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# THEMED ISSUE: GPCR

# RESEARCH PAPER

# Central antinociception induced by μ-opioid receptor agonist morphine, but not δ- or κ-, is mediated by cannabinoid $CB_1$ receptor

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**Background and purpose:** It has been demonstrated that cannabinoids evoke the release of endogenous opioids to produce antinociception; however, no information exists regarding the participation of cannabinoids in the antinociceptive mechanisms of opioids. The aim of the present study was to determine whether endocannabinoids are involved in central antinociception induced by activation of μ-, δ- and κ-opioid receptors.
**Experimental approach:** Nociceptive threshold to thermal stimulation was measured according to the tail-flick test in Swiss mice. Morphine (5 μg), SNC80 (4 μg), bremazocine (4 μg), AM251 (2 and 4 μg), AM630 (2 and 4 μg) and MAFP (0.1 and 0.4 μg) were administered by the intracerebroventricular route.
**Key results:** The $CB_1$-selective cannabinoid receptor antagonist AM251 completely reversed the central antinociception induced by morphine in a dose-dependent manner. In contrast, the $CB_2$-selective cannabinoid receptor antagonist AM630 did not antagonize this effect. Additionally, the administration of the anandamide amidase inhibitor, MAFP, significantly enhanced the antinociception induced by morphine. In contrast, the antinociceptive effects of δ- and κ-opioid receptor agonists were not affected by the cannabinoid antagonists. The antagonists alone caused no hyperalgesic or antinociceptive effects.
**Conclusions and implications:** The results provide evidence for the involvement of cannabinoid $CB_1$ receptors in the central antinociception induced by activation of μ-opioid receptors by the agonist morphine. The release of endocannabinoids appears not to be involved in central antinociception induced by activation of κ- and δ-opioid receptors.
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**Keywords:** morphine; SNC80; bremazocine; $CB_1$ receptor; $CB_2$ receptor; central antinociception

**Abbreviations:** AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone; FAAH, fatty acid amide hydrolase; MAFP, methyl arachidonyl fluorophosphonate/(5Z,8Z,11Z,14Z)-5,8,11,14-eicosatetraenyl-methyl ester phosphonofluoridic acid; SNC80, (+)-4-[(alphaR)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; $\Delta^9$-THC, Δ9-tetrahydrocannabinol

## Introduction

Cannabinoids and opioids are two separate groups of psychoactive drugs that exhibit several similar pharmacological effects, including analgesia, sedation, hypothermia and inhibition of motor activity (Manzaneres *et al.*, 1999; Massi *et al.*, 2001; Varvel *et al.*, 2004). In addition, receptors for both drugs are coupled to similar intracellular signalling mechanisms, mainly to a decrease in cAMP production through the activation of $G_i$ proteins (Bidaut-Russell *et al.*, 1990; Childers, 1991).

Opioids produce their pharmacological effects by acting mainly through three types of opioid receptors, namely μ, δ and κ (Singh *et al.*, 1997). Two types of cannabinoid receptors

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have been identified. $CB_1$ receptors are expressed primarily in central and peripheral neurones, and $CB_2$ receptors, mainly in immune cells (Pertwee, 2001; Howlett *et al.*, 2002). $CB_2$ expression in rat microglial cells (Carrier *et al.*, 2004), cerebral granule cells (Skaper *et al.*, 1996), mast cells (Samson *et al.*, 2003) and adult rat retina (Lu *et al.*, 2000) has also been demonstrated.

