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$GABA_B$ receptors modulate morphine antinociception: Pharmacological and genetic approaches

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ABSTRACT

Previous studies in our laboratory showed an interaction between the GABAergic and opioid systems involved in the analgesic effect of baclofen (BAC). Furthermore, it is known that sex differences exist regarding various pharmacological responses of morphine (MOR) and they are related to an increased sensitivity to MOR effects in males. The aims of the present study were to evaluate the possible involvement of the $GABA_B$ receptors in the antinociceptive responses induced by MOR (1, 3 and 9 mg/kg, s.c.) administration using both pharmacological (BAC 2 mg/kg, i.p.; and 2-OH-saclofen, SAC 0.3 mg/kg, intra cisterna magna) and genetic approaches ($GABA_{B1}$ knockout mice; $GABA_{B1}$ KO) in mice of both sexes. In addition, we explored the alterations in c-Fos expression of different brain areas involved in the antinociceptive effect of MOR using both approaches. The pharmacological approach showed a higher dose-dependent antinociceptive effect of MOR in male mice compared to female mice. BAC and SAC pretreatment potentiated and attenuated the antinociceptive effect of MOR, respectively, in both sexes. The genetic approach revealed a dose-dependent antinociceptive effect of MOR in the wild type mice, but not in the $GABA_{B1}$ KO mice and no sex differences were observed. Additionally, BAC and SAC pretreatment and the lack of $GABA_{B1}$ subunit of the $GABA_B$ receptor prevented the changes observed in c-Fos expression in the cingulate cortex and nucleus accumbens of male mice. Our results suggest that the $GABA_B$ receptors are involved in the MOR antinociceptive effect of both male and female mice.

1. Introduction

Global opioid deaths have increased in men and women from all social economic status and ages (Blum et al., 2018). In particular in the United States, the current opioid-epidemic deaths began in the 1990s and have claimed over 200,000 lives so far (Meldrum, 2016). Even though it is a multi-factorial phenomenon, alternative therapies for treating pain that reduces the incidence of addiction could help decrease opioid abuse and opioid-related deaths.

In previous studies we have shown an interaction between the GABAergic and opioid systems involved in the mechanism of action of the antinociceptive effect of baclofen (selective $GABA_B$ receptor agonist, BAC) (Balerio and Rubio, 2002). Similarly, clinical studies have demonstrated that morphine (μ-opioid receptor agonist, MOR) analgesia could be enhanced by BAC administration (Gordon et al., 1995) and patients who received intrathecal BAC after total knee arthroplasty, reported a significant less opioid use compared to the group of patients that only received opioids (Sanders et al., 2009). A possible explanation could be that the co-activation of opioid and $GABA_B$ receptors induced by exogenous or endogenous agents might produce an enhanced opioid analgesia compared to the analgesia produced by the activation of opioid receptors alone (Gordon et al., 1995). In addition, we have also observed that BAC pretreatment decreased the expression of MOR withdrawal syndrome in mice of either sex (Diaz et al., 2001), which is in agreement with previous results (Zarrindast and Mousa-Ahmadi, 1999). Finally, we have reported that BAC was able to re-establish the decreased dopamine levels during MOR withdrawal in male mice (Diaz et al., 2003). Together, these results suggest that the

Abbreviations: %MPE, maximal possible effect; Acb, nucleus accumbens; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; ANOVA, analysis of variance; BAC, baclofen; BLA, basolateral amygdala; CeA, central amygdala; Cg, cingulate cortex; i.p., intraperitoneal; i.c.m., intra cisterna magna; KO, knockout; MOR, morphine; n.s., non-significant; NAL, naloxone; PBS, phosphate buffered saline; PBS-T, phosphate buffered saline with triton; PCR, polymerase chain reaction; s.c., subcutaneous; SAC, 2-OH-saclofen; SAL, saline solution; SEM, standard error of the mean; WT, wild type

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combination of BAC and MOR for pain treatment could be a good alternative to achieve a successful analgesic treatment with the potential to reduce the negative consequences of opiate use.

MOR is commonly used to treat chronic and acute pain although it has the potential to cause addiction (Lee and Ho, 2013). Several preclinical studies have demonstrated that MOR antinociception tends to be greater in males compared to females in humans and other species (Cepeda and Carr, 2003; Craft et al., 2008; Kest et al., 2000b; Miller and Ernst, 2004). Even though it has been suggested that not all strains of mice show sex differences (Kest et al., 1999) the importance of including both sexes in preclinical studies has been addressed recently by Clayton and Collins (2014). Noteworthy, these sex differences in opiate antinociception are not due to the pharmacokinetics of MOR (Cicero et al., 1996; Cicero et al., 1997; Craft et al., 1996; Sarton et al., 2000). Several studies have demonstrated the role of circulating adult sex hormones in the presence of sex differences in MOR analgesia studies (Ali et al., 1995; Banerjee et al., 1983) but little is known about the organizational effect of sex hormones that could indicate structural brain differences between male and female mice that can be explored using prepubertal mice. In this way, the influence of adult sex hormones on MOR antinociception is avoided. Even though the mechanism of sex differences in MOR analgesia remains to be clarified, they are likely to be the result of differences in receptor density, binding and localization, as well as anatomical and physiological differences in the opiate-responsive neural circuits (Loyd and Murphy, 2006; Loyd et al., 2007, 2008; Loyd et al., 2008).

The early expression gene, c-Fos, is a transcription factor considered to be a marker of neuronal activity (Dragunow and Faull, 1989). It is known that addiction related behaviors are associated to different molecular adaptations, such as gene regulation, which are observed in specific brain areas (Berke and Hyman, 2000; Nestler, 2001). In line with this, several authors have shown that acute MOR (Ziolkowska et al., 2012), naloxone (μ-opioid receptor antagonist, NAL)-precipitated MOR withdrawal (Frenois et al., 2002; Georges et al., 2000; Gracy et al., 2001; Pedrón et al., 2013), MOR self-administration (Madsen et al., 2012) and MOR rewarding effects (Lasheras et al., 2015) alter Fos-like immunoreactivity in diverse brain regions. The interaction between opioid and GABAergic systems in analgesia has been well documented in the spinal cord, but there are only a few studies regarding the contribution of most of the rostral structures to the brainstem in opioid antinociception. Similarly, the role of the periaqueductal gray (PAG) and its connection with the rostral ventromedial medulla and the amygdala in MOR analgesia has been well documented (see Loyd and Murphy, 2014 for a review) but little is known about brain areas such as the central nucleus of the amygdala (CeA) and the cerebral cortex which have been implicated in the opioid induced antinociception (d'Amore et al., 1991; Finnegan et al., 2006; Manning and Mayer, 1995a; Manning and Mayer, 1995b; Matthies and Franklin, 1995). Previous studies have suggested a critical role for the basolateral amygdala (BLA) and CeA in the antinociceptive effect of opioids (Finnegan et al., 2005; Helmstetter et al., 1998; Tershner and Helmstetter, 2000; Zhu and Pan, 2004) BLA is involved in opioid analgesia through a projection to the CeA, which in turn activates the descending modulatory pathway (Helmstetter et al., 1998). Moreover, there is evidence that the activation of presynaptic μ-opioid receptors primarily attenuates GABAergic synaptic inputs to the CeA-projecting neurons in the BLA (Finnegan et al., 2005). Brain areas implicated in the rewarding properties of drugs of abuse, like the nucleus accumbens (Acb) have shown to have a role in pain modulation through an opioidergic mechanism (Gear and Levine, 1995). Interestingly, these authors have suggested that pain could be rewarding under certain conditions (Gear et al., 1999). The evidence suggests that the rostral forebrain could modulate nociception through a descending inhibitory mechanism. In addition, it has been proposed that MOR acting within the forebrain could alter the local processing of nociceptive messages and they are not perceived as painful (Cohen et al., 1984). Considering all these findings, we decided to explore the induction of c-Fos expression in areas that have been recently related to the antinociceptive effect of MOR. These areas have also been associated with the induction of MOR reward (Liu et al., 1994) and dependence (Georges et al., 2000) which is one of the most significant negative secondary effects induced by MOR treatment.

To our present knowledge, there are no previous studies regarding the effect of BAC or 2-OH-saclofen ($GABA_B$ receptor antagonist, SAC) pretreatment in MOR analgesia in male and female mice or assessing the effect of the lack of the $GABA_{B1}$ subunit of the $GABA_B$ receptor in MOR analgesia. Therefore, the aim of the present study was to explore the involvement of $GABA_B$ receptors in the possible alterations induced by MOR (antinociceptive responses and c-Fos expression), using both pharmacological (BAC and SAC) and genetic ($GABA_{B1}$ KO mice) approaches.

