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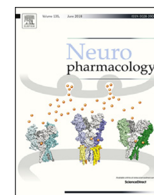
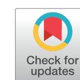


Activation of 5-HT_{2C} (but not 5-HT_{1A}) receptors in the amygdala enhances fear-induced antinociception: Blockade with local 5-HT_{2C} antagonist or systemic fluoxetine



Lígia Renata Rodrigues Tavares [a, b], Daniela Baptista-de-Souza [a, c], Azair Canto-de-Souza [a, b, c, d, *]

[a] Psychobiology Group, Department of Psychology/CECH- Federal University of São Carlos-UFSCar, São Carlos, São Paulo, 13565-905, Brazil
[b] Joint Graduate Program in Physiological Sciences UFSCar/UNESP, São Carlos, São Paulo, 13565-905, Brazil
[c] Neuroscience and Behavioral Institute-IneC, Ribeirão Preto, São Paulo, 14040-901, Brazil
[d] Program in Psychology UFSCar, São Carlos, São Paulo, 13565-905, Brazil

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A B S T R A C T

It is well-known that the exposure of rodents to threatening environments [e.g., the open arm of the elevated-plus maze (EPM)] elicits pain inhibition. Systemic and/or intracerebral [e.g., periaqueductal gray matter, amygdala) injections of antiaversive drugs [e.g., serotonin (5-HT) ligands, selective serotonin reuptake inhibitors (SSRIs)] have been used to change EPM-open arm confinement induced antinociception (OAA). Here, we investigated (i) the role of the 5-HT_{1A} and 5-HT_{2C} receptors located in the amygdaloid complex on OAA as well as (ii) the effects of systemic pretreatment with fluoxetine (an SSRI) on the effects of intra-amygdala injections of 8-OH-DPAT (a 5-HT_{1A} agonist) or MK-212 (a 5-HT_{2C} agonist) on nociception in mice confined to the open arm or enclosed arm of the EPM. Nociception was assessed by the writhing test. Intra-amygdala injections of 8-OH-DPAT (10 nmol) or MK-212 (0.63 nmol) produced a pronociceptive effect and intensified OAA, respectively. Fluoxetine (2.5 mg/kg, intraperitoneally) did not change 8-OH-DPAT effects on nociception but antagonized the enhancement of the OAA produced by MK-212. Interestingly, prior injection of SB 242084 (a selective 5-HT_{2C} antagonist) into the amygdala also blocked the MK-212 effects on OAA. These results indicate that 5-HT may facilitate nociception and intensify OAA, respectively, at 5-HT_{1A} and 5-HT_{2C} receptors located in the amygdala of mice. The impairment produced by systemic fluoxetine on the OAA enhancement provoked by intra-amygdala MK-212 suggests that this type of fear-induced antinociception may be modulated by SSRIs.

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1. Introduction

Evolutionarily, the inhibition of pain can be considered a defense response to an aversive or harmful stimulus (Butler and Finn, 2009). Several emotional responses related to aversive stimulus, such as fear, anxiety, and panic may inhibit or reduce sensitivity to pain (Canto-de-Souza et al., 1998; Nunes-de-Souza et al., 2000). Moreover, the descending inhibitory pain pathway is an important mechanism by which the interaction between processes induced by nociceptive stimuli and fear of stimuli can lead to the inhibition of pain or analgesia (Bolles and Fanselow, 1980; Helmstetter and Fanselow, 1987; Bolles and Fanselow, 1980; Fardin et al., 1984; Miczek et al., 1982; Rodgers, 1995; Siegfried et al., 1990; Terman et al., 1984; Watkins and Mayer, 1982).

From this viewpoint, previous studies have demonstrated that the exposure of rodents to the elevated plus maze (EPM), a widely used test to study anxiety in animals (e.g., Carobrez and Bertoglio, 2005), can also elicit antinociception (Stephens et al., 1986; Lee and Rodgers, 1990; Taukulis and Goggin, 1990; Conceição et al., 1992). We have previously shown that the confinement of mice to the open arm (OA) of the EPM markedly reduces the nociceptive response (Nunes-de-Souza et al., 2000; Baptista et al., 2009; Baptista et al., 2012), a phenomenon described as OA antinociception (OAA).

Systemic and intracerebral injections of anti-anxiety drugs have

* Corresponding author. Department of Psychology, Psychobiology Group/CECH-UFSCar Rod. Washington Luís, km 235 Monjolinho, São Carlos, 13565-905, São Paulo, Brazil.
E-mail address: souzaalm@ufscar.br (A. Canto-de-Souza).

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been used to investigate the OAA underlying mechanisms (Jiménez-Velázquez et al., 2006; Gomes and Nunes-de-Souza, 2009; Nunes-de-Souza et al., 2000; Paul et al., 2002). In this context, considering the role of the amygdaloid complex in the modulation of nociceptive and emotional states (Fields, 2000), this area became one of the most important sites for these pharmacological manipulations (e.g., Nunes-de-Souza et al., 2000; Baptista et al., 2009).

The serotonergic system is one of the several neurotransmitter systems located in the amygdala and is involved in the modulation of defensive responses (File et al., 1981; Graeff, 1981; Hodges et al., 1987). Serotonin, an indoleamine, plays a significant role in nociceptive transmission control, either by increasing or attenuating pain (Heinricher et al., 2009). Several studies have used selective serotonin reuptake inhibitors (SSRIs) as pharmacological tools to demonstrate the serotonergic system role on modulation of the nociceptive behavior (Salerno et al., 2002; Tomkins et al., 2001; McCleane, 2008; Jung et al., 1997; Stone et al., 2003; Duman et al., 2004; Kesim et al., 2005). For instance, it has been demonstrated that fluoxetine, an SSRI, provokes antinociception assessed in the paw formalin (Sawynok et al., 1999; Pedersen et al., 2005) and hot plate tests (Singh et al., 2001; Schreiber and Pick, 2006; Hache et al., 2012). In addition, administration of fluoxetine produced antinociceptive effects in both writhing and tail flick tests (Singh et al., 2001; Pedersen et al., 2005; Sikka et al., 2011).

In brief, serotonin can interact with 7 classes of receptors are differentiated into 14 subtypes (Barnes and Sharp, 1999). Specifically, 5-HT_{1A} and 5-HT_{2C} receptors have been the most widely studied in the modulation of anxiety responses (Deakin et al., 1992; Millan, 2003). However, the studies that have investigated the role of serotonin in pain modulation have reported inconsistent results (Sommer and Kress, 2004; Sommer, 2006).