In recent years, the interaction between cannabinoid and opioid systems in nociceptive effects has been the focus of much attention (Welch and Eads, 1999; Finn *et al.*, 2004). For example, a greater-than-additive interaction between Δ9-tetrahydrocannabinol ($\Delta^9$-THC) and morphine administered intravenously has been demonstrated, because inactive doses of the drugs in combination produced a potent analgesic effect. This combination of drugs produced effects through pathways mediated by both μ-opioid receptors and $CB_1$ cannabinoid receptors, because potentiation was completely blocked by selective $CB_1$ and μ receptor antagonists (Reche *et al.*, 1996). Additionally, other studies have shown that $\Delta^9$-THC significantly enhanced the potency of morphine and codeine in the tail-flick test (Smith *et al.*, 1998; Cichewicz *et al.*, 1999). It has been suggested that endogenous opioids might be involved in the regulation of pain control by cannabinoids. Intrathecally administered $\Delta^9$-THC has been shown to release endogenous opioid peptides (Pugh *et al.*, 1996). Cannabinoids $\Delta^9$-THC and levonantradol appear to enhance the antinociceptive effect of morphine by releasing dynorphin A and dynorphin B, respectively (Welch and Eads, 1999). This hypothesis is supported by a number of studies indicating that opioid receptor antagonists might block cannabinoid-induced antinociception (Cox and Welch, 2004). According to some authors, the influence of $\Delta^9$-THC on endogenous opioid effects depends on basal endogenous opioid tone. For example, the induction of the diabetic state in rats decreases the antinociceptive effect of morphine, an effect temporally related to decreased release of specific endogenous opioids. Conversely, $\Delta^9$-THC retains the ability to release endogenous opioids in diabetic rats, and maintains significant antinociception. Similarly, $\Delta^9$-THC was more active in the tail-flick test in diabetic than in non-diabetic mice (Williams *et al.*, 2008). In addition, the effects of endocannabinoids have been recently reviewed (Maione *et al.*, 2006; Degroot and Nomikos, 2007) indicating a significant role for the endocannabinoid system in various pain states (La Rana *et al.*, 2006; Palazzo *et al.*, 2006).

Anandamide, an endocannabinoid, is produced following intracellular cleavage of *N*-arachidonyl-phosphatidy lethanolamine by phospholipase D, and shows preferential affinity for $CB_1$ receptors (Howlett *et al.*, 2002). It is synthesized on demand instead of being stored in synaptic vesicles, and is hydrolysed into arachidonic acid and ethanolamine by a membrane-bound enzyme called fatty acid amide hydrolase (FAAH) (Hohmann and Suplita, 2006). Research has shown that mice lacking the FAAH gene exhibited enhanced antinociceptive behaviour following exogenous administration of anandamide (Cravatt *et al.*, 2001). Some inhibitors of FAAH have been described, such as MAFP. MAFP reacts irreversibly with FAAH (Deutsch *et al.*, 1997), and thus causes potentiation of the responses induced by endocannabinoids (Ho and Randall, 2007).

The participation of opioids in antinociception induced by cannabinoids has been observed, and our group previously demonstrated, for the first time, the participation of cannabinoids in the peripheral antinociception induced by opioids (Pacheco *et al.*, 2008). Therefore, the aim of the present study was to determine whether endogenous cannabinoids are also involved in central antinociception induced by activation of μ-, δ- and κ-opioid receptors.

## Methods

*Animals*

The experiments were performed on 25–30 g male Swiss mice ($n = 5$ per group) from the CEBIO-UFMG (The Animal Centre of the University of Minas Gerais). The mice were housed in a temperature-controlled room (23 ± 1°C) on an automatic 12 h light/dark cycle (0600–1800 h light phase). All testing was performed during the light phase (0800–1500 h). Food and water were freely available until the onset of the experiments. The algesimetric protocol was approved by the Ethics Committee on Animal Experimentation (CETEA) of the Federal University of Minas Gerais (UFMG).

*Algesimetric method*

The tail-flick test used in the present study was a slight modification of the procedure described by D'Amour and Smith (1941). In brief, a heat source was applied to the tail of the mouse 2 cm from the tip, and the time (s) taken for the mouse to withdraw its tail from the heat source was described as the tail-flick latency. Heat intensity was adjusted so that the baseline latencies were between 3 and 4 s. To avoid tissue damage, a cut-off time was established at 9 s. The baseline latency was obtained for each mouse before drug administration (zero time) and determined from the average of three consecutive trials. To reduce stress, the mice were habituated to the apparatus 1 day prior to the experiments.

*i.c.v. Injections*

The mice were restrained by a special device, and the tops of their heads were then shaved. Next, drugs were injected into the lateral ventricle by i.c.v. route using a 5 μL Hamilton syringe. The injection site was 1 mm either side of the midline on a line drawn through the anterior base of the ears (modified from Haley and McCormick, 1957). The syringe was inserted perpendicularly through the skull into the brain to a depth of 2 mm, and 2 μL of solution was injected. To ascertain the areas in the brain ventricular system into which the drugs penetrated, the drugs were diluted in 0.5% Evans blue, and the brains were sectioned for confirmation after completion of the experiments.

