-A Count Rat Corticosterone kit; Diagnostic Products Corp., Los Angeles, CA, USA). The detection limit was 5.7 ng/ml and the intra-assay and inter-assay coefficients of variation were less than 10%. Serotonin (5-HT) and its metabolite 5-hydroxyindol-3-acetic acid (5-HIAA) were quantified by high-performance liquid chromatography (HPLC) with coulometric detection, as previously described previously [7]. The tissues were sonicated in 500–1000 µl of cold 0.3 N perchloric acid containing 0.4 mM sodium bisulfite, 0.4 mM EDTA, and 200 pmol/ml of isoproterenol as internal standard. The homogenate was centrifuged (13,000 rpm for 3 min) and 25-µl aliquots were injected into the HPLC system. The HPLC system consisted of a Waters pump (Model 590), a pulse dampener and a Rheodyne injection valve with a C18 reversed phase column (125 × 4.6 mm ID, 5 µm particle size). The potentials of the electrodes were +50 and +200 mV for the analytical cells no. 1 and 2, respectively. The mobile phase (flow rate = 1 ml/min) consisted of 100 mM H₃PO₄, 0.1 mM EDTA, 0.4 mM sodium octanesulphonic acid, and 4.5% acetonitrile (pH 3.1). The amount of indolamines in the samples were calculated as the area under the peaks, and expressed as pmol/mg wet weight of

tissue. The ratios 5-HIAA/5-HT were calculated and used as a metabolic index of neural activity.

Statistical Analysis

The data from the nociceptive test and the plus-maze was analysed by one-way analysis of variance (ANOVA). The Tukey test with a level of significance set at $p < 0.05$ was used for *post-hoc* comparisons. The results from the endocrine and neurochemical determinations were analysed by a Student's *t*-test.

RESULTS

Nociceptive Test

The ANOVA rendered significant effects of the pharmacological treatments on the vocalization during stimulus, $F(3,32) = 6.4$, $p < 0.01$, and on the vocalization after discharge $F(3,32) = 9.9$, $p < 0.001$, with this latter response being the most markedly affected by the drugs. As Fig. 1 shows, morphine (5 mg/kg) and HS 198 (30 $\mu\text{g}/\text{kg}$) had a similar effect on both vocalization reactions, with both drugs significantly increasing the thresholds for these responses. Significant differences were found between the two doses of HS 198 for the vocalization after discharge. The tail withdrawal (motor response) was not affected by any of the pharmacological treatments.

Plus-maze

The ANOVA revealed significant effects of the pharmacological treatments for the four parameters recorded: number of entries $F(3,48) = 4.7$, $p < 0.01$ and time $F(3,48) = 15.5$, $p < 0.001$ in the open arms; number of entries $F(3,48) = 3$, $p < 0.05$ and time $F(3,48) = 3.7$, $p < 0.05$ in the closed arms. As Table 1 shows, the frequency of visits to the open arms was increased by the higher dose of HS 198 (30 $\mu\text{g}/\text{kg}$), whereas the duration of visits to these arms was increased by the two doses of HS 198 (30 and 20 $\mu\text{g}/\text{kg}$) as well as by morphine (5 mg/kg). The number of closed arm entries was significantly reduced by morphine, which also decreased the time spent in this type of arms. An overall effect of the pharmacological treatments was also found for the total time in arms $F(3,48) = 11.7$, $p < 0.001$, and *post-hoc* comparisons showed that the three treatments induced a similar increase in this parameter (Table 1). The percentage of entries and time in the open arms, were significantly increased by the pharmacological treatments [$F(3,48) = 5.8$, $p < 0.01$ and $F(3,48) = 12.3$, $p < 0.001$, respectively], and no significant differences between the effect of morphine (5 mg/kg) and HS 198 (20 and 30 $\mu\text{g}/\text{kg}$) were found (Table 2).

Endocrine and Neurochemical Determinations

The results showed that HS 198 (30 $\mu\text{g}/\text{kg}$) induced a significant increase in the corticosterone levels, $t(17) = -3.6$, $p < 0.01$ [mean \pm SEM; saline = 338.9 ± 29.5 , $n = 10$; HS 198 = 500.1 ± 34.5 , $n = 9$]. With respect to the effects of HS 198 on the diverse serotonergic systems studied, the analysis revealed that the dose of 30 $\mu\text{g}/\text{kg}$ induced a significant increase in the hypothalamic 5-HT content, $t(16) = -2.4$, $p < 0.05$. The drug also induced modest although significant changes in the 5-HIAA contents of hippocampus $t(16) = -2.3$, $p < 0.05$ and midbrain $t(14) = 2.5$, $p < 0.05$. No significant effects were found in any of the other parameters analysed (Fig. 2).