2. Experimental procedure

2.1. Animals

2.1.1. Pharmacological approach: in Swiss Webster albino mice

We used Swiss Webster prepubertal (indicated by vaginal smears) male and female mice obtained from Bioterio Central (Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Argentina) weighing 15–20 g and housed five per cage (n = 210). All animal were 23–25 days old at the beginning of the experiment. All experiments were performed with the investigators being blind to treatment conditions.

2.1.2. Genetic approach: in $GABA_{B1}$ knockout mice and their wild-type littermates

Mice lacking the $GABA_{B1}$ subunit of the $GABA_B$ receptor were generated at Dr. Bernhard Bettler's laboratory, Department of Physiology, University of Basel, Switzerland (Schuler et al., 2001). We have developed our own $GABA_{B1}$ KO mice colony in the Institute of Pharmacological Research (CONICET) located at the Faculty of Pharmacy and Biochemistry. The $GABA_{B1}$ KO mice and their wild-type littermates were obtained by intercrossing heterozygous animals (Balb/c strain). All mice were genotyped by polymerase chain reaction (PCR) analysis, as described previously (Schuler et al., 2001). Male and female prepubertal mice (23–25 days old) weighing 15–20 g were housed five per cage (n = 150). All the experiments were performed with the investigators being blind to genotype and treatment conditions.

2.1.3. Determination of estrous cycle stage

After each female mouse was challenged in the hot plate, a vaginal smear was obtained to determine the stage of the estrous cycle (Caligioni, 2009). Lavages were collected, placed onto glass slides and stained with methylene blue for observation under a light microscope. 98% of female mice were on anestrous (absence of cornified cells and a small amount of neutrophils), which indicated their prepubertal status. The remaining 2% was not taken into account for data analysis.

2.2. Care and handling conditions

Both groups of animals (pharmacological and genetic approaches) were acclimatized to the laboratory conditions according to local regulation (SENASA, 2002) (12-h light:12-h dark cycle, 21 ± 0.5 °C room temperature, 65 ± 10% humidity). Mice were handled and habituated to the injections four times a day for three days prior to the experiment in order to reduce stress. Habituation to the injection consisted in restraining the animals and emulating an injection pressure over the skin without using a needle. Food and water were available *ad libitum*. Behavioral tests and animal care were conducted following the standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609/EEC and 2001-486/EEC). All

experimental protocols were approved by the Animal Care Committee from the Faculty of Medicine, University of Buenos Aires (Res. CS n° 4081/04).

2.3. Drugs

MOR hydrochloride (Chemotecnica Sintyal, Buenos Aires, Argentina), NAL (Sigma, Aldrich, Saint Louise, Missouri), (±) BAC (Novartis, Basel, Switzerland) and SAC (Sigma Chemical Co., Buenos Aires, Argentina) were used in this study. MOR hydrochloride and BAC were dissolved in isotonic (NaCl 0.9%) saline solution (SAL) and administered subcutaneously (s.c.) and intraperitoneally (i.p.), respectively. The MOR dose refers to the salt form. SAC was dissolved in isotonic (5%) glucose solution immediately before use and administered intra cisterna magna (i.c.m.) because SAC does not cross the blood brain barrier. MOR and BAC were administered in a volume of 0.1 ml/10 g body weight of the animals. An i.c.m. injection was performed during short ether anesthesia. In this case, 10 µl of solution per mouse were injected slowly into the cisterna magna (Ueda et al., 1979). Different groups of drug-naïve animals were used for each experiment. The doses of MOR (1, 3 and 9 mg/kg, s.c.) were chosen based on a previous study by Celerier et al. (2003). The dose of BAC (2 mg/kg, i.p.) and SAC (0,3 mg/kg, i.c.m.) were chosen based on previous studies in our laboratory (Balerio and Rubio, 2002).

For the pharmacological approach, BAC (2 mg/kg) or SAL was administered i.p. 45 min before MOR (1, 3 and 9 mg/kg, s.c.) or SAL injection in Swiss Webster male and female mice. SAC (0.3 µg/kg) or glucose 5% was administered i.c.m. 10 min before MOR (1, 3 and 9 mg/kg, s.c.) or SAL injection in Swiss Webster mice. For the genetic approach, $GABA_{B1}$ KO male and female mice and their wild-type littermates were injected s.c. with MOR (1, 3 and 9 mg/kg) or SAL.

2.4. Locomotor activity

Locomotor responses to MOR (1, 3 and 9 mg/kg, s.c.) were evaluated by using a locomotor activity box (22 × 44 × 44 cm) (Infra Red ACTIMETER, Panlab, Spain). The box contained a line of photocells 2 cm above the floor to measure horizontal movements, and another line located 6 cm above the floor to measure vertical activity (rearing). Mice were individually placed in the box immediately after MOR or SAL injection without previous exposure to the box. The horizontal and vertical activity was recorded for a period of 15 min in a low-luminosity environment (5 lx).

2.5. Antinociceptive responses to morphine

Two different nociceptive tests were performed to evaluate the antinociceptive responses elicited by MOR: the tail-immersion and the hot-plate tests. The number of animals employed per sex per group for these experiments were 8–11 for the pharmacological approach and 6–13 for the genetic approach.

2.5.1. Tail-immersion

The tail-immersion test was conducted as previously described (Simonin et al., 1998), 15 min after MOR (1, 3 or 9 mg/kg, s.c.) or SAL administration. The water temperature was maintained at 50 ± 0.5 °C using a thermo-regulated water circuit-plating pump (Clifton, North Somerset UK). The trial was terminated once the animal flicked its tail and the baseline latencies were between 4 and 10 s. In the absence of tail-flick, a 30 s cut-off was used to prevent tissue damage. The control reading was the averaged response latency of a group of saline-treated mice. The analgesic responses obtained from the above mentioned method were calculated as the percentage of the maximum possible effect (MPE). The following formula (Harris and Pierson, 1964) was used for the calculation:

$$\%\mathrm{MPE} = \frac{\mathrm{Testreading(s)} - \mathrm{controlreading(s)}}{\mathrm{Cutofftime(s)} - \mathrm{controlreading(s)}} \times 100$$

2.5.2. Hot-plate

The hot-plate test was performed as previously described (Simonin et al., 1998), 16 min after MOR (1, 3 or 9 mg/kg, s.c.) or SAL injection. The heated surface of the plate was kept at a temperature of 52 ± 0.1 °C (Ugo Basile, Italy, Model-DS 37). The end point used was the licking of forepaws or a jumping response and the baseline latencies were between 3 and 8 s. In absence of paw-licking or jump, a 30 s cut-off was used to prevent tissue damage. The control reading was the averaged response latency of a group of saline-treated mice. The analgesic responses obtained according to the above method were calculated as the percentage of the MPE.

2.6. c-Fos experiments

2.6.1. Tissue preparation

Mice were deeply anesthetized 18 min after the MOR or SAL injection using a mixture of ketamine (70 mg/kg, Holliday-Scott S.A., Argentina) and xylazine (10 mg/kg, König, Argentina). They were then perfused transcardially with heparinized PBS (0.1 M phosphate buffered saline, pH 7.4), followed by a cold solution of 4% paraformaldehyde delivered with a peristaltic pump. Brains were removed, postfixed for 2 h in the same fixative, and cryoprotected overnight in a 30% sucrose solution. Coronal frozen sections were cut at 40 µm on a freezing microtome. They were collected in three serial groups of free-floating sections and stored at 4 °C. The number of animals per group was 3–4 for both experimental approaches.

2.6.2. c-Fos immunohistochemistry

The procedure was adapted from previously described protocols (Bester et al., 2001). All reactions were performed on floating sections agitated on a shaker. Sections from different experimental groups were processed in parallel to minimize variations in immunohistochemical labelling. Free-floating sections were rinsed in 0.1 M phosphate buffered saline with 0.15% Triton X-100 (PBS-T; pH 7.4) and then incubated with 3% hydrogen peroxide in PBS-T for a period of 30 min to remove endogenous peroxidase activity. After a second rinsing in PBS-T, sections were incubated for 30 min in 2% normal goat serum in PBS-T. Then, sections were incubated overnight in rabbit polyclonal antibody anti-c-Fos (SC-52; Santa Cruz Biotechnology, USA) (1:1000 in PBS 0.1 M, thimerosal 0.02%, normal goat serum 1%) at 4 °C. Sections were then rinsed and incubated for 2 h in goat anti-rabbit biotinylated antibody (Vector Laboratories, USA) (1:250 in PBS-T). After being rinsed, sections were incubated for 2 h in avidin-biotinylated horseradish peroxidase complex (1:125, ABC kit, Vector Laboratories). After successive washes in PBS-T and Tris buffer (0.25 M; pH 7.4), the antibody-antigen complex was developed with 0.05% 3,3′-diaminobenzidine (Sigma, USA) and 0.015% H_2O_2 in 20 ml Tris buffer (0.1 M; pH 7.4). Sections were mounted on gelatin-coated slides, dehydrated and coverslipped. Controls for the specificity of the primary antibody were performed in a previous study (Wada et al., 2010).