*Experimental protocol*

SNC80 (δ-opioid agonist), morphine (μ-opioid agonist) and bremazocine (κ-opioid agonist) were administered i.c.v. into the lateral ventricle. Dose–response curves were obtained for all opioid receptor agonists to determine effective doses for

this study (data not shown). AM251 ($CB_1$ receptor antagonist) and AM630 ($CB_2$ receptor antagonist) were injected i.c.v. 1 min prior to the opioid agonists. MAFP (anandamide amidase inhibitor) was administered by the i.c.v. route 1 min prior to the morphine.

The nociceptive threshold was always measured in the tail of the mouse. The protocol was assessed in pilot experiments to determine the best moment for the injection of each substance. In such experiments, the animals were measured at intervals of 5 min until the optimum effect for the test substance was observed.

*Statistical analysis*

Data were analysed statistically by one-way analysis of variance with *post hoc* Bonferroni test for multiple comparisons. Probabilities of less than 5% ($P < 0.05$) were considered to be statistically significant.

*Chemicals*

The following drugs and chemicals were used: morphine (Merck, Darmstadt, Germany); SNC80 (Tocris, Ellisville, MO, USA); bremazocine (RBI, Natick, MA, USA); AM251 (Tocris); AM630 (Tocris); and MAFP (Tocris). The drugs were dissolved as follows: morphine and bremazocine (saline); SNC80, AM251 and AM630 (20% DMSO in saline); MAFP (10% DMSO in saline), and injected in a volume of 2 µL into the lateral ventricle. The saline used for dilution of all drugs contained 0.5% Evans blue.

## Results

*Antagonism of morphine-induced antinociception by AM251*

Intracerebroventricular administration of the $CB_1$ receptor antagonist AM251 (2 and 4 µg) inhibited the morphine-induced central antinociception (5 µg) in a dose-dependent manner (Figure 1). The highest dose of AM251 did not alter the tail-flick latency.

*Effect of AM630 on morphine-induced antinociception*

The $CB_2$ receptor antagonist AM630 (2 and 4 µg) did not modify the central antinociception of morphine (5 µg; Figure 2). In addition, this drug had no significant effect on the nociceptive threshold in control mice.

*Effect of AM251 and AM630 on antinociception induced by SNC80 or bremazocine*

Neither AM251 (4 µg) nor AM630 (4 µg) reduced the central antinociceptive effect of SNC80 (4 µg; Figure 3A). In addition, AM251 (4 µg) and AM630 (4 µg) had no effect on the central antinociception induced by bremazocine (4 µg; Figure 3B).

*Increase of morphine-induced antinociception by MAFP*

MAFP (0.1 and 0.2 µg) administration progressively enhanced the antinociception induced by a low dose of morphine (2.5 µg; Figure 4). MAFP alone had no antinociceptive effect.

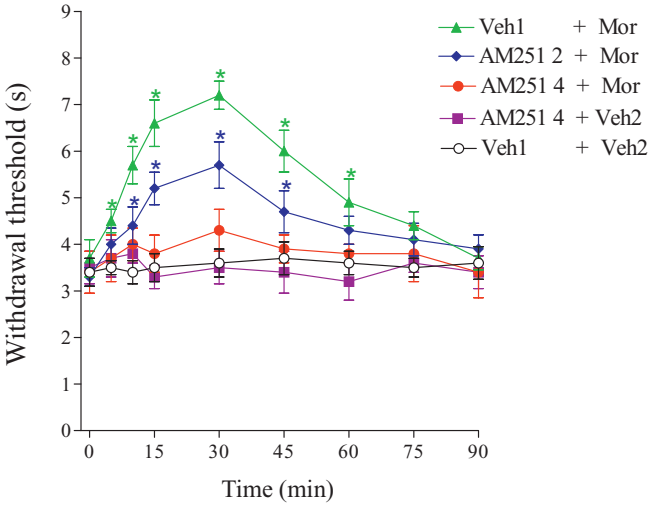


**Figure 1** Antagonism induced by i.c.v. administration of AM251 on the central antinociception produced by morphine. AM251 (2 and 4 µg) was administered 1 min prior to morphine (Mor, 5 µg). This antagonist did not significantly modify the nociceptive threshold in control mice. Each point represents the mean ± SEM for five mice per group. *Indicates a significant difference compared to Veh1 + Veh2-injected group (analysis of variance + Bonferroni test, $P < 0.05$). Veh1, vehicle1 (20% DMSO in saline); Veh2, vehicle2 (saline).

