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μ-Opioid and 5-HT1A receptors in the dorsomedial hypothalamus interact for the regulation of panic-related defensive responses

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Abstract

The dorsomedial hypothalamus (DMH) and the dorsal periaqueductal gray (DPAG) have been implicated in the genesis and regulation of panic-related defensive behaviors, such as escape. Previous results point to an interaction between serotonergic and opioidergic systems within the DPAG to inhibit escape, involving μ-opioid and 5-HT1A receptors (5-HT1AR). In the present study we explore this interaction in the DMH, using escape elicited by electrical stimulation of this area as a panic attack index. The obtained results show that intra-DMH administration of the non-selective opioid receptor antagonist naloxone (0.5 nmol) prevented the panicolytic-like effect of a local injection of serotonin (20 nmol). Pretreatment with the selective μ-opioid receptor (MOR) antagonist CTOP (1 nmol) blocked the panicolytic-like effect of the 5-HT1AR agonist 8-OHDPAT (8 nmol). Intra-DMH injection of the selective MOR agonist DAMGO (0.3 nmol) also inhibited escape behavior, and a previous injection of the 5-HT1AR antagonist WAY-100635 (0.37 nmol) counteracted this panicolytic-like effect. These results offer the first evidence that serotonergic and opioidergic systems work together within the DMH to inhibit panic-like behavior through an interaction between μ-opioid and 5-HT1A receptors, as previously described in the DPAG.

Keywords

Panic, endogenous opioid, serotonin, dorsomedial hypothalamus, electrical stimulation

Introduction

Electrical stimulation of the dorsal periaqueductal gray (DPAG) matter elicits escape behavior in experimental animals as well as subjective and neurovegetative manifestations that resemble a panic attack in humans (Del-Ben and Graeff, 2009; Graeff et al., 1993; Jenck et al. 1995; Lovick, 2000), and has been validated as an animal model of panic (Moreira et al., 2013; Schenberg et al., 2001). The neurons that regulate panic-like responses in the DPAG are modulated by several neurotransmitters, among which serotonin (5-HT) and endogenous opioid peptides exert a major role (Eichenberger et al., 2002; Graeff et al., 2015; Ribeiro et al., 2005).

Employing electrical stimulation of the rat DPAG, we obtained results indicating an interplay between 5-HT and endogenous opioids in the DPAG to control panic-like behavior, effected through a cooperative interaction between the 5-HT1A receptor (5-HT1AR) and the μ-opioid receptor (MOR). Particularly with the help of selective ligands of these receptors, it has been shown that both the 5-HT1AR agonist 8-OHDPAT and the MOR agonist DAMGO increased the threshold electrical current intensity applied into the DPAG necessary to elicit escape. Moreover, there was reciprocal antagonism between the two receptors, in such a way that the MOR antagonist CTOP blocked not only the anti-escape effect of DAMGO, but also that of 8-OHDPAT; conversely, the 5HT1AR antagonist WAY-100635 blocked the anti-escape effect of both 8-OHDPAT and DAMGO. Furthermore, the combination of sub-effective doses of 8-OHDPAT and DAMGO had a significant anti-escape effect

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(Rangel et al., 2014; Roncon et al., 2013). Similar results have been obtained with another well-validated panic model, the escape task performed by rats in the elevated T-maze (Roncon et al., 2013).

As in the DPAG, electrical and chemical stimulation of the medial hypothalamus (MH) have been shown to elicit panic-like responses in laboratory animals (De Freitas et al., 2013, 2014; Freitas et al., 2009; Milani and Graeff, 1987; Silveira and Graeff, 1992; Ullah et al., 2015, 2017). Similar to findings in the DPAG (Nogueira and Graeff, 1995), stimulation of 5-HT1AR and 5-HT2AR within the dorsomedial hypothalamus (DMH) impair the escape response evoked by electrical stimulation of this region (De Bortoli et al., 2013), as well as the escape response performed in the elevated T-maze (Nascimento et al., 2014). In addition, rats chronically infused with a GABA synthesis inhibitor into the DMH become vulnerable to panic-like manifestations induced by systemic injection of sodium lactate, similar to panic patients (Liebowitz et al., 1984). This vulnerability was attributed to the lack of activation of 5-HT-containing neurons localized in the lateral wings of the dorsal raphe nucleus and neighboring ventrolateral PAG column that project rostrally to the DPAG and caudally to sympathomotor control areas in the rostral ventrolateral medulla oblongata (Johnson et al., 2004). These projections are supposed to inhibit the behavioral and neurovegetative manifestations of the panic attack (Johnson et al., 2008).