2.6.3. Data quantification

For quantitative analysis, cells positive for c-Fos immunoreactivity were identified by the presence of dense immunohistochemical staining within the nuclei, under a light microscope. Digital images of the selected sections were taken at 100× on a Nikon Microscope (Eclipse 55i) equipped with a digital camera (Nikon DS, Control Unit DS-L1).

For every area, the number of Fos-positive nuclei was counted within a grid under ImageJ 1.36 b, provided by National Institutes of Health, USA (public domain software). The counting was performed bilaterally in each brain area by an observer blind to drug treatment. These counts were averaged into a single score for each region of each

animal and finally the group mean ± standard error of the mean (SEM) was calculated. Fos-positive cells were quantified in the following brain regions, identified according to the anatomic atlas of Paxinos and Franklin (Paxinos and Franklin, 2004): nucleus accumbens shell (AcbSh) and core (AcbC), cingulate cortex (Cg) 1 and 2, caudate putamen, bed nucleus of the stria terminalis, the basolateral amygdaloid nucleus, central nucleus of the amygdale, dentate gyrus, and CA1 and CA3 areas of the hippocampus.

2.7. Statistical analysis

To determine differences between the experimental groups in behavioral and immunohistochemical responses for the pharmacological approach, data were analyzed with a three-way ANOVA with pretreatment, MOR dose and sex as main factors. In all cases, when a significant interaction between the three factors was observed, subsequent two-way ANOVAs and Tukey's *post hoc* test were applied. A three-way ANOVA was performed using sex, treatment and genotype as factors for the genetic approach. Calculations were performed using the SPSS 20.0 statistical package (SPSS Inc., Chicago, Illinois, USA). In all cases, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of MOR (1, 3 and 9 mg/kg, s.c.) in locomotor activity

We did not observe changes in locomotor activity in male and female mice compared to the control groups after the administration of MOR 1 or 3 mg/kg, s.c. However, we observed an increase in horizontal locomotor activity in both male and female mice compared to their control groups ($p < 0.05$ and $p < 0.01$, respectively) after MOR 9 mg/kg administration (Fig. 1). These results indicate that MOR administration does not induced sedation.

3.2. Pharmacological approach: in Swiss Webster albino mice

3.2.1. Effect of baclofen and 2-OH-saclofen on antinociceptive responses induced by morphine: behavioral changes

A three-way ANOVA conducted on the %MPE of the tail immersion test revealed a significant pretreatment × MOR dose × sex interaction [$F(4,72) = 7.73$, $p < 0.001$]. Taking this into account, we performed a two-way ANOVA with pretreatment and MOR dose as main factors. The statistical analysis is reported in Table 1 (two and one-way ANOVAs).

In male mice, MOR (1, 3 and 9 mg/kg, i.p.) showed a dose-response antinociceptive relationship in the tail immersion test ($p < 0.001$), respectively (Tukey's *post hoc*). In addition, *post hoc* test revealed that these effects were potentiated by BAC (2 mg/kg) when administered before MOR 3 ($p < 0.05$) and 9 mg/kg ($p < 0.01$) whereas these effects were blocked by SAC (0.3 mg/kg) pretreatment when administered before MOR at the dose 9 mg/kg ($p < 0.001$) (Fig. 2A).

In female mice, MOR (1, 3 and 9 mg/kg, i.p.) also showed a dose-response antinociceptive relationship in the tail immersion test ($p < 0.001$), respectively (Tukey's *post hoc*). These effects were not significantly altered by BAC pretreatment. Conversely, these effects were blocked by SAC (0.3 mg/kg) pretreatment when administered before MOR 3 (n.s.) and 9 mg/kg ($p < 0.001$) (Fig. 2A).

A three-way ANOVA conducted on the %MPE of the hot plate test revealed a significant pretreatment × MOR dose × sex interaction [$F(4,97) = 5.42$, $p < 0.001$]. In male mice, MOR (1, 3 and 9 mg/kg, i.p.) showed a dose-response antinociceptive relationship in the hot-plate test ($p < 0.001$), respectively (Tukey's *post hoc*). In addition, *post hoc* test showed that these effects were potentiated by BAC (2 mg/kg) when administered before MOR 3 ($p < 0.05$) and 9 mg/kg ($p < 0.001$) while the antinociceptive effect of MOR was blocked by SAC (0.3 mg/kg) when administered before MOR 9 mg/kg ($p < 0.05$) according to Tukey's *post hoc* test. (Fig. 2B).

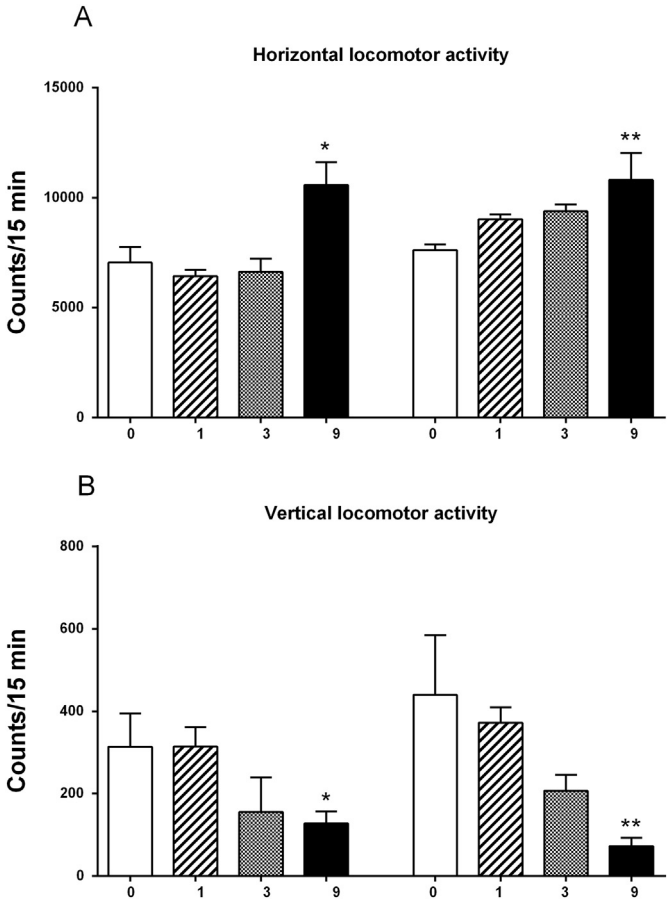

Fig. 1. Morphine (MOR) 9 mg/kg administration increased horizontal locomotor activity (A) and decreased vertical locomotor activity (B) in both male and female mice. Locomotor activity was measured for 15 min immediately after morphine (MOR; 1, 3 and 9 mg/kg; s.c.) or saline injection, respectively. Results are expressed as mean ± SEM (n = 8–11 mice for each sex/group) of the number of counts per 15 min. *$p < 0.05$, **$p < 0.01$, compared with the corresponding saline treated group.

In females, MOR (1, 3 and 9 mg/kg, i.p.) alone also showed a dose-response antinociceptive relationship in the hot plate test ($p < 0.05$), respectively (Tukey's *post hoc*). In addition, *post hoc* showed that these effects were potentiated by BAC (2 mg/kg) only when administered before MOR 9 mg/kg ($p < 0.05$). On the contrary, the antinociceptive effect of MOR was blocked by SAC (0.3 mg/kg) pretreatment when administered before MOR 3 (n.s.) and 9 mg/kg (n.s.) (Fig. 2B).

3.2.2. Effect of baclofen and 2-OH-saclofen on antinociception induced by morphine: c-Fos expression

A three-way ANOVA conducted on the %MPE of the hot plate revealed a pretreatment × MOR dose interaction in Cg [$F(6,65) = 2.92$, $p < 0.05$] and AcbSh [$F(6,64) = 2.19$, $p < 0.05$]; a pretreatment × sex interaction in Cg [$F(3,65) = 2.94$, $p < 0.05$] and AcbSh [$F(3,64) = 2.85$, $p < 0.05$] and a MOR dose × sex interaction in Cg [$F(3,65) = 3.50$, $p < 0.05$] and AcbSh [$F(3,64) = 3.79$, $p < 0.05$]. We did not observe significant pretreatment × MOR dose × sex interactions in any the areas studied. The statistical analysis is reported in Table 2 (two and one-way ANOVAs).