DISCUSSION

14-Methoxymetopon (HS 198), is a potent opioid-agonist [15, 37] which displays an analgesic action far more potent than mor-

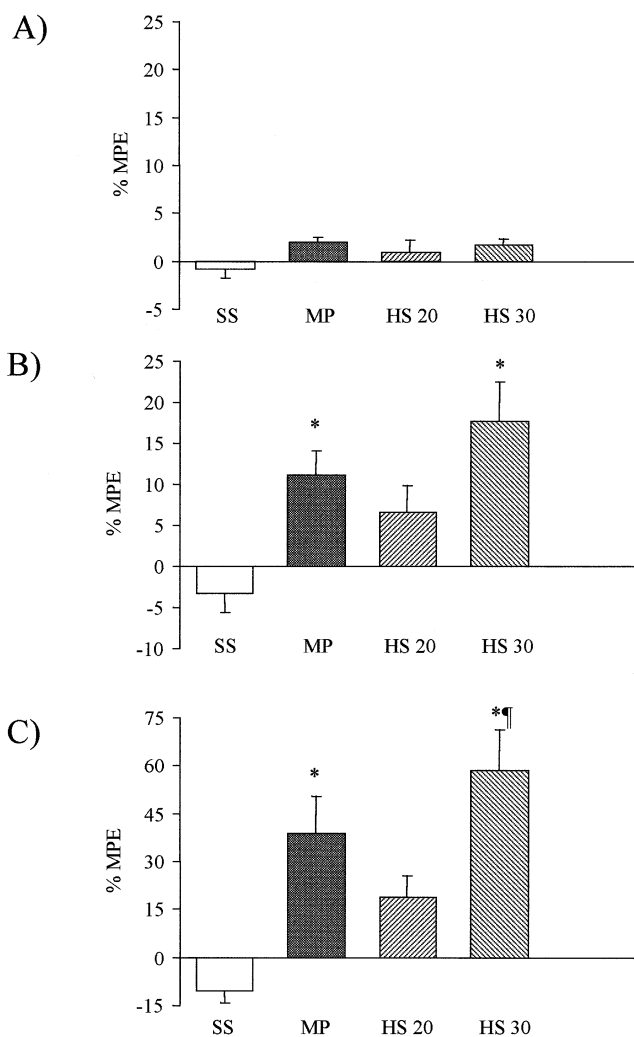


FIG. 1. Effects of morphine (MP) (5 mg/kg, subcutaneous [s.c.]) and 14-methoxymetopon (HS 198) (HS) (20 and 30 $\mu\text{g}/\text{kg}$, s.c.) on the responses of adult male rats in the tail electric stimulation test: (A) tail withdrawal (motor response), (B) vocalization during stimulus, and (C) vocalization after discharge. Nociceptive responses were recorded immediately before acute administration of the corresponding drug or saline (SS), and 20 min after treatment. % MPE (percentage maximum possible effect) = (post-treatment latency-baseline latency/cut off-baseline latency) \times 100. Histograms represent the mean \pm SEM of nine animals. Tukey test: * $p < 0.05$ vs. the control SS group; † $p < 0.05$ vs. the group injected with HS 198 (20 $\mu\text{g}/\text{kg}$).

phine in diverse nociceptive tests in rats and mice [15,31,37] and produces adverse side effects to a much lesser extent than conventional opioid analgesics [13,15]. The problem with fentanyl and other narcotics used in neuroleptic analgesia is the life threatening respiratory depression after surgery, whereas it has been recently reported that HS 198 does not show respiratory depression in the dog [13], which suggests that an important contribution to drug safety and adequate treatment of severe pain could be accomplished with this compound. The nociceptive test used in this work allows the evaluation of three nociceptive responses that appear to be related to pain regulating systems existing at distinct levels of the central nervous system. Thus, the motor response and the vocalization during stimulus have been suggested to be integrated

TABLE 1
EFFECTS OF MORPHINE AND 14-METHOXIMETOPON (HS 198) ON PLUS-MAZE ACTIVITY IN MALE RATS

	Open Arms		Closed Arms		Both types of Arms	
	Frequency	Duration (s)	Frequency	Duration (s)	Frequency	Duration (s)
SS	2.50 ± 0.34	36.38 ± 6.81	7.86 ± 0.74	125.32 ± 12.18	10.36 ± 0.63	161.70 ± 10.01
MP	4.78 ± 0.72	165.81 ± 16.08*	5.11 ± 0.79*	71.81 ± 7.65*	9.89 ± 1.23	237.61 ± 10.09*
HS 20	4.27 ± 0.38	108.84 ± 0.09*†	6.07 ± 0.36	98.34 ± 7.81	10.33 ± 0.59	207.18 ± 7.75*
HS 30	5.36 ± 0.82*	119.98 ± 17.75*	6.14 ± 0.68	105.90 ± 12.05	11.50 ± 1.30	225.88 ± 10.35*