Because of the reciprocal anatomical connections between the medial and posterior hypothalamic nuclei and the PAG (Falconi-Sobrinho et al., 2017; Ullah et al., 2017), it is likely that the two diencephalic and mesencephalic structures work together to control panic-like responses in experimental animals and, supposedly, panic attacks in patients diagnosed with panic disorder (Canteras, 2002; Canteras and Graeff, 2014; De Bortoli et al., 2013). This possibility prompted us to further explore the neurotransmitter regulation of escape responses in the DMH. As a consequence, the present study aimed to explore the interaction between 5-HT and opioids in the DMH by using electrical stimulation and intra-cerebral drug injections of receptor ligands. First, we verified whether the non-selective opioid receptor antagonist naloxone is able to block the anti-escape effect of 5-HT. Then, we investigated whether the 5-HT1AR agonist 8-OHDPAT and the MOR agonist DAMGO have an anti-escape effect, and, if so, whether this effect is reciprocally blocked by the 5-HT1AR antagonist WAY-100635 and the MOR antagonist CTOP.

Materials and methods

Animals

Male Wistar rats (260–280 g) from Ribeirão Preto Medical School of the University of São Paulo, Brazil (FMRP-USP) were housed in groups of 4−5 per cage under a 12h light/dark cycle (lights on at 07:00) at 22 ± 1°C, with free access to food and water, except during tests. All experiments were performed in accordance with the Brazilian Society of Neuroscience and Behavior (SBNeC) for the care and use of laboratory animals and were approved by the Experimental Animal Ethical Committee of the Ribeirão Preto Medical School, University of São Paulo, Brazil (protocol number: 153/2012). Efforts were made to minimize animal suffering and to reduce the number of animals employed.

Apparatus

Escape behavior induced by electrical stimulation of the DMH was evaluated in a 40 cm diameter circular arena surrounded by 40 cm high walls made of transparent Plexiglas®. A sine-wave stimulator generated the electrical stimulation (Marseillan, 1977). The stimulation current (peak to peak) was monitored on the screen of an oscilloscope (Minipa, Brazil). The brain electrode was connected to the stimulator by means of an electromechanical swivel and a flexible cable, allowing ample movement of the animal inside the experimental cage.

Drugs

The following drugs were used: 5-hydroxytryptamine creatinine sulfate (5-HT; Sigma, USA), (±)-8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide (8-OHDPAT; Sigma, USA), D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP; Tocris, UK), [D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin acetate salt (DAMGO; Sigma, USA), Naloxone hydrochloride (NAL; Sigma, USA), N-(2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl)-N-2-pyridinyl cyclohexanecarboxamide maleate (WAY-100635; Sigma, USA). All drugs were dissolved in sterile saline (0.9%) and freshly prepared before testing

Surgery

Rats were anesthetized with 2,2,2 tribromoethanol (250 mg/kg, i.p.) and scalp local anesthesia (2% lidocaine with a vasoconstrictor; Harvey, Brazil) and fixed in a stereotaxic frame (Stoelting, USA) for the implant of a chemitrode into the DMH.

The chemitrode was made of a stainless-steel guide cannula (outside diameter 0.6 mm, 16 mm long) glued to a brain electrode made of stainless-steel wire (diameter 0.25 mm), that was enamel insulated, except at the cross-section of the tip, reaching 1.5 mm below the lower end of the cannula. The electrode wire was connected to a male pin, parallel to the outer end of the cannula, which could be plugged into a socket at the end of a flexible electrical cable for brain stimulation. The chemitrode was implanted using coordinates according to the atlas of Paxinos and Watson (2007).

With the incisor bar 3.5 mm below the horizontal plane, the chemitrode was vertically and unilaterally introduced using the following coordinates, with bregma serving as the reference: anteroposterior −2.8 mm; mediolateral ± 0.3 mm, and dorsoventral −8.7 mm.

The chemitrodes were attached to the skull by means of acrylic resin and two stainless-steel screws. A stylet with the same length as the guide cannula was introduced inside it to prevent obstruction.

At the end of the surgery, all animals were intramuscularly injected (0.1 mL/100 g) with pentabiotic preparation (benzylpenicillin and streptomycin; Forte Dodge, Brazil) to prevent possible infections. In addition, flunixin meglumine (Schering Plough, Brazil; 0.01 mL/100 g), a drug with analgesic, antipyretic and anti-inflammatory properties, was administered subcutaneously for post-surgery analgesia. The animals were left undisturbed for 5–7 days after the surgery, with the exception of handling for cage cleaning.