The 5-HT_{1A}, an inhibitory G-coupled protein receptor (GCPR), and 5-HT_{2C}, a stimulatory GCPR (Azmitia, 2007; Pytliak et al., 2011; Shih et al., 1991), are widely distributed in brain regions involved in defensive behaviors, including the amygdala (Pompeiano et al., 1994; Hannon and Hoyer, 2008; Artigas, 2013). In this context, previous studies have emphasized the role of the amygdala 5-HT_1 and 5-HT_2 receptors in the modulation of defensive responses. Specifically, intra-amygdala injection of 8-OH-DPAT (a 5-HT_{1A} receptor agonist) elicits anxiogenic-like effects but fails to alter OAA responses (Nunes-de-Souza et al., 2000). Regarding the involvement of 5-HT_2 receptors located in the amygdala on defensive responses, local injections of 5-HT_{2C} agonists have induced anxiogenic-like effects in rats (Vicente and Zangrossi, 2012; de Melo Cruz et al., 2005; Christianson et al., 2010).

In the last two decades, there has been an increasing interest to investigate the interaction between serotonergic receptors and SSRIs. In this context, previous findings have suggested that SSRIs can change the anxiolytic effects induced by intracerebral injections of serotonergic agonists in rats (de Bortoli et al., 2006; Vicente and Zangrossi, 2012, 2014; Zanoveli et al., 2007, 2010). However, it remains to be determined whether fluoxetine is capable to change the effects of 5-HT_{1A} and 5-HT_{2C} receptor activation in the amygdaloid complex on nociception.

Thus, it is relevant to evaluate the role of the amygdala 5-HT_{1A} and 5-HT_{2C} receptors in OAA, as well as to investigate whether fluoxetine can alter the effects of intra-amygdala injections of 5-HT1A or 5-HT2C agonists on this type of defensive response. Thereby, our hypothesis is that fluoxetine can change the effects of intra-amygdala injections of $5HT_{1A}$ or $5HT_{2C}$ receptor agonists on OAA. To study that, we investigated the effects of (i) intra-amygdala injections of 8-OH-DPAT, a 5-HT1A receptor agonist, (ii) the combined systemic fluoxetine and intra-amygdala injections of 8-OH-DPAT, (iii) intra-amygdala injections of MK-212, a 5-HT2C receptor agonist, (iv) the combined systemic fluoxetine and intra-amygdala injections of the MK-212, and (v) the combined intra-amygdala injections of SB-242084 (a 5-HT_{2C} antagonist) and MK-212 on EPM-OAA in mice.

2. Material and methods

2.1. Subjects and ethics

Adult male albino Swiss mice from the animal facility of the Federal University of São Carlos, SP, Brazil, were used. The mice, weighing approximately 25–30 g at the beginning of the experiments, were housed in groups of 10 per cage (cage size: 41 cm × 34 cm × 16 cm) and were maintained under controlled conditions (23 °C ± 1 °C; 12-h light:dark cycle, lights on at 7:00 a.m.), with *ad libitum* food and water. The experiments were performed during the light phase of the light:dark cycle (9:00 a.m.–4:00 p.m.).

All experiments in this study were conducted in accordance with the recommendations of the Brazilian Guidelines for Care and Use of Animals for Scientific and Educational Purposes, created by The National Council for Control of Animal Experimentation. All procedures were approved by the Federal University of São Carlos' Ethics Committee on the Use of Animals (CEUA/UFSCar 029/2014). All endeavors were made to reduce animal suffering and to minimize the number of animals used.

2.2. Drugs

The following drugs were used for intra-amygdala microinjection: 8-OH-DPAT [(±)-8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide; 5.6 or 10 nmol/0.1 μL; Sigma-Aldrich] and MK-212 [6-chloro-2-(1-piperazinyl) pyrazine hydrochloride; 0.21 or 0.63 nmol/0.1 μL; Tocris Cookson Inc.], SB 242084 [6-chloro-2,3-dihydro-5-methyl-*N*-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1H-indole-1-carboxyamide dihydrochloride] (0.1 nmol/0.1 μL). Doses used for the intra-amygdala treatments were based on previous studies (Nunes-de-Souza et al., 2000; Gomes and Nunes-De-Souza, 2009; Vicente and Zangrossi, 2012; Baptista-de-Souza et al., 2017, unpublished results). The drugs were prepared in a vehicle of physiological sterile saline with 2% of Tween 80. The same vehicle was injected intra-amygdala into animals in the control group (vehicle group).

The drug used for systemic treatment was fluoxetine hydrochloride [Sigma (2.5 mg kg^{-1}; subcutaneously, s. c.)], prepared in physiological sterile saline (0.9% NaCl). The fluoxetine dose used in this protocol was based on a previous study that reported ineffective dose intrinsically in antinociception [see discussion section] (Baptista-de-Souza et al., 2017, unpublished results).

2.3. Surgery and microinjection

Mice were anesthetized with ketamine and xylazine [100 mg/kg and 10 mg/kg intraperitoneally (i.p.), respectively] and fixed in a stereotaxic frame (Insight Instruments, Brazil). Bilateral stainless steel guide cannulae (25-gauge x 7 mm; Insight Instruments) were then implanted and fixed to the skull by using dental acrylic and jeweler's screws. The bregma was considered the reference point, and the following coordinates were used to locate the target site in the amygdaloid complex: anterior/posterior, −1.3 mm; medial/lateral, 3.3 mm; dorsal/ventral, 2.8 mm (Paxinos and Franklin, 2001). To reduce the incidence of occlusion, each guide cannula was sealed with a stainless steel wire to protect it from blockage at the time of surgery. At the end of the stereotaxic surgery, the animals received an intramuscular injection of the anti-inflammatory

and analgesic drug ketoprofen (benzene acetic acid, 5 mg/kg) (Lu et al., 2004) and an intramuscular injection of the antibiotic ceftriaxone (ceftriaxone sodium hemipentahydrate, 4 mg/kg) (Garber et al., 2011; Stepanovic-Petrovic et al., 2014). Subsequently, the mice were allowed to recover from the surgical procedure for 4–5 days. Solutions were injected into the amygdala by using a microinjection unit (33-gauge stainless steel cannula; Insight Instruments) that extended 2 mm beyond the tip of the bilateral guide cannulae.

The microinjection units were connected to a 10-μL Hamilton microsyringe through polyethylene tubing (PE-10), and the flow rate was controlled with an infusion pump (BI, 2000–Insight Instruments) programmed to deliver 0.1 μL of each solution for 60 s. The injection needle was introduced through the guide cannula until its lower end reached 2 mm below the cannula. The microinjection procedure included gently restraining the mice, inserting the injection unit, and infusing the solution for 60 s with an additional 90 s to maximize diffusion from the needle tip.