Since we did not observe changes in c-Fos expression after the administration of MOR 1 and 3 mg/kg (data not shown), we only describe herein the results corresponding to the c-Fos expression induced by MOR 9 mg/kg (Table 2).

In male mice, Tukey's *post hoc* showed that MOR 9 mg/kg reduced the number of c-Fos positive nuclei in the Cg ($p < 0.001$), AcbC

Table 1
Effect of sex and BAC or SAC pretreatment on the antinociceptive responses to MOR.

	Two way ANOVA						One way ANOVA			
	Treatment		Sex		Interaction		Males		Females	
	F value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value
BAC										
Hot plate	$F(5, 72) = 43.84$	< 0.001	$F(1, 72) = 16.04$	< 0.001	$F(5, 72) = 4.95$	$= 0.001$	$F(5, 41) = 36.11$	< 0.001	$F(5, 41) = 11.95$	< 0.001
Tail immersion	$F(5, 72) = 43.45$	< 0.001	$F(1, 72) = 5.34$	< 0.05	$F(5, 72) = 2.62$	< 0.05	$F(5, 41) = 32.84$	< 0.001	$F(5, 41) = 12.70$	< 0.001
SAC										
Hot plate	$F(5, 92) = 10.84$	< 0.001	$F(1, 92) = 4.61$	< 0.05	$F(5, 92) = 3.70$	< 0.01	$F(5, 54) = 17.87$	< 0.001	$F(5, 51) = 5.03$	< 0.001
Tail immersion	$F(5, 73) = 21.46$	< 0.001	$F(1, 73) = 0.01$	n.s.	$F(5, 73) = 0.78$	n.s.	$F(5, 41) = 15.97$	< 0.001	$F(5, 42) = 7.15$	< 0.001

Two-way ANOVA with drug treatment and sex as main factors. When an interaction was observed, one-way ANOVA for males and females followed by Tukey's *post hoc* was performed ($n = 8$–11).

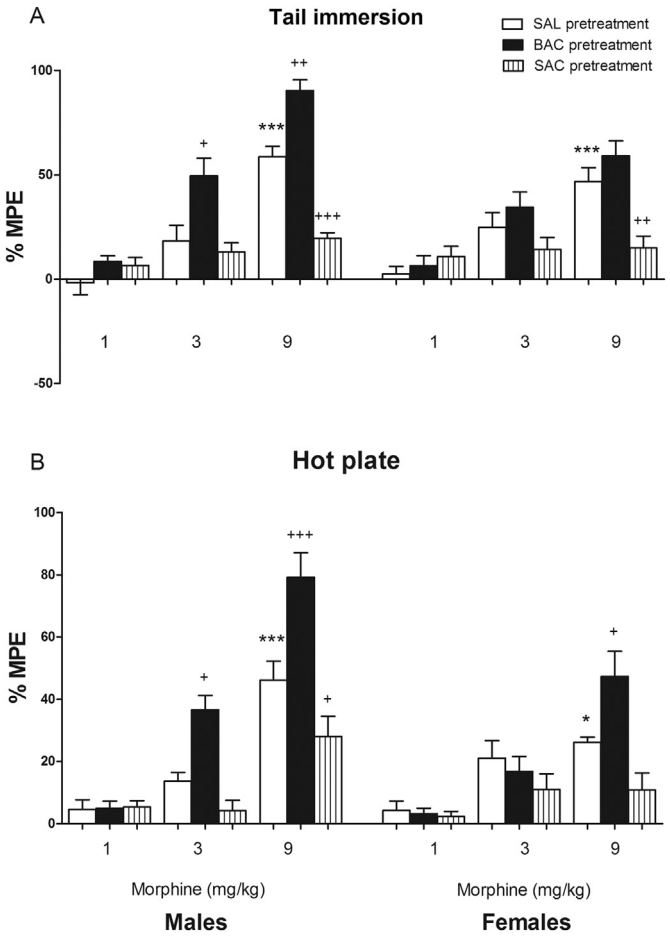

Fig. 2. Baclofen (BAC) (2 mg/kg, i.p.) potentiated the antinociceptive responses induced by morphine while 2-OH-saclofen (SAC) (0.3 mg/kg, i.c.m.) blocked these responses in male and female mice. Antinociceptive responses in the tail immersion (A) and the hot plate (B) tests were measured 15 and 16 min after morphine (MOR; 1, 3 and 9 mg/kg; s.c.) or saline injection, respectively. BAC or vehicle (SAL) was injected 45 min before MOR or vehicle injection. SAC or vehicle (glucose) was injected 10 min before morphine or vehicle injection. Results are expressed as mean ± SEM ($n = 8$–11 mice for each sex/group) of the percentage of maximal possible effect (%MPE). Bars represent vehicle pretreated (white bars), BAC pretreated (black bars) and SAC pretreated male and female mice (striped bars). $^{*}p < 0.05$, $^{***}p < 0.001$, compared with saline pretreated groups administered with lower dose of MOR; $+p < 0.05$; $++p < 0.01$; $+++p < 0.001$ compared with saline pretreated groups administered with the same dose of MOR.

($p < 0.05$) and AcbSh ($p < 0.05$). BAC pretreatment was able to prevent this reduction only in the Cg ($p < 0.05$). SAC pretreatment reestablished the decrease in the number of c-Fos positive nuclei in the AcbC and AcbSh induced by MOR 9 mg/kg administration but it had no effect in c-Fos positive nuclei reduction observed in the Cg after MOR 9 mg/kg administration (Fig. 3).

In female mice, MOR 9 mg/kg decreased the number of c-Fos positive nuclei ($p < 0.01$) in the AcbSh only and neither BAC nor SAC pretreatment were able to prevent these responses (Fig. 3).

On the other hand, we did not observe any changes in c-Fos expression in the other brain areas studied of both male and female mice (Table 2).

3.3. Genetic approach: in Balb/c $GABA_{B1}$ knockout mice and their wild-type littermates

3.3.1. Antinociceptive responses induced by morphine: behavioral changes

A three-way ANOVA conducted on the %MPE of the tail immersion test revealed a significant genotype × group interaction [$F(2,97) = 18.10$, $p < 0.001$] but there was no significant sex × genotype × group interaction [$F(2,97) = 0.768$, $p = 0.467$]. Taking this into account, we performed a two-way ANOVA with genotype and treatment as main factors. The statistical analysis is reported in Table 3 (two and one-way ANOVAs). In male mice, MOR (1, 3 and 9 mg/kg, i.p.) showed a dose-response relationship in the tail immersion in WT mice. MOR 3 and 9 mg/kg administration showed a significant lower % MPE ($p < 0.01$, $p < 0.001$, respectively) in $GABA_{B1}$ KO mice compared with their WT littermates. In female mice, MOR (1, 3 and 9 mg/kg, i.p.) alone also showed a dose-response relationship in the hot plate test. MOR 3 and 9 mg/kg administration showed significant lower % MPEs ($p < 0.01$ and $p < 0.001$, respectively) in $GABA_{B1}$ KO female mice compared with their WT littermates (Fig. 4A).

A three-way ANOVA conducted on the %MPE in the hot plate revealed a significant effect of sex [$F(1,97) = 9.94$, $p < 0.01$], a genotype × treatment interaction [$F(2,97) = 5.45$, $p < 0.001$] but there was no significant sex × genotype × treatment interaction [$F(2,97) = 0.431$, $p = 0.651$].

In wild type (WT) male mice, MOR 1, 3 and 9 mg/kg showed a dose-response relationship in the hot plate test. After MOR 3 mg/kg administration, $GABA_{B1}$ KO mice had a significant lower %MPE compared with their WT littermates ($p < 0.05$). In female mice, MOR 1, 3 and 9 mg/kg also showed a dose-response relationship in the hot plate test. After MOR 3 mg/kg administration, $GABA_{B1}$ KO mice showed a significant lower %MPE, compared to their WT littermates ($p < 0.05$) (Fig. 4B).

3.3.2. Antinociceptive responses induced by morphine: c-Fos expression

A three-way ANOVA conducted on c-Fos expression revealed a significant treatment × sex interaction in Cg [$F(3,43) = 11.60$, $p < 0.001$] and AcbC [$F(3,43) = 2.38$, $p < 0.05$] and a

Table 2
Effect of sex and BAC or SAC pretreatment on c-Fos expression in different brain areas after MOR administration.