Procedures

Intra-DMH injections. All of the drugs were injected into the DMH. A needle (outside diameter, 0.3 mm, 17.5 mm length) was introduced through the guide cannula until its tip was 1.5 mm below the cannula end. A total volume of 0.2 μL was injected for 120 s using a 10 μL microsyringe (Hamilton 701-RN, USA) attached to a microinfusion pump (KD Scientific, USA). The displacement of an air bubble inside the polyethylene catheter connecting the syringe needle to the intra-cerebral needle was used to monitor the microinjection. The needle was removed 60 s after the injection was finished.

Escape threshold determination. Animals were gently handled by the experimenter for 5 min, 48 and 24 h before the tests. On test day, animals were placed into the experimental cage, and the escape threshold was determined through an electrical stimulus (AC, 60 Hz, 10 s) presented through the implanted chemitrode. The inter-stimulus interval was 10 s. The current intensity started at a level of 20 μA and was increased by steps of 4 μA until the rat presented running or jumping reactions, characterizing the escape behavior. When these behaviors were observed, the experimenter interrupted application of electrical stimulation to the DMH. The basal escape threshold was defined as the lowest current intensity that evoked escape in three successive trials of electrical stimulation. Animals with basal thresholds above 152 μA were excluded from the study. Drug effects were determined as the difference between post- and pretreatment escape thresholds. An increase in this value was taken as a panicolytic-like effect. All the treatment groups were independent and rats were tested only once. In addition, the experimenters were blind to the treatment conditions during the tests, which were conducted in a room with light intensity at 60 lux.

Experiment 1. We investigated whether the panicolytic-like effect of 5-HT is blocked by naloxone. To this end, 10 min after the determination of the basal escape threshold, rats were pretreated with naloxone (0.5 nmol) or saline and 10 min later with 5-HT (20 nmol) or saline. Thus, the following groups (n = 7–9) were formed: saline/saline, naloxone/saline, saline/5-HT and naloxone/5-HT. At 10 min after the last injection, the escape threshold was redetermined.

Experiment 2. The effects of CTOP and DAMGO were assessed. After 10 min from the determination of basal escape threshold, rats were microinjected with saline or CTOP (1.0 or 2.0 nmol; experiment 2a; n = 8) and saline or DAMGO (0.1 nmol or 0.3 nmol; experiment 2b; n = 6–7) into the DMH, and the escape threshold were redetermined 20 or 10 min later for CTOP and DAMGO, respectively.

Experiment 3. We evaluated whether MOR and 5-HT1AR interact. In experiment 3a, 10 min after the determination of the basal escape threshold, rats were injected into the DMH with CTOP (1 nmol) or saline and 10 min later with 8-OHDPAT (8 nmol) or saline. Thus, the following groups (n = 5–7) were formed: saline/saline, saline/DPAT, CTOP/saline and CTOP/DPAT. At 10 min after the last injection, the escape threshold was determined again. In experiment 3b, 10 min after the determination of the basal escape threshold, rats were pretreated with the 5HT1AR antagonist, WAY-100635 (0.37 nmol) or saline and 10 min later with DAMGO (0.3 nmol) or saline. Thus, the following groups (n = 5–6) were formed: saline/saline, WAY/saline, saline/DAMGO and WAY/DAMGO. After 10 min from the last injection, the escape threshold was redetermined.

The doses of the drugs used in all these experiments were selected on the basis of previous results with the DPAG (De Bortoli et al., 2013; Rangel et al., 2014; Roncon et al., 2012, 2013).

Histology. After the experiments, animals were sacrificed under deep anesthesia with chloral hydrate. Each brain was perfused through the heart with saline solution followed by 10% formalin solution, before being removed and fixed in 10% formalin. Serial 60-μm coronal sections were cut using a cryostat to localize the positions of the chemitrode tips according to the atlas of Paxinos and Watson (2007). Only data from rats having chemitrode tips inside the DMH were included in the statistical analysis.

Statistical analysis

Data from experiments 1, 3a and 3b were analyzed by two-way analysis of variance (ANOVA), where pretreatment (antagonists or saline) and treatment (agonists or saline) were considered as independent factors. Data from experiments 2a and 2b were analyzed by one-way ANOVA with the treatment as the only independent factor. When appropriate, post-hoc comparisons were performed by Tukey's test. The level of significance was set at $p < 0.05$. The Statistica Six Sigma (StatSoft Inc., Tulsa, USA) software was used for the statistical analysis.

Results

Figure 1 depicts the sites of chemitrode placements in the DMH of animals tested and included in the current study.

Experiment 1: Antagonism of anti-escape effect of 5-HT by naloxone

Figure 2 shows that 5-HT injection into the DMH increased the escape threshold, and this panicolytic-like effect was blocked by the pretreatment with naloxone. A two-way ANOVA revealed significant effects of pretreatment with naloxone ($F_{(1,27)} = 17.59$, $p < 0.01$) and treatment with 5-HT ($F_{(1,27)} = 28.56$, $p < 0.01$), as well as a pretreatment × treatment interaction ($F_{(1,27)} = 23.19$, $p < 0.01$). Post-hoc comparisons showed that 5-HT significantly increased the escape threshold compared with control and this effect disappeared when 5-HT was given after naloxone.