2.4. Apparatus and general procedure

The basic EPM design was similar to the originally validated design for mice (Lister, 1987). It comprised two OAs (30 cm × 5 cm x 0.25 cm) and two enclosed arms (EAs: 30 cm × 5 cm × 15 cm) that extended in a cross from a common central platform (5 cm × 5 cm), with the entire maze raised to 38.5 cm above the floor level. The confinement to an OA or EA was achieved by placing an easily removable gate at the proximal end of each arm of the EPM. All testing was conducted under moderate illumination (77 lux, measured from the central platform of the EPM) during the light phase of the light:dark cycle.

Nociceptive behavior was assessed using the writhing test, as previously described (Vander Wende and Margolin, 1956). In the present study, writhing was induced by injecting 0.1 mL/10 g body weight (b.w.) of 0.6% acetic acid i. p. immediately after the intra-amygdala drug injection. The mice were individually confined to either an OA or EA of the EPM for 5 min, during which the number of writhes was recorded. Between subjects, the maze was thoroughly cleaned with 20% ethanol and dried with a cloth. All sessions were video-recorded with a camera that was connected to a monitor in an adjacent laboratory. This experimental protocol was repeated in all experiments described below.

2.5. Experimental procedures

2.5.1. Experiment 1. Effects of bilateral intra-amygdala microinjections of 8-OH-DPAT on OAA in mice

This protocol aimed to investigate the involvement of the 5-HT_{1A} receptor within the amygdala on OAA. Sixty-six mice received different doses of 8-OH-DPAT (5.6 or 10 nmol/0.1 μL) or vehicle via bilateral microinjections into the amygdala. Simultaneously, the mice received an i. p. injection of 0.6% acetic acid, (0.1 mL/10 g b. w.). Immediately after acetic acid injection, each mouse was confined either in the OA or EA of the EPM to record the number of writhes. A similar experimental protocol was repeated in the following experiments (Supporting Information Fig. 1A).

2.5.2. Experiment 2. Effects of combined treatment with systemic fluoxetine injection and bilateral intra-amygdala microinjection of 8-OH-DPAT on OAA in mice

This protocol aimed to investigate the effects of the interaction of fluoxetine and 5-HT_{1A} receptor within the amygdala on OAA. For this, seventy-eight mice received a subcutaneous injection of fluoxetine (2.5 mg/kg s. c.) or saline. Twenty-eight minutes later, the mice received a bilateral intra-amygdala microinjection of 8-OH-DPAT (10 nmol/0.1 μL, dose selected in experiment 1) or vehicle and a simultaneous i. p. injection of 0.6% acetic acid (0.1 mL/10 g b. w.). Immediately after acetic acid injection, each mouse was confined either in the OA or EA of the EPM to record the number of writhes (Supporting Information Fig. 1B).

2.5.3. Experiment 3. effects of bilateral intra-amygdala microinjection of MK-212 on OAA in mice

This protocol aimed to investigate the involvement of the 5-HT_{2C} receptor within the amygdala on OAA. Sixty-five mice received bilateral intra-amygdala microinjections of different doses of MK-212 (0.21 or 0.63 nmol/0.1 μL) or vehicle and an i. p. injection of 0.6% acetic acid (0.1 mL/10 g b. w.). Immediately after acetic acid injection, each mouse was confined either in the OA or EA of the EPM to record the number of writhes (Supporting Information

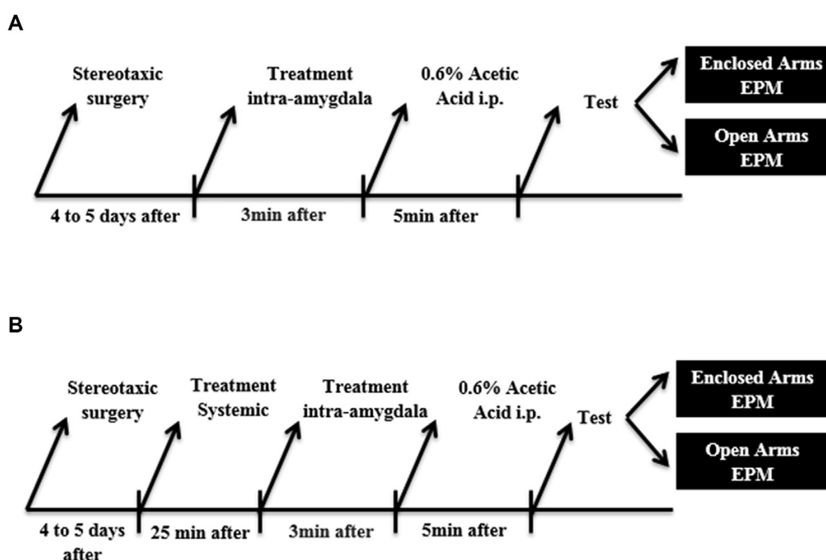

Fig. 1. (A) Timeline of the writhing test showing the intra-amygdala treatment in mice exposed to the enclosed or open arms of the elevated plus maze (EPM). **(B)** Timeline of the writhing test showing the treatment with systemic fluoxetine followed by intra-amygdala treatment in mice exposed to the enclosed or open arms of the EPM.

Fig. 1A).

2.5.4. Experiment 4. Effects of combined treatment with systemic fluoxetine and bilateral microinjection of MK-212 into the amygdala on OAA in mice

This protocol aimed to investigate the effects of the interaction of fluoxetine and 5-HT_{2C} receptor within the amygdala on OAA. For this, seventy-six mice received a subcutaneous injection of fluoxetine (2.5 mg/kg s. c.) or saline. Twenty-five minutes later, each mouse received a bilateral intra-amygdala microinjection of MK-212 (0.63 nmol/0.1 μL, dose selected in experiment 3) or vehicle and an i. p. injection of 0.6% acetic acid (0.1 mL/10 g b. w.). Immediately after acetic acid injection, each mouse was confined either in the OA or EA of the EPM to record the number of writhes (Supporting Information Fig. 1B).

2.5.5. Experiment 5. Effects of combined treatment of SB 242084 and MK-212 both into the amygdala on OAA in mice

This protocol aimed to investigate whether the enhancement of OAA provoked by intra-amygdala injection of MK-212 would be changed by prior local injection of SB 242084, a selective 5-HT_{2C} receptor antagonist. For this, fifty-three mice received bilateral intra-amygdala microinjections of SB-242084 (0.1 nmol) or saline and 5 min later intra-amygdala injection of MK-212 (0.63 nmol) or vehicle and an i. p. injection of 0.6% acetic acid (0.1 mL/10 g b. w.). Immediately after acetic acid injection, each mouse was confined either in the OA or EA of the EPM to record the number of writhes (Supporting Information Fig. 1B).