Brain region	Two-way ANOVA						One-way ANOVA, *post hoc* Tukey			
	Treatment		Sex		Interaction		Males		Females	
	F	*p* value	*F*	*p* value	*F*	*p* value	*F*	*p* value	*F*	*p* value
BAC										
Cingulate cortex	$F_{(7, 40)} = 4.44$	< 0.001	$F_{(1, 40)} = 16.28$	< 0.001	$F_{(7, 40)} = 2.60$	< 0.05	$F_{(7, 20)} = 5.59$	< 0.001	$F_{(7, 20)} = 1.15$	n.s.
Caudate putamen	$F_{(7, 40)} = 3.37$	n.s.	$F_{(1, 37)} = 1.08$	n.s.	$F_{(7, 37)} = 7.31$	< 0.001	$F_{(7, 17)} = 16.16$	< 0.001	$F_{(7, 20)} = 1.16$	n.s.
Nucleus accumbens core	$F_{(7, 39)} = 4.86$	< 0.001	$F_{(1, 39)} = 10.53$	< 0.001	$F_{(7, 39)} = 1.86$	< 0.05	$F_{(7, 20)} = 5.73$	< 0.001	$F_{(7, 19)} = 1.80$	n.s.
Nucleus accumbens shell	$F_{(7, 39)} = 3.37$	< 0.01	$F_{(1, 39)} = 0.21$	n.s.	$F_{(7, 39)} = 2.81$	< 0.01	$F_{(7, 20)} = 3.78$	< 0.01	$F_{(7, 29)} = 2.70$	< 0.05
Bed nucleus of the stria terminalis	$F_{(7, 53)} = 3.50$	< 0.001	$F_{(1, 53)} = 0.11$	n.s.	$F_{(7, 53)} = 0.45$	n.s.	–		–	
Basolateral amygdala	$F_{(7, 39)} = 1.39$	n.s.	$F_{(1, 39)} = 7.68$	< 0.01	$F_{(7, 39)} = 1.03$	n.s.	–		–	
Central amygdala	$F_{(7, 40)} = 1.80$	n.s.	$F_{(1, 40)} = 5.45$	< 0.01	$F_{(7, 40)} = 0.57$	n.s.	–		–	
Dentate gyrus	$F_{(7, 54)} = 0.80$	n.s.	$F_{(1, 54)} = 4.53$	< 0.05	$F_{(7, 54)} = 1.48$	n.s.	–		–	
CA3	$F_{(7, 46)} = 1.29$	n.s.	$F_{(1, 46)} = 0.21$	n.s.	$F_{(7, 46)} = 1.35$	n.s.	–		–	
CA1	$F_{(7, 46)} = 1.26$	n.s.	$F_{(1, 46)} = 0.81$	n.s.	$F_{(7, 46)} = 0.56$	n.s.	–		–	
SAC										
Cingulate cortex	$F_{(8, 46)} = 5.20$	< 0.001	$F_{(1, 46)} = 5.00$	< 0.05	$F_{(8, 46)} = 2.53$	< 0.05	$F_{(8, 14)} = 4.80$	< 0.01	$F_{(8, 15)} = 1.84$	n.s.
Caudate putamen	$F_{(8, 46)} = 2.05$	n.s.	$F_{(1, 46)} = 2.45$	n.s.	$F_{(8, 46)} = 1.24$	n.s.	–		–	
Nucleus accumbens core	$F_{(8, 45)} = 5.18$	< 0.001	$F_{(1, 45)} = 5.37$	< 0.05	$F_{(8, 45)} = 1.92$	n.s.	–		–	
Nucleus accumbens shell	$F_{(8, 45)} = 4.59$	< 0.001	$F_{(1, 45)} = 3.19$	n.s.	$F_{(8, 45)} = 3.67$	< 0.01	$F_{(8, 22)} = 3.61$	< 0.5	$F_{(8, 22)} = 3.61$	< 0.01
Bed nucleus of the stria terminalis	$F_{(8, 47)} = 5.19$	< 0.001	$F_{(1, 47)} = 1,13$	n.s.	$F_{(8, 47)} = 1,44$	n.s.	–		–	
Basolateral amygdala	$F_{(8, 45)} = 1.46$	n.s.	$F_{(1, 45)} = 1.90$	n.s.	$F_{(8, 45)} = 0.90$	n.s.	–		–	
Central amygdala	$F_{(8, 45)} = 2.33$	n.s.	$F_{(1, 45)} = 1.17$	n.s.	$F_{(8, 45)} = 0.68$	n.s.	–		–	
Dentate gyrus	$F_{(8, 54)} = 0.98$	n.s.	$F_{(1, 54)} = 1.14$	n.s.	$F_{(8, 54)} = 1.53$	n.s.	–			
CA3	$F_{(8, 49)} = 1.02$	n.s.	$F_{(1, 49)} = 0.05$	n.s.	$F_{(8, 49)} = 1.14$	n.s.				
CA1	$F_{(8, 49)} = 1.53$	n.s.	$F_{(1, 49)} = 1.53$	n.s.	$F_{(8, 49)} = 0.55$	n.s.				

Two-way ANOVA with drug treatment and sex as main factors. When an interaction was observed, one-way ANOVA for males and females followed by Tukey's *post hoc* was performed (n = 3–4).

genotype × treatment interaction in Cg [$F(3,43) = 4.04$, $p < 0.05$]. We only observed a significant sex × genotype × group interaction in the AcbC [$F(3,43) = 7.61$, $p < 0.001$]. We performed a two-way ANOVA with genotype and treatment as main factors. The statistical analysis is reported in Table 4 (two and one-way ANOVAs).

In male mice, Tukey's *post hoc* showed that MOR 9 mg/kg increased the number of c-Fos positive nuclei in the Cg ($p < 0.05$) and the AcbC ($p < 0.05$) but not in the AcbSh ($p < 0.05$) of WT male mice. Instead, in the $GABA_{B1}$ KO mice, the administration of MOR 9 mg/kg decreased de number of c-Fos positive nuclei in the Cg ($p < 0.01$) and in the AcbC ($p < 0.001$) and had no effect in the AcbSh. There were no significant differences between experimental groups in female mice (Fig. 5).

4. Discussion

In the present study, the pharmacological approach revealed more antinociceptive responses of MOR in male than in female mice and that BAC pretreatment potentiated these responses in male mice. In female mice, BAC pretreatment only potentiated the antinociceptive response in the hot plate after MOR 9 mg/kg. In addition, SAC pretreatment attenuated the antinociceptive response of MOR in both male and female mice. Likewise, the genetic approach showed that the lack of $GABA_{B1}$ subunit of the $GABA_B$ receptor blocked the antinociceptive responses induced by MOR administration in both sexes. On the other hand, BAC was able to prevent the decrease in c-Fos expression induced by MOR administration in the Cg of male, but not in female mice. Moreover, SAC was able to prevent the decrease in c-Fos expression observed in the AcbC and the AcbSh after MOR administration in male, but was not able in female mice. The lack of the $GABA_{B1}$ subunit of the $GABA_B$ receptor prevented the increase in the number of c-Fos positive nuclei observed in the Cg and AcbC of wild-type male mice after MOR administration.

MOR antinociceptive responses were evaluated in the tail immersion and hot-plate, two tests with different neuronal pathways involved in the processing of nociceptive signals. The tail-immersion mainly evokes a response mediated by a spinal reflex (Caggiula et al., 1995), whereas responses to the hot-plate require supraspinal integration of the nociceptive stimuli (Caggiula et al., 1995; Rubinstein et al., 1996). The pharmacological and genetic approaches showed that male and female mice displayed a dose-dependent antinociceptive response to MOR acute administration in both tests, as previously reported (Ahmadi et al., 2013; Cicero et al., 1996, 1997). Moreover, male mice displayed a greater antinociceptive response compared to female mice in agreement with previous studies (Cicero et al., 1996, 1997; Kest et al., 2000a; Loyd et al., 2007). After testing locomotor activity in male and female mice, we only observed an increase in locomotor activity in both male and female mice compared to their control groups after MOR 9 mg/kg administration. Our results are in agreement with a previous study by Celerier et al. (2003) which showed an increase in the ambulatory activity of mice after MOR 9 mg/kg administration. On the contrary, several studies have reported a decrease in locomotor activity or motor coordination (Craft et al., 2006; Shen et al., 2013). In the present study, we evaluated locomotor activity for 15 min immediately after MOR administration, accordingly to Celerier et al. (2003). Conversely, the studies that reported a sedative effect of MOR measured the locomotor activity 30 o 60 min after MOR administration (Craft et al., 2006; Shen et al., 2013). These protocol differences could explain contradictory results regarding locomotor activity. In conclusion, our results rule out the possibility that the antinociceptive responses observed in the present study were caused by a sedative effect of MOR. In adult rodents, sex differences regarding MOR analgesia have extensively been studied (Cicero et al., 1996, 1997) and adult sex hormones have shown to play an important role in the pharmacological effects of MOR (see Loyd and Murphy, 2014 for review). Previous studies using prepubertal mice showed that male and female mice respond differently to MOR acute administration (Sternberg et al., 2004), suggesting that sex differences are present before adult sex hormones start circulating. Furthermore, a previous study reported that the sex-related differences observed in MOR antinociceptive activity cannot be explained by differences in the pharmacokinetics of MOR, suggesting that sex differences in MOR-induced antinociception could be related to inherent differences in the brain sensitivity to MOR (Cicero et al., 1997). In this context, Cicero et al. (1996) hypothesized that the number or affinity of the opiate receptors involved in antinociceptive responses or the biochemical