The animals were tested 20 min after subcutaneous administration of saline (SS) (control group), morphine (MP) (5 mg/kg) or HS 198 (HS) (20 and 30 µg/kg). Values represent the mean ± SEM of 9–15 animals. Tukey test: **p* < 0.05 versus SS, †*p* < 0.05 versus MP.

at the spinal cord and caudal brainstem levels respectively, whereas the vocalization afterdischarge is considered to be organised in diencephalic or limbic structures and to reflect the affective component of pain [4,24]. In accord with our previous study [9], morphine at a dose of 5 mg/kg significantly increased the thresholds for both vocalization reactions without affecting the motor response. The present results also show that HS 198 (30 µg/kg) significantly affected both vocalization responses, whereas no significant effects were observed on the tail withdrawal response. The vocalization afterdischarge was the most sensitive to the effects of HS 198 and morphine. Other studies also indicate that the most sensitive response to modulation of enkephalin-metabolising enzymes is the vocalization afterdischarge, whereas the spinal reflex appears to be the least modified by these compounds [24,25,34]. It has been proposed that the specific effect of morphine on the affective pain response may be related to human descriptions of morphine analgesia, in which the affective component of pain is more potently modulated than the sensory component [17]. Taking into account the doses of morphine and HS 198 used in this work, the results indicate that HS 198 may exert an effect far more potent than morphine on the affective component of pain, which is of special relevance for its possible future clinical applications. Isolated tissue studies and binding assays support µ-opioid receptor activation as the mechanism of action of HS 198 [15], and there is substantial evidence indicating that the effects of both, endogenous and exogenous opioids, on the vocalization responses to electrical stimulation is mediated by µ-receptor without the contribution of δ- or κ-receptors [9,21–23,25]. Thus, it is likely that the specific effect of HS 198 on the affective pain response is mediated by µ-receptors located in limbic structures.

Previous results have shown that morphine exerts an anxiolytic effect in the plus-maze, when it is administered either peripherally

TABLE 2

EFFECTS OF MORPHINE AND 14-METHOXIMETOPON (HS 198) ON PLUS-MAZE ACTIVITY IN MALE RATS

	% Open Arms Entries	% Time in Open Arms
SS	26.1 ± 3.8	24.6 ± 4.7
MP	49.1 ± 4.7*	68.6 ± 4.0*
HS 20	40.6 ± 2.7*	51.8 ± 4.0*
HS 30	43.5 ± 4.7*	51.0 ± 6.2*

The animals were tested 20 min after subcutaneous administration of saline (SS) (control group), morphine (MP) (5 mg/kg), or HS 198 (HS) (20 and 30 µg/kg). Values represent the mean ± SEM of 9–15 animals. Tukey test: **p* < 0.05 versus SS.

(0.1 and 0.3 mg/kg) or at low doses in the dorsal periaqueductal gray [2,18,26], an effect that appears to be mediated by both, opioid and non-opioid systems [18]. The lack of an anxiolytic effect at the dose of 2.5 mg/kg was attributed to its reducing effects on locomotor activity (measured in a specific motility test) [18].