2.6. Histology

After each experiment, all animals received an intra-amygdala 0.1-μL infusion of Evans blue (1%) by using the microinjection procedure. The mice were euthanized in a CO_2 chamber. The brain was removed from the skull and maintained in paraformaldehyde; thereafter, it was cryoprotected in 30% sucrose until soaked. Subsequently, coronal 60-μm slices were cut and mounted on gelatin-coated slides. This allowed the injection sites to be verified histologically against the Paxinos and Franklin Mouse Brain Atlas (2001). Data from animals with injection sites outside the amygdala were excluded from the study.

2.7. Statistical analysis

For experiments 1 and 3, the data were analyzed using a two-way analysis of variance (ANOVA; treatment x type of confinement). For experiments 2 and 4 the data were analyzed using three-way ANOVA (systemic treatment x intra-amygdala treatment x type of confinement). For experiment 5, the data was analyzed using three-way ANOVA (prior intra-amygdala treatment x intra-amygdala treatment x type of confinement). Significant F values were followed up by Duncan's multiple range test. $P \leq 0.05$ was considered statistically significant.

3. Results

Only mice with microinjection sites located bilaterally within the amygdala were included in the study. Histological analysis confirmed that 338 mice had accurate microinjection the amygdala. Cannula placements were within the basolateral nucleus (60%), the lateral nucleus (14%), the central nucleus (12%) and in the basomedial nucleus (14%) of the animals. The following groups were formed: 66 animals were used to investigate the effects of intra-amygdala microinjection of 8-OH-DPAT (vehicle, 5.6 or 10 nmol) (Exp1). In experiment 2, 78 mice were used to evaluate the effects of systemic fluoxetine (2.5 mg/kg s. c.) and intra-amygdala injections of 8-OH-DPAT (10 nmol). 65 mice were used to assess the effects of intra-amygdala microinjections of MK-212 (vehicle, 0.21 or 0.63 nmol) in experiment 3. In experiment 4, 76 mice were necessary to reveal the effects of systemic fluoxetine (2.5 mg/kg s. c.) and intra-amygdala microinjections of MK-212 (0.63 nmol). In experiment 5, 53 mice were required to reveal the effects of intra-amygdala microinjections of SB-242084 (0.1 nmol) and intra-amygdala microinjection of MK-212 (0.63 nmol). The sample sizes for each experimental group are shown in Table 1.

Fig. 2A shows a schematic representation of histological results according to the Franklin and Paxinos Mouse Brain Atlas (2001). The black circles represent the sites of drug infusion that were on-target within the amygdala. Gray circles represent the animals that had infusion locations outside the amygdala. Fig. 2B shows a photomicrograph of a midbrain coronal section of a representative subject showing an injection site within the amygdala.

Table 1
Pharmacological treatments and sample sizes of each experimental group.

Experimental groups	Treatment	Confinamenton the EPM	
		OA	EA
Experiment 1	Vehicle	10	10
	8-OH-DPAT (5.6 nmol/0.1 μl)	12	12
	8-OH-DPAT (10 nmol/0.1 μl)	12	12
Experiment 2	Saline + Vehicle	9	10
	Saline + 8-OH-DPAT (10 nmol/0.1 μl)	11	12
	Fluoxetine 2.5 (mg/Kg) + Vehicle	8	10
	Fluoxetine (2.5 mg/kg) + 8-OH-DPAT (10 nmol/0.1 μl)	9	9
Experiment 3	Vehicle	8	13
	MK-212 (0.21 nmol/0.1 μl)	11	11
	MK-212 (0.63 nmol/0.1 μl)	11	11
Experiment 4	Saline + Vehicle	9	12
	Saline + MK-212 (0.63 nmol/0.1 μl)	10	10
	Fluoxetine (2.5 mg/kg) + Vehicle	9	9
	Fluoxetine (2.5 mg/kg) + MK-212 (0.63 nmol/0.1 μl)	9	8
Experiment 5	Saline + Vehicle	6	7
	Saline + MK-212 (0.63 nmol/0.1 μL)	6	7
	SB-242084 (0.1 nmol/0.1 μL) + Vehicle	7	6
	SB-242084 (0.1 nmol/0.1 μL) + MK-212 (0.63 nmol/0.1 μL)	6	8

EPM: elevated plus-maze; OA: open arm; EA: enclosed arm.

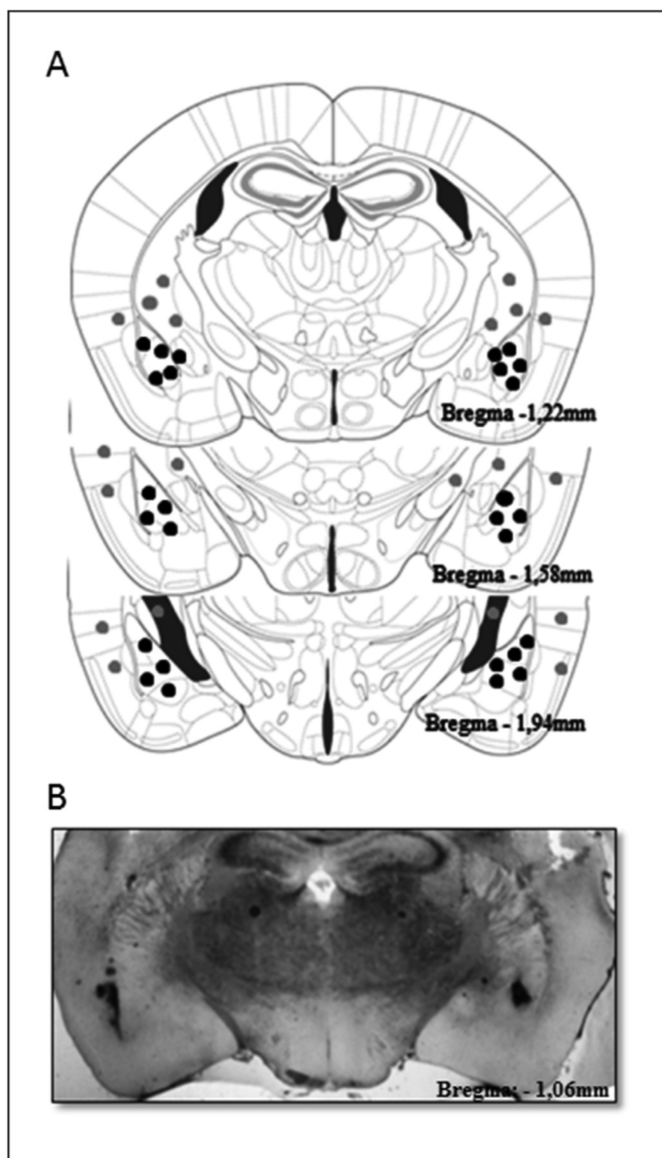

Fig. 2. **(A)** Target sites for microinjection into the amygdala. Schematic representation of microinjections sites within the amygdala. The black circles represent the sites of drug infusion that were on-target within the amygdala. The gray circles represent the locations in which the infusion occurred outside the amygdala. The number of dots in the figure is fewer than the actual number of animals used because of data overlapping. **(B)** Photomicrograph of a coronal section of a representative subject showing an injection site within the mice amygdala.