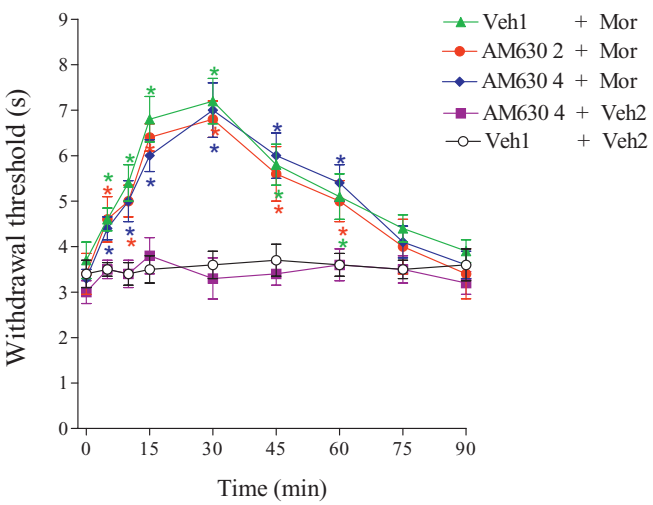


**Figure 2** Effect of i.c.v. administration of AM630 on the central antinociception produced by morphine. AM630 (2 and 4 µg) was administered 1 min prior to morphine (Mor, 5 µg). Each point represents the mean ± SEM for five mice per group. *Indicates a significant difference compared to Veh1 + Veh2-injected group (analysis of variance + Bonferroni test, $P < 0.05$). Veh1, vehicle1 (20% DMSO in saline); Veh2, vehicle2 (saline).

## Discussion

Recent papers have suggested the reciprocal importance of endogenous opioid and cannabinoid systems in both the acute and chronic effects of these two systems. Evidence exists that cannabinoid-induced antinociception may depend, to