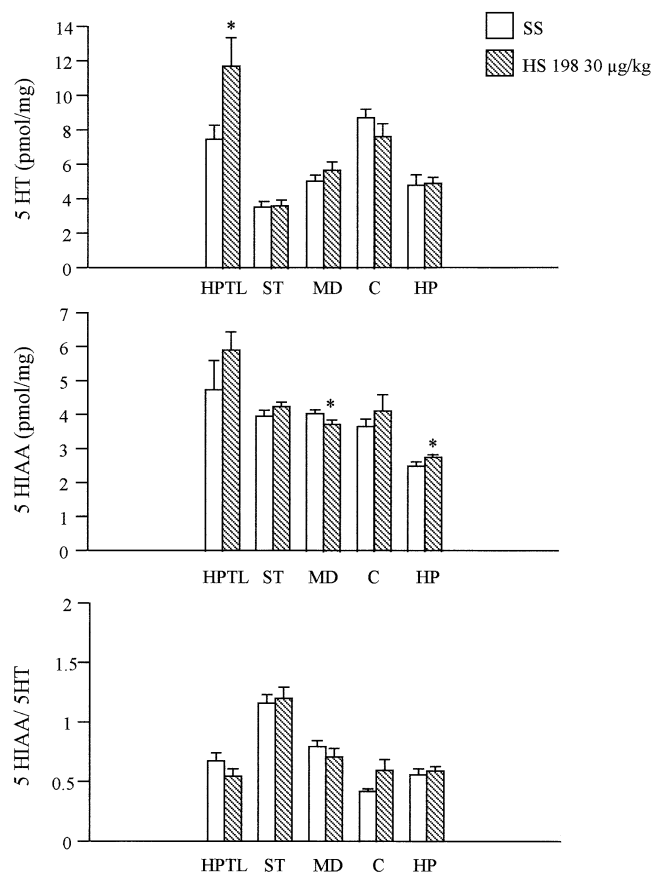


FIG. 2. Effects of 14-methoxymetopon (HS 198) (30 µg/kg, subcutaneous [s.c.]) on the levels of serotonin (5-HT), its metabolite hydroxyindol-3-acetic acid (5-HIAA) and the 5-HIAA/5-HT ratio in hypothalamus (HPTL), striatum (ST), midbrain (MD), cortex (C), and hippocampus (HP). The animals were sacrificed by decapitation 20 min after receiving HS 198 or saline (SS) (control group). The contents of 5-HT and 5-HIAA were determined by high-performance liquid chromatography with coulometric detection. Histograms represent the mean ± SEM of 8–10 animals. Tukey test: **p* < 0.05 vs. the control SS group.

The present results show that morphine at a dose of 5 mg/kg induced a significant increase in the percentages of entries and time in the open arms, which clearly indicates an anxiolytic effect [10,11,27]. No significant differences were found between the effect of morphine (5 mg/kg) and HS 198 (20 and 30 μ g/kg) on these parameters, which indicates that HS 198 may be far more potent than morphine in inducing an anxiolytic effect. All the experimental groups showed a similar number of total entries, indicating that the different pharmacological treatments did not induce any significant modification of the global activity in the plus-maze. However, we have previously found that HS 198 at a dose of 30 μ g/kg decreased the motor activity in the holeboard (unpublished data). The data indicates that the present experimental conditions might not be adequate for detecting changes in general motor activity. As for other opioid and non-opioid compounds, this specific effect of HS 198 can be more easily detected in other tests, such as the holeboard, which are designed to provide independent measures of motor activity and exploration [10].

The present results indicate that HS 198 at a dose of 30 μ g/kg induced a significant increase in the serum corticosterone levels. The stimulatory effects of opioids on the hypothalamic-pituitary-adrenal (HPA) axis is relatively well documented [3,14,20,32]. In previous studies morphine has been shown to induce clear corticosterone responses at doses of 20–30 mg/kg [14,32], whereas no significant effect of morphine on corticosterone levels was found at doses of 5 or 10 mg/kg [32]. Thus, HS 198 appears to be far more potent than morphine in inducing this neuroendocrine effect. In parallel with the activation of the HPA system, HS 198 (30 μ g/kg) also induced a significant increase in the hypothalamic content of 5-HT. There is evidence about the existence of reciprocal influences between the serotonergic system and the HPA axis. Some reports have shown that 5-HT nerve terminals synapses with corticotrophin releasing factor (CRF) neurons in the hypothalamus, and 5-HT stimulates the HPA axis [5,8]. Moreover, a number of changes have been shown in central serotonergic systems associated with either CRF or glucocorticoid administration [5,29]. We are currently performing additional experiments (administration of specific antagonists for CRF, serotonin and opioid receptors) in order to elucidate the specific mechanisms mediating these effects. There is substantial evidence about the involvement of the serotonergic system in the modulation of anxiety [11,12,16,33]. The present results show that HS 198, which induced a clear anxiolytic effect in the plus-maze, also induced a number of changes in the serotonergic systems of hypothalamus, midbrain and hippocampus. It is likely that the anxiolytic effect of HS 198 is related with its effect on the hypothalamic 5-HT content. In fact, a decreased hypothalamic content of 5-HT has been correlated with an increased level of anxiety/emotionality [35] and there have been reported reductions in 5-HT in diverse brain regions including the hypothalamus, in relation to several stressful situations [1,28].

In conclusion, this is the first report showing that HS 198 may be far more potent than morphine in reducing the emotional/affective component of pain and in inducing an anxiolytic effect, which may be of additional interest for a potential clinical use of the compound. In parallel with these behavioural effects, HS 198 induced a potent stimulatory effect on adrenocortical activity together with an increase in the hypothalamic 5-HT content. Further research is needed to elucidate the specific opioid (and non opioid) receptors involved in these actions.

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