3.1. Experiment 1. Intra-amygdala 8-OH-DPAT reversed OAA in mice

In experiment 1, a two-way ANOVA (type of confinement factor x treatment factor) revealed statistically significant main effects for the type of confinement factor ($F_{1,\ 60} = 266.55$, $P < 0.05$) and treatment factor ($F_{2,\ 60} = 60.37$, $P < 0.05$). The interaction between treatment and type of confinement was also statistically significant ($F_{2,\ 60} = 5.81$, $P < 0.05$). The post-hoc Duncan test indicated a significantly lower number of writhes in the OA-confined group than in the EA-confined group. The animals that received 10 nmol of 8-OH-DPAT showed an increase in the number of writhes compared with those that received a vehicle, regardless of whether they were confined to the OA or EA (Fig. 3).

3.2. Experiment 2. Systemic fluoxetine did not change the attenuation of OAA induced by intra-amygdala 8-OH-DPAT in mice

A three-way ANOVA (type of confinement factor x systemic treatment factor x intra-amygdala treatment) revealed statistically significant main effects for the type of confinement factor ($F_{1,\ 70} = 101.08$, $P < 0.05$) and intra-amygdala treatment ($F_{1,\ 70} = 62.93$, $P < 0.05$). The post hoc Duncan test indicated a significantly lower number of writhes in the OA-confined group than in the EA-confined group. Similar to experiment 1, the animals confined to the OA or EA that received 8-OH-DPAT (10 nmol) showed an increase in the number of writhes compared with those that received a vehicle (Fig. 4). The animals treated with fluoxetine (2.5 mg/kg) and vehicle did not indicated significant effect when compared to the saline/vehicle group, regardless of whether they were OA or EA confined, and when they received fluoxetine (2.5 mg/kg) + 8-OH-DPAT (10 nmol) did not show significant effect when compared to the saline/8-OH-DPAT group (Fig. 4). The animals treated with fluoxetine (2.5 mg/kg) and vehicle were not significantly different compared to the saline/vehicle group ($F_{1,\ 70} = 0.17$, $P > 0.05$), regardless of whether they were OA or EA confined, and when they received fluoxetine (2.5 mg/kg) + 8-OH-DPAT (10 nmol) did not show significant effect when compared to the saline/8-OH-DPAT group ($F_{1,\ 70} = 0.085$, $P > 0.05$), (Fig. 4).

3.3. Experiment 3. Intra-amygdala MK-212 enhanced OAA in mice

A two-way ANOVA (type of confinement factor x treatment factor) revealed statistically significant main effects for the type of confinement factor ($F_{1,\ 59} = 334.32$, $P < 0.05$) and treatment factor ($F_{2,\ 59} = 6.48$, $P < 0.05$). The post-hoc Duncan test indicated that the number of writhes was significantly lower in the OA-confined group than in the EA-confined group. Additionally, OA-confined animals injected with 0.63 nmol of MK-212 displayed a lower number of writhes than did their respective controls (Fig. 5).

3.4. Experiment 4. Systemic fluoxetine attenuated the enhancement of OAA induced by intra-amygdala MK-212 bilateral microinjection in mice

In experiment 4, three-way ANOVA (type of confinement factor x systemic treatment factor x intra-amygdala treatment) revealed statistically significant main effects for type of confinement factor ($F_{1,\ 68} = 292.34$, $P < 0.05$) and systemic treatment factor ($F_{1,\ 68} = 6.53$, $P < 0.05$). The interaction effects for systemic treatment factor × intra-amygdala treatment were also statistically significant ($F_{1,\ 68} = 9.74$, $P < 0.05$). The post-hoc Duncan test indicated a significantly lower number of writhes in the OA-confined group than in the EA-confined group. However, the animals treated with both fluoxetine + MK-212 and confined to the OA showed no statistical difference in the number of writhes compared to their respective controls (saline + vehicle). Overall, these data indicate that the enhancement of the OA-induced antinociception, observed in mice treated with MK-212 in experiment 3, was blocked by fluoxetine pretreatment (Fig. 6).

3.5. Experiment 5. Intra-amygdala SB-242084 attenuated the enhancement of OAA induced by intra-amygdala MK-212 bilateral microinjection in mice

In experiment 5, three-way ANOVA (type of confinement factor x prior intra-amygdala treatment factor x intra-amygdala treatment) revealed statistically significant main effects for type of

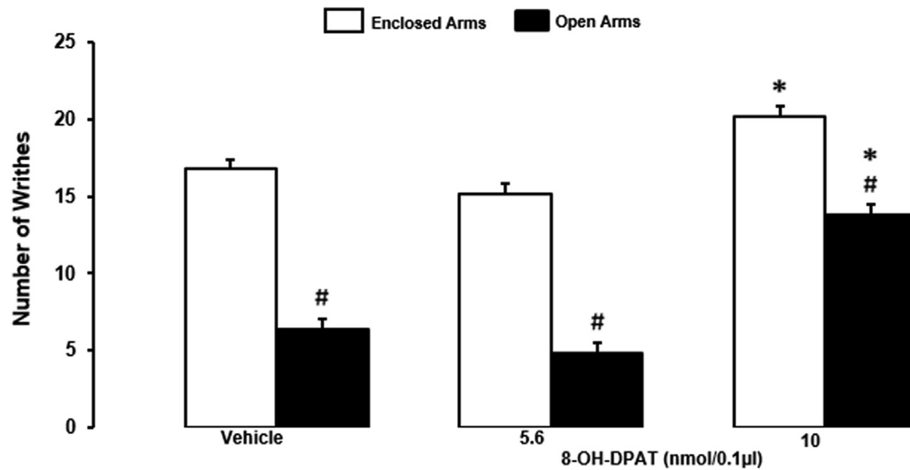

Fig. 3. Effects of bilateral intra-amygdala microinjections of 8-OH-DPAT (5.6 or 10 nmol/0.1 μL) on open-arm antinociception (OAA) in mice. All data are presented as mean ± standard error of mean (SEM; n = 10–12). #$P < 0.05$ compared with the EA-confined group. *$P < 0.05$ compared with the respective vehicle group. Two-way ANOVA, followed by Duncan's post-hoc test.