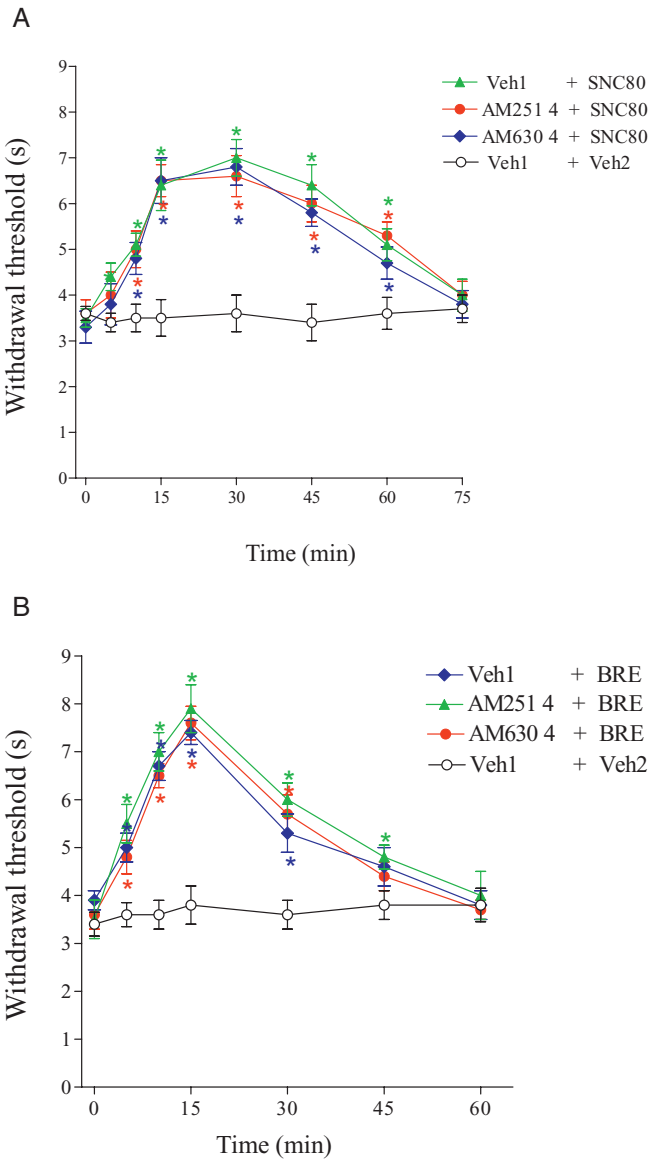


**Figure 3** Effect of i.c.v. administration of AM251 and AM630 on the central antinociception produced by SNC80 (A) or bremazocine (B). AM251 (4 μg) or AM630 (4 μg) was administered 1 min prior to SNC80 (4 μg) or bremazocine (BRE; 4 μg). Each point represents the mean ± SEM for five mice per group. *Indicates a significant difference compared to Veh1 + Veh2-injected group (analysis of variance + Bonferroni test, $P < 0.05$). Veh1, vehicle1 (20% DMSO in saline); Veh2, vehicle2 [20% DMSO in saline (A) or saline (B)].

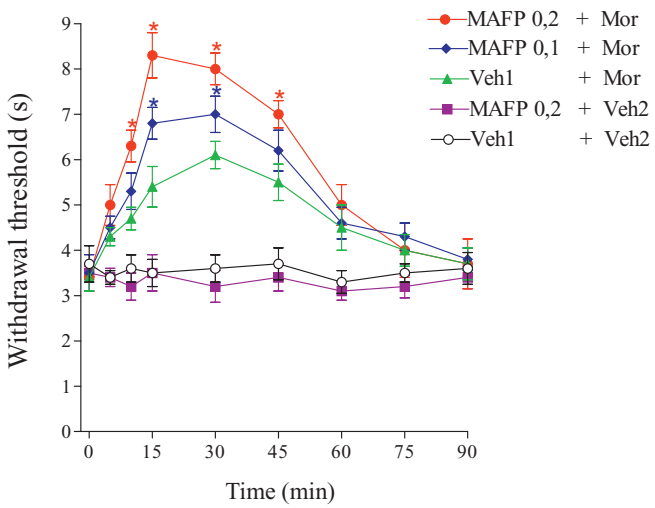


**Figure 4** Potentiation of morphine-induced antinociception by MAFP. The MAFP (0.1 and 0.2 μg) was administered 1 min prior to morphine (2.5 μg). This drug alone (0.2 μg) did not induce any effect. Each point represents the mean ± SEM for five mice per group. *Indicates a significant difference compared to Veh1 + Veh2-injected group (analysis of variance + Bonferroni test, $P < 0.05$). Veh1, vehicle1 (10% DMSO in saline); Veh2, vehicle 2 (saline).

some extent, on the release of opioid peptides (Reche *et al.*, 1996). Given the lack of information regarding the participation of endogenous cannabinoids in the analgesic mechanism of opioids, the present work used AM251 ($CB_1$ receptor antagonist) and AM630 ($CB_2$ receptor antagonist) to characterize the role of endocannabinoids in the central antinociception induced by opioids.

Initially, the ability of the μ-, δ- and κ-opioid receptor agonists morphine, SNC80 and bremazocine, respectively, to induce central antinociception in the tail-flick test was investigated. Analysis of the results showed that the agonists produced a central antinociceptive effect in a dose-dependent manner (data not shown), with doses of 5, 4 and 4 μg of morphine, SNC80 and bremazocine, respectively, chosen for the present study.