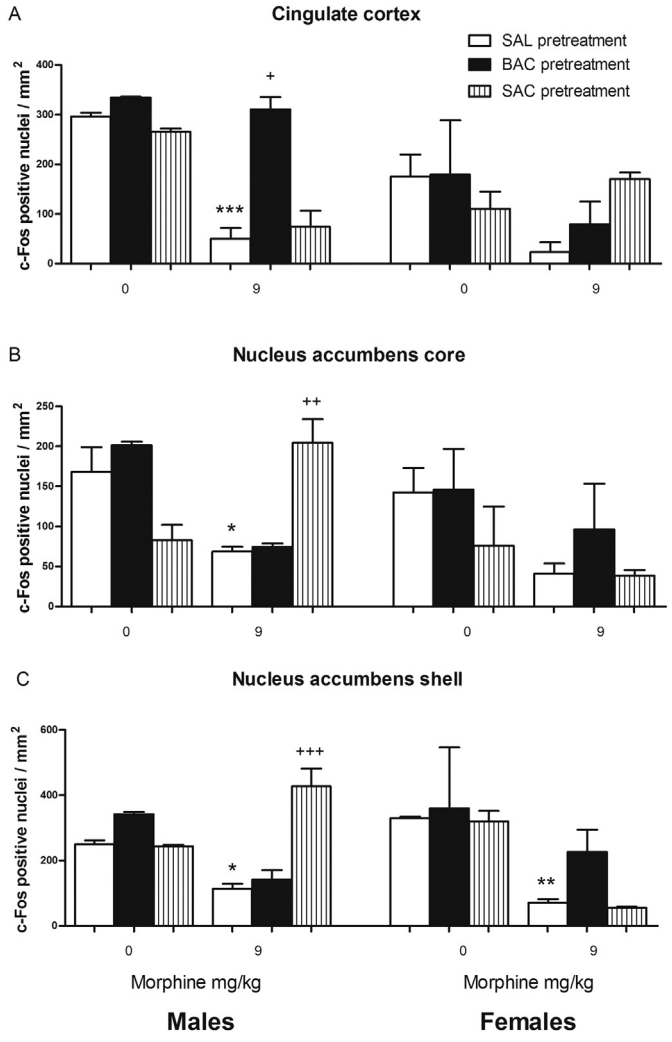

Fig. 3. Baclofen (BAC) 2 mg/kg i.p. was able to prevent the reduction in c-Fos expression induced by morphine (MOR) 9 mg/kg s.c. administration observed in the cingulate cortex (Cg) (A) but not in the nucleus accumbens core (AcbC) (B) and shell (AcbSh) (C) of male mice. 2-OH-saclofen (SAC) 0,3 mg/kg i.c.m. was able to prevent the reduction in c-Fos expression observed in the nucleus accumbens core (AcbC) (B) and shell (AcbSh) (C) but not in the cingulate cortex (Cg) (A) induced by morphine (MOR) 9 mg/kg s.c. administration in male mice. In female mice, neither BAC nor SAC administration were able to prevent the reduction in c-Fos expression observed in the AcbSh (C) of female mice after MOR 9 mg/kg, s.c. administration. Results are expressed as mean ± SEM (n = 3–4 mice for each group) of number of c-Fos positive nuclei/mm^2. Bars represent vehicle pretreated (white bars), BAC pretreated (black bars) and SAC pretreated (striped bars) male and female mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared to saline control group; +$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$, compared to saline pretreated group. Only the photographs of sections from male mice are shown.

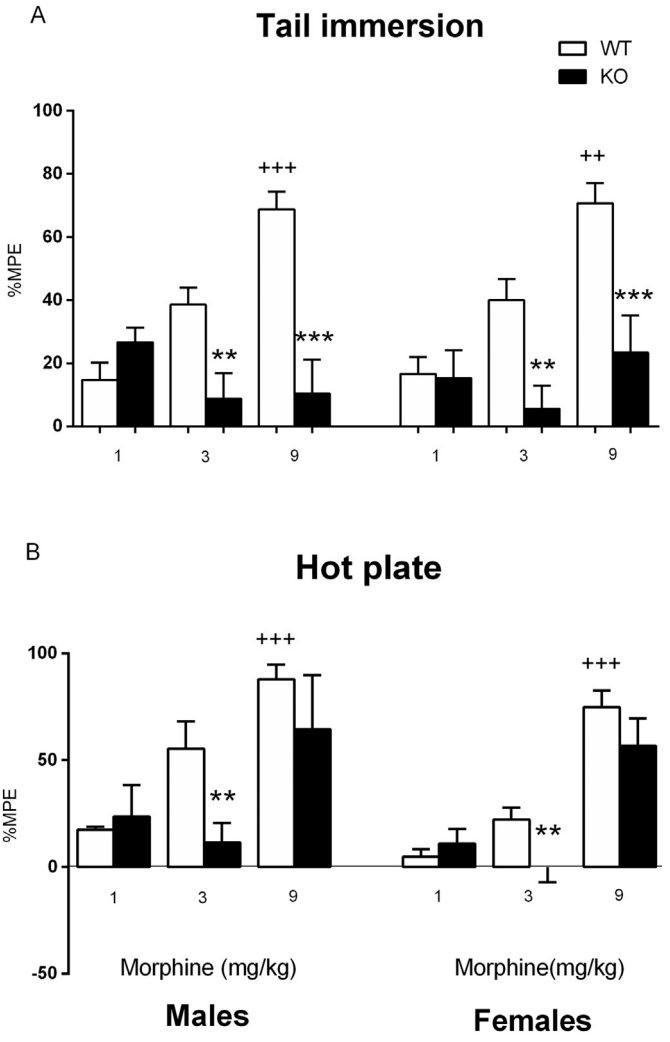

Fig. 4. Morphine (MOR) antinociception was abolished in $GABA_{B1}$ KO mice. Antinociceptive responses in the tail-immersion (A) and hot-plate (B) test were measured 15 and 16 min respectively after MOR administration (1, 3 and 9 mg/kg, s.c.). Results are expressed as mean ± SEM of latency time (in seconds) in wild-type (WT) (white bars) (n = 12–13) and $GABA_{B1}$ knockout (KO) (black bars) (n = 6–8) mice. Statistical analysis was performed using two-way ANOVA with treatment (between subjects) and genotype (between subjects) as factors of variation, followed by corresponding one-way ANOVA and *post hoc* comparisons using the Tukey test. ++$p < 0.01$, +++$p < 0.001$, compared to SAL-MOR 1 mg/kg group; **$p < 0.01$, ***$p < 0.001$, compared to wild-type mice.

reactions induced by receptor occupancy might be sex dependent. This could be the result of an organizational hormone effect in brain morphology and neurobiology (Breedlove, 1992, 1994). In fact, early studies suggested large sex-linked differences in the number and regional

Table 3
Effect of genotype and treatment on the antinociceptive responses to MOR.

	Two way ANOVA						One way ANOVA			
	Treatment		Genotype		Interaction		WT		KO	
	F value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value
Hot plate	$F(2, 82) = 46.72$	< 0.001	$F(1, 82) = 8.10$	< 0.01	$F(2, 82) = 4.95$	< 0.05	$F(2, 58) = 54.55$	< 0.001	$F(2, 24) = 13.43$	< 0.001
Tail immersion	$F(2, 94) = 12.78$	< 0.001	$F(1, 94) = 36.95$	< 0.001	$F(2, 94) = 13.2$	< 0.001	$F(2, 70) = 45.77$	< 0.001	$F(2, 24) = 1.07$	n.s.

Two-way ANOVA with treatment and genotype as main factors. When an interaction was observed, one-way ANOVA for WT and KO followed by Tukey's *post hoc* was performed (n = 6–13).

Table 4
Effect of treatment and genotype on c-Fos expression in different brain areas after MOR administration in male and female mice.