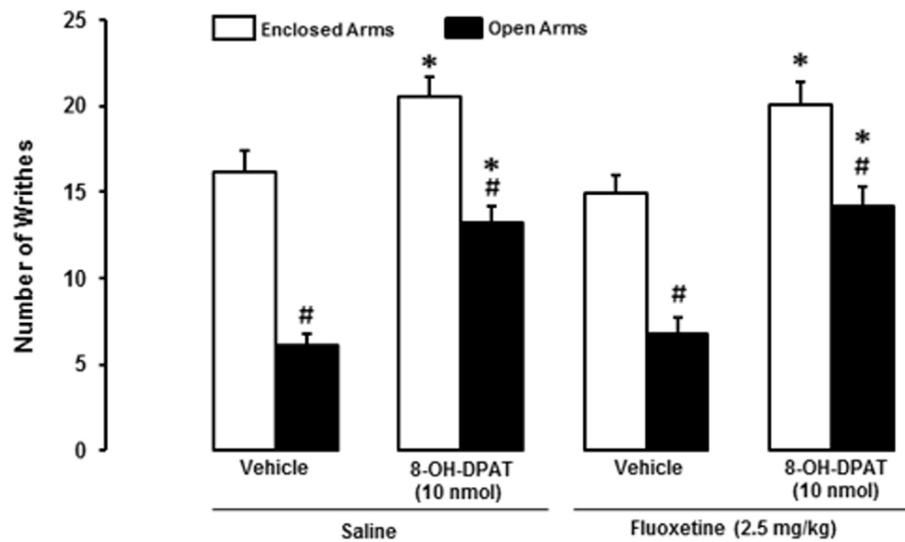

Fig. 4. Effects of the combined injections of acute systemic fluoxetine (2.5 mg/kg) followed by bilateral intra-amygdala microinjections of 8-OH-DPAT (10 nmol/0.1 μL) on OAA in mice. All data are presented as mean ± SEM (n = 8–12). #$P < 0.05$ compared with the EA-confined group. *$P < 0.05$ compared with the respective vehicle group. Three-way ANOVA, followed by Duncan's post-hoc test.

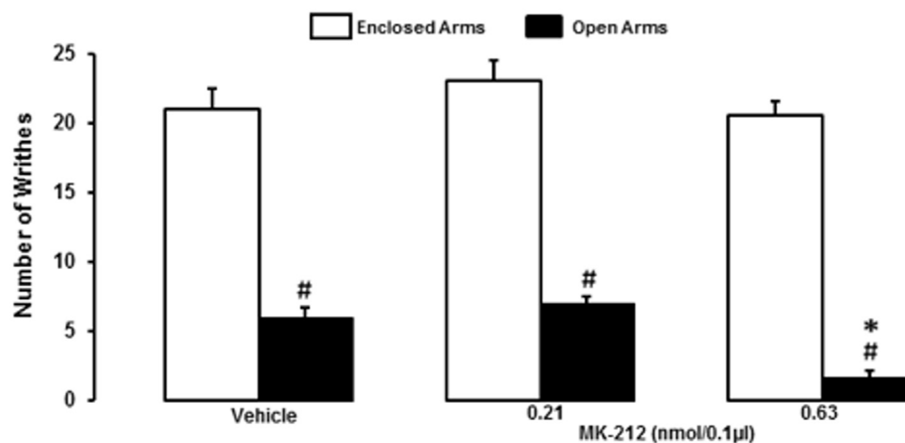

Fig. 5. Effects of the bilateral intra-amygdala microinjections of MK-212 (0.21 or 0.63 nmol/0.1 μL) on OAA in mice. All data are presented as mean ± SEM (n = 8–13). #$P < 0.05$ compared with the EA-confined group. *$P < 0.05$ compared with the respective vehicle group. Two-way ANOVA, followed by Duncan's post-hoc test.

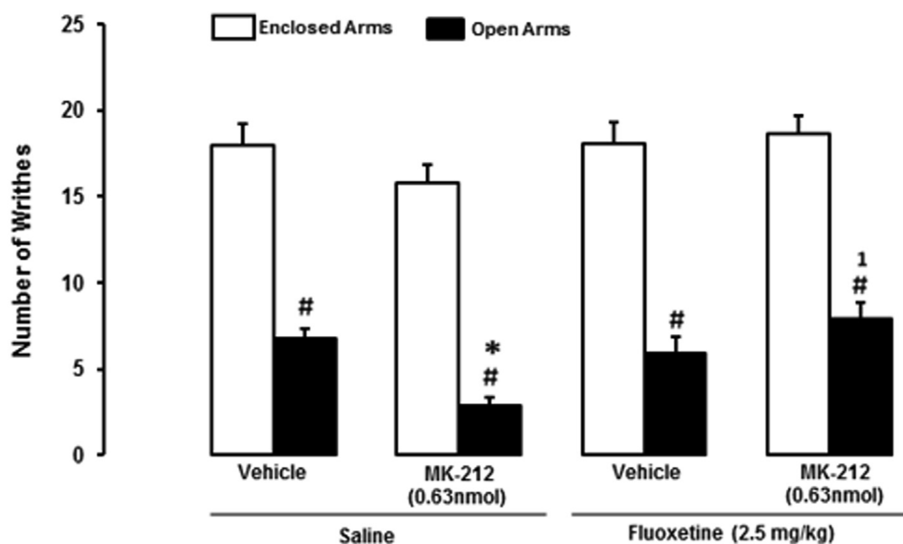

Fig. 6. Effects of the combined injections of acute systemic fluoxetine (2.5 mg/kg) and bilateral intra-amygdala microinjections of MK-212 (0.63 nmol/0.1 μL) on OAA in mice. All data are presented as mean ± SEM (n = 8–12). #$P < 0.05$ compared with the EA-confined group. *$P < 0.05$ compared with the respective vehicle group. [1]$P < 0.05$ compared with the saline + MK-212 group. Three-way ANOVA, followed by Duncan's post-hoc test.