AM251 was able to prevent the central antinociception induced by morphine, in a dose-dependent manner. Likewise, our group previously demonstrated that AM251 reversed the peripheral antinociception induced by morphine (Pacheco *et al.*, 2008). AM251 is a potent $CB_1$ receptor antagonist, 306-fold selective over $CB_2$ receptors (Gatley *et al.*, 1997; Lan *et al.*, 1999). High levels of the $CB_1$ receptor are expressed in the central nervous system (CNS), where cannabinoids act at presynaptic $CB_1$ receptors to elicit changes in the synaptic efficacy of neuronal circuits (Freund *et al.*, 2003). It has been verified that the activation of $CB_1$ receptors present at peripheral, spinal and supraspinal sites produced antinociceptive effects (Hohmann, 2002; Hohmann and Suplita, 2006). Other $CB_1$ antagonist compounds include SR141716A, AM281 and LY320135. It is noteworthy that although AM251 and AM281 are structurally very similar to SR141716A, and share its ability to block $CB_1$ receptors and to produce inverse cannabimimetic effects, several pharmacological differences between SR141716A and either or both AM251 and AM281 have unexpectedly been detected *in vitro*, for example in experiments with cardiovascular tissue (Pertwee, 2004) and with rat hippocampal slices (Hájos and Freund, 2002). In addition, SR141716A was reported to increase the synaptic concentration of biogenic amines, that is, enhance the synaptic availability of the monoamine neurotransmitters noradrenalin, 5-hydroxytryptamine and dopamine in the brain (Witkin *et al.*, 2005). AM251, in contrast to SR141716A, is devoid of vanilloid activity and is more selective for the $CB_1$ receptor [*Ki* ($CB_1$ vs. $CB_2$) = 7.5 nM vs. >2 μM] than SR141716A [*Ki* ($CB_1$ vs. $CB_2$) = 5.6 nM vs. >1 μM] (Hohmann and Suplita, 2006).

The interaction between cannabinoids and opioids has been extensively studied, and numerous authors have

reported that cannabinoids enhance the antinociception of morphine through the release of opioid peptides, for example: the cannabinoid $\Delta^9$-THC produced an increase in morphine antinociception by dynorphin A release (Welch and Eads, 1999); another study demonstrated that naloxone blocked the synergistic antinociception produced by low oral doses of $\Delta^9$-THC and morphine, indicating the involvement of the μ receptor in this effect (Cichewicz *et al.*, 1999); and the potentiation between morphine and $\Delta^9$-THC was reversed by the μ-opioid receptor antagonist β-funaltrexamine, administered by i.c.v. route. The authors of the last study speculated that both cannabinoid and μ-supraspinal opioid receptors activate similar descending inhibitory pathways regulating the release of neurotransmitters involved in nociceptive transmission at a spinal level. Thus, a combination of both $\Delta^9$-THC and morphine may result in the sequential activation of spinal and supraspinal mechanisms, leading to antinociception (Reche *et al.*, 1996). It has also been found that pretreatment with cannabinoids enhances the antinociceptive effect of a micro-injection of morphine into the ventral PAG, suggesting that alternating opioid and cannabinoid treatment could be therapeutically advantageous by preventing the development of tolerance and enhancing morphine antinociception (Wilson *et al.*, 2008). Recently, it was suggested that $CB_1$ and μ-opioid receptors form heterodimers (Rios *et al.*, 2006). Heterodimer formation is needed for the function of certain G-protein-coupled receptors, such as the $GABA_B$ receptor (Ong and Kerr, 2000).

The $CB_2$ receptor antagonist AM630 did not block the central antinociception induced by morphine. AM630 is a $CB_2$ ligand 165-fold selective over $CB_1$ receptors (Ross *et al.*, 1999). The $CB_2$ receptor is primarily located on immune cells in the periphery (Galiègue *et al.*, 1995), although studies have demonstrated the presence of $CB_2$ receptors in a number of brain regions, contrary to the prevailing view that they are restricted to peripheral tissues (Sickle *et al.*, 2005; Gong *et al.*, 2006; Onaivi *et al.*, 2006). It has also been suggested that supraspinal $CB_2$ in the thalamus may contribute to the modulation of neuropathic pain responses (Jhaveri *et al.*, 2006). However, the $CB_2$ receptor protein has not been located on central neurones, and the effects of endocannabinoids in the brain have always been attributed to an action at $CB_1$ receptors. Moreover, many studies have shown that $CB_2$ receptor-selective agonists produce peripheral antinociception, but do not cause CNS effects produced by non-selective cannabinoid receptor agonists, suggesting that selective activation of $CB_2$ receptors may achieve the goal of peripheral pain relief without CNS effects (Malan *et al.*, 2001). These receptors have not been found on peripheral neurones, suggesting that the activation of $CB_2$ receptors produces antinociception indirectly, by causing the release of mediators from non-neuronal cells that alter the responsiveness of primary afferent neurones to noxious stimuli. One cell type that might mediate the actions of $CB_2$ receptor-selective agonists is the keratinocytes, which have been reported to express $CB_2$ receptors (Casanova *et al.*, 2003) and to contain endogenous opioid peptides (Kauser *et al.*, 2003). It has been demonstrated that antinociception produced by $CB_2$ receptor-selective agonists may be mediated by the stimulation of β-endorphin release from cells expressing $CB_2$ receptors. The β-endorphin released thus appears to act at μ-opioid receptors, probably on the terminals of primary afferent neurones, to produce peripheral antinociception (Ibrahim *et al.*, 2005).