Brain region	Two-way ANOVA						One-way ANOVA, *post hoc* Tukey			
	Treatment		Genotype		Interaction		WT		KO	
	F	*p* value	*F*	*p* value	*F*	*p* value	*F*	*p* value	*F*	*p* value
Males										
Cingulate cortex	$F_{(3,\ 12)} = 6.80$	< 0.01	$F_{(1,\ 12)} = 10.62$	< 0.01	$F_{(3,\ 12)} = 4.09$	< 0.05	$F_{(3,\ 9)} = 3.26$	n.s.	$F_{(3,\ 9)} = 14.25$	< 0.01
Caudate putamen	$F_{(3,\ 12)} = 7.11$	< 0.05	$F_{(1,\ 12)} = 0.85$	n.s.	$F_{(3,\ 12)} = 4.01$	n.s.	–		–	
Nucleus accumbens core	$F_{(3,\ 12)} = 4.07$	< 0.05	$F_{(1,\ 12)} = 0.2$	n.s.	$F_{(3,\ 12)} = 7.19$	< 0.01	$F_{(3,\ 9)} = 5.73$	n.s.	$F_{(3,\ 9)} = 10.83$	< 0.01
Nucleus accumbens shell	$F_{(3,\ 12)} = 1.03$	n.s.	$F_{(1,\ 12)} = 3.08$	n.s.	$F_{(3,\ 12)} = 2.78$	n.s.	–		–	
Bed nucleus of the stria terminalis	$F_{(3,\ 12)} = 0.78$	n.s.	$F_{(1,\ 12)} = 2.02$	n.s.	$F_{(3,\ 12)} = 0.45$	n.s.	–		–	
Basolateral amygdala	$F_{(3,\ 12)} = 1.30$	n.s.	$F_{(1,\ 12)} = 7.68$	< 0.01	$F_{(3,\ 12)} = 1.03$	n.s.	–		–	
Central amygdala	$F_{(3,\ 12)} = 1.02$	n.s.	$F_{(1,\ 12)} = 5.45$	< 0.01	$F_{(3,\ 12)} = 0.57$	n.s.	–		–	
Dentate gyrus	$F_{(3,\ 12)} = 4.54$	< 0.05	$F_{(1,\ 12)} = 9.38$	< 0.01	$F_{(3,\ 12)} = 2.53$	n.s.	–		–	
CA3	$F_{(3,\ 12)} = 1.35$	n.s.	$F_{(1,\ 12)} = 0.37$	n.s.	$F_{(3,\ 12)} = 1.91$	n.s.	–		–	
CA1	$F_{(3,\ 12)} = 1.30$	n.s.	$F_{(1,\ 12)} = 0.93$	n.s.	$F_{(3,\ 12)} = 2.97$	n.s.	–		–	
Females										
Cingulate cortex	$F_{(3,\ 16)} = 4.97$	< 0.05	$F_{(1,\ 12)} = 4.60$	< 0.05	$F_{(3,\ 12)} = 2.08$	n.s.	–		–	
Caudate putamen	$F_{(3,\ 16)} = 0.15$	n.s.	$F_{(1,\ 16)} = 3.68$	n.s.	$F_{(3,\ 16)} = 0.85$	n.s.	–		–	
Nucleus accumbens core	$F_{(3,\ 16)} = 0.63$	n.s.	$F_{(1,\ 16)} = 6.59$	< 0.05	$F_{(3,\ 16)} = 2.15$	n.s.	–		–	
Nucleus accumbens shell	$F_{(1,\ 16)} = 2.23$	n.s.	$F_{(1,\ 16)} = 0.09$	n.s.	$F_{(3,\ 16)} = 0.10$	n.s.	–		–	
Bed nucleus of the stria terminalis	$F_{(3,\ 14)} = 5.58$	< 0.01	$F_{(1,\ 14)} = 31.8$	< 0.001	$F_{(3,\ 16)} = 1,44$	n.s.	–		–	
Basolateral amygdala	$F_{(3,\ 16)} = 1.46$	n.s.	$F_{(1,\ 16)} = 1.09$	n.s.	$F_{(3,\ 16)} = 0.90$	n.s.	–		–	
Central amygdala	$F_{(3,\ 16)} = 0.33$	n.s.	$F_{(1,\ 16)} = 1.17$	n.s.	$F_{(3,\ 16)} = 0.68$	n.s.	–		–	
Dentate gyrus	$F_{(3,\ 15)} = 1.73$	n.s.	$F_{(1,\ 15)} = 0.78$	n.s.	$F_{(3,\ 15)} = 0.25$	n.s.	–		–	
CA3	$F_{(3,\ 15)} = 2.94$	n.s.	$F_{(1,\ 15)} = 2.11$	n.s.	$F_{(3,\ 15)} = 1.14$	n.s.	–		–	
CA1	$F_{(3,\ 15)} = 1.71$	n.s.	$F_{(1,\ 15)} = 5.06$	< 0.05	$F_{(3,\ 15)} = 1.55$	n.s.	–		–	

Two-way ANOVA with drug treatment and genotype as main factors. When an interaction was observed, one-way ANOVA for males and females followed by Tukey's *post hoc* was performed (n = 3–4).

distribution of opiate receptors (Hammer, 1984, 1985, 1990; Hammer et al., 1994). Taken altogether, we used prepubertal mice (indicated by vaginal smears) in the pharmacological and the genetic approaches of this study in order to rule out the influence of circulating adult gonadal hormones in MOR antinociception which has been reported in previous studies (Ali et al., 1995; Banerjee et al., 1983). In this sense, the use of prepubertal mice allows us to indicate that the sex differences observed in the present study would be caused by organizational hormone effects (Craft & Ulibarri, 2009). Therefore, the sex differences observed in the antinociceptive response of male and female mice could be attributed to sex differences in the receptor density and affinity as suggested by Cicero et al. (1996). However, we did not observe sex differences in the antinociceptive response to the doses of MOR (1, 3 and 9 mg/kg) employed in the present study for the genetic approach. Variables such as opiate receptor specificity, route and dose of drug administration, type of analgesiometric test employed, species and strain of the animal tested have been shown to influence the pharmacodynamics of opiate analgesia even though preclinical behavioral tests of analgesia have shown to be quite consistent (see Mogil, 2012 for review). In line with this, a previous study from Duman et al. (2006) did not observe sex differences in MOR antinociception in Balb/c mice in agreement with our results. Taken altogether, our results support the hypothesis proposed by Kest et al. (1999) that sex differences in MOR analgesia are strain dependent.

Regarding the pharmacological approach, our results showed that the pretreatment with the $GABA_B$ agonist, BAC (2 mg/kg), was able to potentiate the MOR-induced antinociception and the pretreatment with the $GABA_B$ receptor antagonist, SAC (0,3 μg/kg), attenuated or even blocked the MOR-induce antinociceptive responses. These results could not be attributed to motor impairment since the dose of BAC and SAC chosen for the present study do not induce motor effects, as previously reported by our laboratory (Balerio and Rubio, 2002; Varani et al., 2014). Several studies have reported interactions between the GABAergic and opioidergic systems in antinociceptive responses. Aley and Kulkarni (1989) and Balerio and Rubio (2002) observed that systemic administration of BAC potentiated MOR induced antinociception and that NAL pretreatment reversed the antinociceptive effect of BAC. In the same way, the genetic approach showed that MOR (1 and 3 mg/kg) did not induce an antinociceptive effect in the $GABA_{B1}$ KO mice compared to their WT littermates. Nevertheless, we did observe an antinociceptive effect after MOR 9 mg/kg administration in both male and female mice. These responses could be the result of compensatory mechanisms to the lack of the $GABA_{B1}$ subunit of the $GABA_B$ receptor.

One possible site for GABAergic-opioid interaction is the spinal cord. Morphological studies demonstrated the coexistence of immunoreactivity in the superficial dorsal horn neurons for GABA and enkephalin (Todd et al., 1992) and the existence of GABAergic neurons connected to primary afferents in the dorsal horn (Barber et al., 1978). Moreover, it has been shown that in the superficial dorsal horn, μ-opioid receptor-expressing neurons are postsynaptic to GABAergic axon terminals. BAC acts in $GABA_B$ receptors and is thought to inhibit neurotransmitter release by increasing potassium currents and/or decreasing calcium currents. Lamina II of the spinal cord dorsal horn, an important site for nociceptive processing, has been shown to be rich in $GABA_B$ receptors and up to 50% of these receptors disappear after dorsal rhizotomy or capsaicin treatment suggesting that many of these $GABA_B$ receptors are located on the spinal terminals of primary afferent nociceptors. Since opioid receptors have also been found on the terminals of primary afferent nociceptors and have also been shown to inhibit neurotransmitter release, it is possible that an interaction between opioids and BAC at the level of the medullary dorsal horn underlies the enhanced analgesia induced by BAC pretreatment. In line with this, a supraspinal site for GABAergic opioid interaction is also possible. Moreover, BAC microinjected into the periaqueductal gray matter or into the rostroventral medulla produces antinociception. Since $GABA_B$ receptors have been shown to act as autoreceptors at GABAergic synapses, BAC might inhibit GABA release (Gordon et al., 1995) which could result in an increase in antinociceptive response.