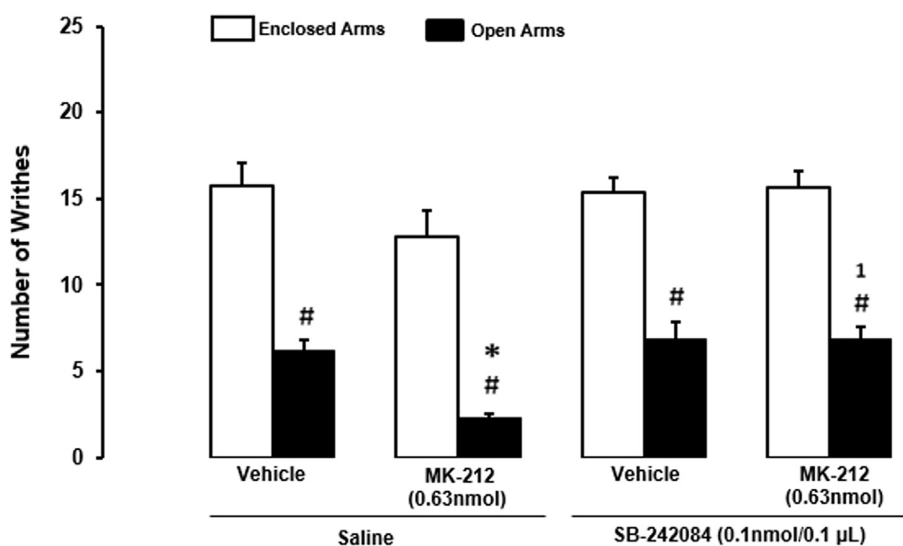

Fig. 7. Effects of the combined bilateral intra-amygdala microinjections of SB-242084 (0.1 nmol/0.1 μL) and bilateral intra-amygdala microinjections of MK-212 (0.63 nmol/0.1 μL) on OAA in mice. All data are presented as mean ± SEM (n = 6–8). #$P < 0.05$ compared with the EA-confined group. *$P < 0.05$ compared with the respective vehicle group. [1]$P < 0.05$ compared with the saline + MK-212 group. Three-way ANOVA, followed by Duncan's post-hoc test.

confinement factor ($F_{1, 45} = 180.18$, $P < 0.05$), prior intra-amygdala treatment factor ($F_{1, 45} = 7.42$, $P < 0.05$) and intra-amygdala treatment factor ($F_{1,45} = 5.33$, $P < 0.05$) The interaction effects for prior intra-amygdala treatment factor × intra-amygdala treatment were also statistically significant ($F_{1, 45} = 6.25$, $P < 0.05$). The post-hoc Duncan test indicated a significantly lower number of writhes in the OA-confined group than in the EA-confined group. However, the animals treated with both SB-242084 + MK-212 and confined to the OA showed no statistical difference in the number of writhes compared to their respective controls (saline + vehicle). Overall, these data indicate that the enhancement of the OA-induced antinociception, observed in mice treated with MK-212 in experiment 3, was blocked by intra-amygdala pretreatment with SB-242084 (Fig. 7).

4. Discussion

The present findings show that while 5-HT_{1A} receptors activation in the amygdala accentuated nociceptive behavior, increasing the number of writhes in the OA and EA, the 5-HT_{2C} receptors activation intensified antinociception. Prior systemic treatment with fluoxetine, at a dose devoid of intrinsic activity on nociception (2.5 mg/kg), did not change the hypernociceptive effect provoked by intra-amygdala injection of 8-OH-DPAT (5-HT_{1A} agonist), but this SSRI blocked the OAA enhancement induced by intra-amygdala activation of 5-HT_{2C}. Importantly, the effects of intra-amygdala injection of MK-212 (5-HT_{2C} agonist) were also antagonized by local injection of SB-242084, a selective 5-HT_{2C} receptor antagonist.

The present results corroborate previous studies wherein the

confinement of mice to the OA of the EPM elicits antinociception, a phenomenon known as OAA (Nunes-de-Souza et al., 2000; Baptista et al., 2009). Nunes-de-Souza et al. (2000) demonstrated that intra-amygdala microinjections of 5.6 nmol of 8-OH-DPAT did not change OAA; however, this dose produced an anxiogenic effect in mice exposed to the EPM. Based on these findings, the authors suggested a possible dissociation between the mechanisms involved in the modulation of anxiety and nociception control in the amygdaloid complex.

However, our results demonstrated that intra-amygdala injection of a higher dose of 8-OH-DPAT, i. e, 10 nmol, leads to an increasing in the number of writhes regardless of confinement type (OA or EA), suggesting a direct modulation of this serotonergic receptor subtype in the nociceptive pathways. Previous findings have been shown that systemic serotonin administration produces a dose-dependent hyperalgesic effect, and this effect would be the result of a direct excitatory effect of the primary afferent neuron (Taiwo and Levine, 1992). This increase in nociceptive behavior, observed in Sprague–Dawley rats subjected to the paw withdrawal test, was shown through the administration of 5-HT_{1A} selective agonists, such as 8-OH-DPAT and DP-5-CT (Taiwo and Levine, 1992). Altogether, these evidence confirm the already described role of 5-HT_{1A} receptors in facilitate the hypernociception (Sommer, 2006).

The hypernociceptive role of 5-HT_{1A} receptors have been shown in various experimental protocols. For instance, Canto-de-Souza and colleagues (1998) demonstrated the blockade of social-defeat analgesia through intra-dPAG injection of BAY-R 1531, a 5-HT_{1A} full agonist, whereas local injection of the 5-HT_{1A} antagonist, WAY-100135, enhanced this type of environmentally induced pain inhibition. This antagonist also blocked the increase in the visceromotor response to colorectal distension induced by 8-OH-DPAT (Mickle et al., 2012). The pro-nociceptive property of 8-OH-DPAT and the antinociceptive effect of WAY-100135 have already been reported by Millan and colleagues (1995).

Considering that the 5-HT_{1A} receptor is coupled to an inhibitory Gi protein, it is probable the binding to 8-OH-DPAT causes an efflux of potassium, thereby promoting cell hyperpolarization, and consequently a neuronal inhibition (Fox and Sorenson, 1994; Raymond et al., 2001; Artigas, 2013) within the amygdaloid complex, which in turn would result in a pro-nociceptive effect.

In this context, several studies have shown that amygdala lesions are able to increase pain-related responses in different animal models (Nakagawa et al., 2003; Tanimoto et al., 2003). Recently, our research group have observed that amygdala inhibition through local injection of cobalt chloride, a non-selective synaptic inhibitor (Canto-de-Souza et al., 2014), produced an increase in the number of writhes induced by abdominal injection of 0.6% acetic acid in mice.

Given these findings above, we hypothesized that hypernociception produced by intra-amygdala 8-OH-DPAT is result of hyperpolarization in this area. This neuronal inhibition of the amygdaloid complex might result in an inactivation of crucial neurotransmitter systems of the descending inhibitory pain circuitry (Fields, 2004; McGaraughty et al., 2004), triggering an exacerbation of the sensory nociceptive response.