Several putative endocannabinoids have been isolated in the brain, including anandamide, 2-arachidonoylglycerol (2-AG), noladin ether, virodhamine and *N*-arachidonoyl dopamine (NADA). Three of these five putative endocannabinoids, anandamide, 2-AG and NADA, are susceptible to degradation by FAAH. In order to confirm the participation of endocannabinoids in the central antinociceptive effects of morphine, MAFP was used, an irreversible inhibitor of FAAH. Additionally, the compound MAFP, which is commonly used to inhibit FAAH, has been found to be a potent inhibitor of monoacylglycerol lipase (MGL) activity (Dinh *et al.*, 2002). The crucial role of FAAH and MGL in the inactivation of anandamide suggests that inhibitors of these enzymes could be used to enhance endocannabinoid activity (Ho and Raldall, 2007). It was demonstrated that the combination of URB597 (inhibitor of FAAH) and anandamide produced maximal antinociception in the mouse tail-flick test versus either substance alone. The combination of URB597/anandamide was not active in $CB_1^{-/-}$ knock-out mice, but retained activity in $MOR^{-/-}$ knock-out mice. These data are the first to demonstrate that anandamide, if protected from degradation, acts via the $CB_1$ receptor to interact with the κ-opioid receptors to induce opioid-mediated analgesia (Haller *et al.*, 2008). On the other hand, the present results demonstrated that MAFP administration increased the central antinociception produced by a low dose of morphine (2.5 μg), suggesting that the activation of μ-opioid receptors releases cannabinoids. Anandamide is an agonist at $CB_1$ and $CB_2$ receptors, but presents greater affinity for $CB_1$ receptors (Howlett *et al.*, 2002), and the present work showed that the antinociceptive effect of morphine was completely reversed by the $CB_1$ receptor antagonist AM251. Only a few investigators have evaluated the role of the endocannabinoid systems in modulating opioid systems in behavioural studies. Cannabinoid receptor $CB_1^{-/-}$ knock-out mice are unable to learn to self-administer morphine, suggesting a reduction in morphine's reinforcing property in these mice (Cossu *et al.*, 2001). Morphine-induced place preference is also abolished in cannabinoid $CB_1^{-/-}$ receptor knock-out mice (Martin *et al.*, 2000).

Currently, the identification of endocannabinoids involved in pain modulation is obtained directly by microdialysis, and liquid and/or gas chromatography mass spectrometry (Cravatt *et al.*, 2001; Cravatt and Lichtman, 2002), and indirectly by administration of pharmacological agents that regulate endocannabinoid uptake or degradation (Hohmann and Suplita, 2006). The present study focused on the indirect approach.

In contrast to morphine, AM251 and AM630 did not exert an effect on the central antinociception induced by SNC80 or bremazocine at doses effective on morphine. Also, higher doses were tested without success. However, some studies have demonstrated that intrathecally administered cannabinoids evoke the release of endogenous opioids that stimulate δ- and κ-opioid receptors to produce antinociception (Welch, 1993; Pugh *et al.*, 1996). Other studies have shown that μ- and, particularly, κ-, but not δ-receptors, are involved in the antinociceptive action of $\Delta^9$-THC (Reche *et al.*, 1996). At

present, no studies showing the participation of cannabinoids in the activation of κ- and δ-opioid receptors exist.

In conclusion, the results presented in this paper do not show directly that μ-opioid receptor activation releases endocannabinoids, but the blockade of morphine-induced antinociception by $CB_1$ receptor antagonist and its potentiation by protection of anandamide degradation are strong evidence that the central antinociceptive effect of morphine is mediated by a cannabinoid system.

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## Conflict of interest

The authors state no conflict of interest.

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