On the other hand, MOR (9 mg/kg) was able to induce a decrease in the number of c-Fos positive nuclei in the Cg and AcbC of male mice and in the AcbSh of both sexes. c-Fos is a transcription factor considered to be a marker of neuronal activity (Dragunow and Faull, 1989), a decrease in its expression can be interpreted as a decline of this activity. In agreement with this finding, studies from Bereiter and Bereiter

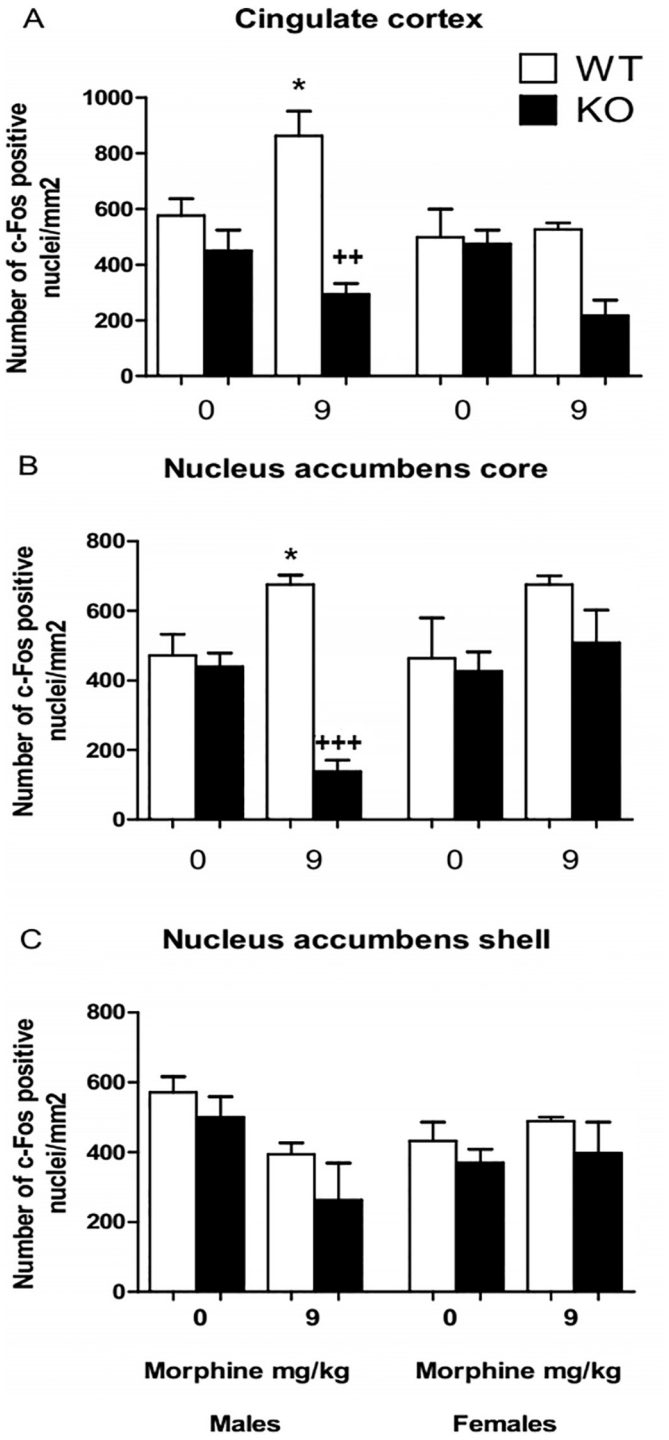

Fig. 5. In male mice, c-Fos expression increased in the Cg and AcbC of in the wild-type but not in the $GABA_{B1}$ knockout mice after MOR 9 mg/kg administration. Results are expressed as mean ± SEM of number of c-Fos positive nuclei/mm^2 in wild-type (WT) (white bars) (n = 3–4) and $GABA_{B1}$ knockout (KO) (black bars) (n = 3–4) mice. Statistical analysis was performed using two-way ANOVA with treatment (between subjects) and genotype (between subjects) as factors of variation, followed by corresponding one-way ANOVA and *post hoc* comparisons using the Tukey test. *$p < 0.05$, compared to SAL-MOR 0 mg/kg group; ++$p < 0.01$, +++$p < 0.001$, compared to wild-type mice. Only the photographs of sections of male mice are shown.

(2000) showed a decrease in Fos-like immunoreactivity in the spinal trigeminal nucleus when MOR was administered after an acute injury in the temporomandibular joint. MOR administration may interfere with the neurotransmission of the pain stimuli resulting in a reduced neuronal activity, reflected in the reduction of c-Fos positive nuclei. The anterior Cg is involved in processing sensorial afferents (Vogt, 1993) and the sensory information related to pain in humans (Hutchison et al., 1999). The nucleus accumbens is a limbic structure within the ventral striatum and is involved in reward and pain processing and also plays an important role in sensorimotor integration (Altier and Stewart, 1999). In our study, BAC was able to prevent the decrease in c-Fos expression in the Cg of male mice. In addition, SAC administration prevented the decrease in c-Fos expression in the AcbC and AcbSh of male mice but neither BAC nor SAC affected the decrease in c-Fos expression observed in the AcbSh of female mice. Interestingly, both the $GABA_B$ receptor agonist (BAC) and the $GABA_B$ receptor antagonist (SAC) had the same preventive effect on c-Fos expression. It has been previously reported that SAC can act as a partial agonist (Urwyler et al., 2005). Therefore, the effect of SAC is equivalent to BAC because SAC had an agonistic effect on c-Fos expression. In contrast, in the genetic approach, MOR administration increased the number of c-Fos positive nuclei in the Cg and in the AcbC of wild-type mice, while in mice lacking the $GABA_{B1}$ subunit of the $GABA_B$ receptor, was not observed significant changes in c-Fos expression. This discrepancy between the results in the genetic and pharmacologic approaches could indicate different mechanisms for inducing c-Fos expression after MOR administration but further studies are needed to clarify this point. Forebrain areas are involved in both opiate and non-opiate pain modulation. Although peripheral and spinal actions of opiates are important for analgesia, receptors in the Cg may be particularly important for opiate-related changes in the emotional aspects of pain. Other chemicals in the brain, such as dopamine, also play a role in pain modulation. Modulation of pain derived from psychological factors, such as attentional, emotional state, or expectation is manifested by changes in pain-evoked activity in the cerebral cortex and most likely involves intrinsic descending modulatory circuits (Cousins, 1986). Moreover, some human pain imaging studies indicate activity in the nucleus accumbens and amygdala (Baliki et al., 2010; Becerra et al., 2001), and this activity is probably a reflection of nociceptive transmission through spino–parabrachial–amygdala projections (Bernard et al., 1996). The PAG has also been observed to be active in human imaging studies of somatic and visceral pain, especially when the brain stem is specifically studied (Dunckley et al., 2005).

Our results indicate that MOR interferes with Cg, AcbC or AcbSh activation to induce its analgesic effect and BAC, SAC or the lack of the $GABA_{B1}$ subunit of the $GABA_B$ receptor prevent these changes providing evidence of a possible role of $GABA_B$ receptors in MOR antinociceptive effects. Taking into account that Cg and Acb are involved in the rewarding effect of MOR, our present results could not only indicate that BAC administration can potentiate the analgesic effect of MOR but they could also suggest a possible effect on the rewarding properties of MOR. Moreover, further research using double label immunohistochemistry could provide important information regarding the cells in which c-Fos is modulated by MOR and $GABA_B$ agonists and antagonist.

In summary, the present results provide evidence supporting the involvement of the $GABA_B$ receptors in the analgesic response induced by acute MOR administration. Our behavioral and neurochemical findings highlight some of the neurobiological substrates involved in the interaction between the GABAergic and opioidergic systems and regarding sex differences in the antinociceptive effect of MOR administration.

Authors' contributions

Valeria T. Pedrón carried out the experiments, analyzed the results and wrote the manuscript. Andrés P. Varani carried out the experiments and corrected the first drafts of the manuscript. Bernhard Bettler kindly provided the heterozygous mice to start our own $GABA_{B1}$ mice colony. Graciela N. Balerio conceived the original idea, supervised the project

and corrected the final version of the manuscript.

Conflict of interest

None.

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