In the other hand, some studies have suggested that the therapeutic effects of fluoxetine treatment are result of this SSRI interaction with the subtypes of serotonergic receptors, mainly 5-HT_{1A} and 5-HT_{2C} (Chen et al., 1995; De Vry et al., 2004). In order to investigate this possible interaction, we choose a dose of fluoxetine devoid of intrinsic effect on nociceptive response (2.5 mg/kg), as previously observed by our group (Baptista-de-Souza et al., 2014), to investigate its potential effects to alter the OAA when combined with intra-amygdala injections of 5-HT receptor ligands. However, in experiment 2, we observed that fluoxetine (2.5 mg/kg) did not alter the effects of 5-HT_{1A} agonist in the amygdaloid complex.

In experiment 3, we evaluated the effect of the intra-amygdala injection of MK-212 on nociceptive response. Different to that observed with the 5-HT_{1A} agonist, intra-amygdala injection of MK-212 (0.63 nmol) intensified OAA. A similar result was previously demonstrated with the activation of 5-HT_{2C} receptors in the midbrain periaqueductal gray matter (PAG) (Baptista et al., 2012). Despite this apparent function convergences, in that the 5-HT2C receptors of the amygdaloid complex and PAG play similar roles in the modulation of nociceptive response, their activations seem to produce quite different effects in the control of anxiety-like behaviors (Deakin and Graeff, 1991; Graeff et al., 1997). In brief, previous studies have reported that while intra-PAG injections of 5-HT_{2C} agonists exert anxiolytic-like effect, stimulation of the same receptor subtype in the amygdaloid complex seems to elicit anxiogenesis (Cornélio and Nunes-de-Souza et al., 2007; de Mello Cruz et al., 2005; Gomes and Nunes-De-Souza, 2009).

Also with regard the role of PAG 5-HT2 receptors in the modulation of nociception, microinjections of ritanserin, a $5HT_{2A/2C}$ antagonist, into the superior colliculus and dorsolateral PAG decreased the innate fear-induced antinociception (de Oliveira et al., 2017). Similar to the results found by Coimbra and Brandão (1997), de Oliveira et al. (2017) demonstrated that fear-induced analgesia was inhibited by microinjections of a 5-HT_2 blocker ketanserin, in the midbrain tectum.

Thereby, we suggest that the increase in OAA observed in response to the highest dose of MK-212 is directly related to exacerbation of anxiety produced by the activation of 5-HT_{2C} receptors in this prosencephalic structure. Notably, this relationship is distinct from that observed in the PAG, which performs a different modulation in the pain and anxiety responses (Baptista et al., 2012).

Interestingly, systemic treatment with fluoxetine prevented OAA enhancement induced by intra-amygdala injection of MK-212 (experiment 4). However, these results are different from those reported in previous studies wherein have been suggested that SSRIs provoke an exacerbation of anxiolytic and panicolytic responses induced by 5-HT_2 agonists in the dorsal PAG (dPAG) (de Bortoli et al., 2006; Zanoveli et al., 2007, 2010).

Considering the accentuation of antinociception induced by MK-212, we expected that an increase in synaptic serotonin levels triggered by the acute treatment of fluoxetine (Carlsson, 1970) would cause an exacerbation of antinociception. However, we observed the attenuation the effect of the 5-HT_{2C} agonist induced by inactive dose of fluoxetine, and thus did not accentuate antinociception, as we have hypothesized. In this context, previous studies have suggested that fluoxetine can also act as an antagonist of 5-HT_{2C} receptors (Ni and Miledi, 1997; Palvimaki et al., 1999). Through electrophysiological techniques, Ni and Miledi (1997) demonstrated that fluoxetine inhibits the binding of serotonin to 5-HT_{2C} receptors expressed in the membranes of cortical cells in rats. This observation suggests that fluoxetine can act as a reversible and competitive antagonist of 5-HT_{2C} receptors.

Subsequently, Palvimaki et al. (1999) corroborated Ni and Miledi's study by demonstrating that treatment with fluoxetine leads to 43% occupancy of the 5-HT_{2C} receptors. Moreover, the affinity of fluoxetine for $5HT_{2C}$ receptors (Ki 65 nM) is close to its affinity for 5-HT transporters (Ki 33 nM) (Ni and Miledi, 1997). Similarly, a study reported that chronic treatment with fluoxetine induced an increase in the 5-HT_{2C} protein expression levels within the amygdala (Baptista-de-Souza et al., 2014). This effect appears to be associated with the antinociceptive feature of fluoxetine, as well as the analgesic effects of SSRIs that occur because of the blockade of 5-HT_{2C} receptors in the amygdala of rats exposed to an arthritis pain test (Grégoire and Neugebauer, 2013).

In our fifth experiment, in order to clarify the possible fluoxetine actions as an antagonist of 5-HT_{2C} receptors, we proceeded with intra-amygdala injections of SB-242084 [a selective 5-HT_{2C} receptor antagonist (0.1 nmol, a dose without intrinsic effects on nociceptive response; see results of Exp. 5)] combined with MK-212. We observed that similar to systemic fluoxetine, intra-amygdala SB-242084 prevented the increase in antinociception induced by MK-212. Altogether, these results seem to strengthen the hypothesis that fluoxetine acts on 5-HT_{2C} receptors, blocking the OAA enhancement induced by activation of this serotonin receptor subtype.

In the same way, the influence of this SSRI on anxiety responses induced by MK-212 intra-amygdala was demonstrated by Vicente and Zangrossi (2014). Those authors demonstrated that chronic treatment with systemic fluoxetine successfully inhibited the anxiogenic effects of MK-212, suggesting that this effect is mediated by the 5-HT_{2C} receptor.

5. Conclusion

The present study demonstrates that serotonin neurotransmission in the amygdaloid complex modulates the OAA. Specifically, we found that 5-HT_{1A} receptors in the amygdala may be modulating antinociception through a direct action on nociceptive pathways. In this context, while intra-amygdala activation of 5-HT_{1A} receptor attenuated the OAA (despite producing an intrinsic effect on nociceptive response in EA-confined animals), local injection of MK-212 (i.e. a drug that activates 5-HT_{2C} receptor) selectively enhanced OAA. Interestingly, while prior systemic injection of fluoxetine did not change the effects of 8-OH-DPAT on nociceptive response, this SSRI prevented the OAA enhancement induced by intra-amygdala injection of MK-212, suggesting that fluoxetine may have acted as a 5-HT_{2C} receptor antagonist. This hypothesis seems to be strengthened by the similar effect obtained with combined intra-amygdala injections of SB 242084, a selective 5-HT_{2C} receptor antagonist, and MK-212.

Disclosure

The authors declare no conflicts of interest related to the research or manuscript.

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