Experiment 2: Assessment of CTOP and DAMGO

In the experiment 2a, one-way ANOVA showed no significant effect of intra-DMH treatment with CTOP (Figure 3, panel A) on the escape threshold (treatment effect $F_{(2,21)} = 2.78$; NS). Figure 3 (panel B) shows that intra-DMH microinjection of DAMGO significantly increased escape threshold (treatment effect $F_{(2,16)} = 13.67$, $p < 0.01$). The post-hoc test revealed that DAMGO 0.3

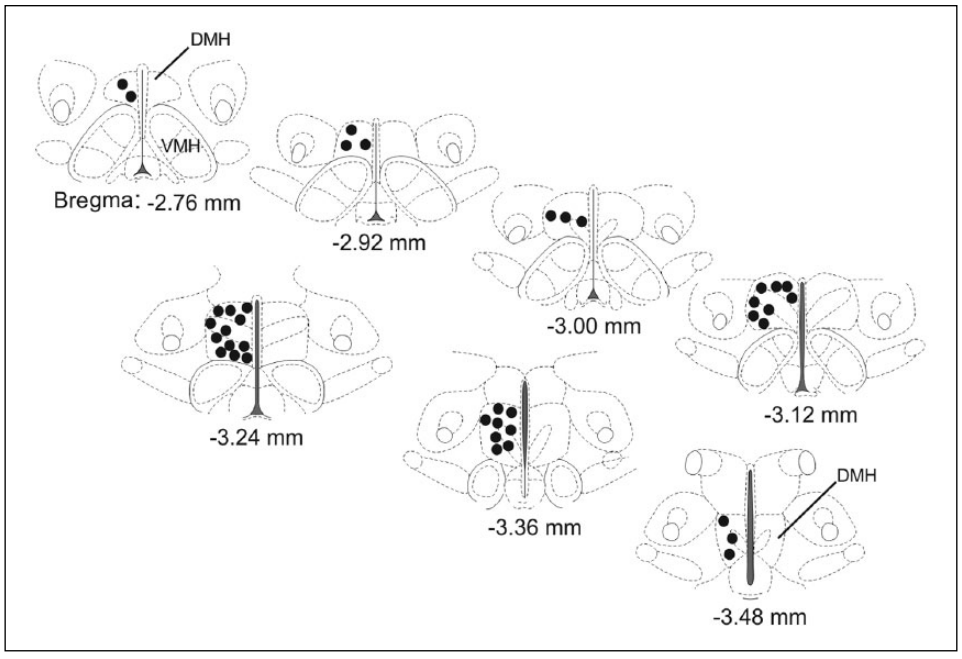

Figure 1. Diagrammatic representation of coronal sections of the rat brain showing the location of chemitrodes and injection sites (dark circles) in the DMH. The figures represent coordinates from the Paxinos and Watson (2007) rat brain atlas. The number of points shown is fewer than the total number of rats used because of several instances of overlap.
DMH: dorsomedial hypothalamus; VMH: ventromedial nucleus of the hypothalamus.

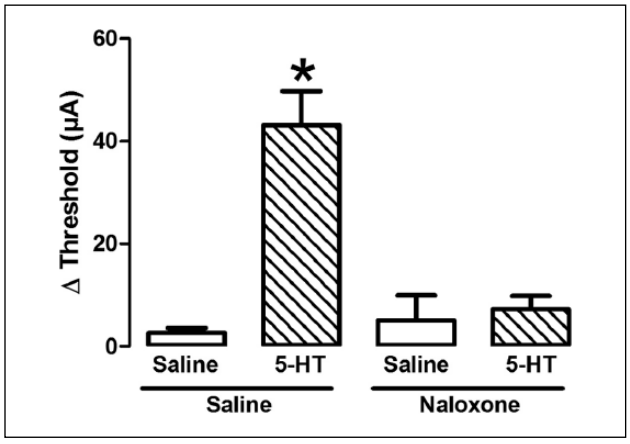

Figure 2. Antagonism of the anti-escape effect of 5-HT (20 nmol) by naloxone (0.5 nmol) in the DMH. The data are expressed as mean + SEM. The change in threshold (Δ) is the difference between escape threshold values (μA) obtained pre- and post-administration of the drugs. *$p < 0.01$, compared with all treatment groups.
DMH: dorsomedial hypothalamus; SEM: standard error of the mean.

nmol significantly increased the escape threshold compared with the control group.

Experiment 3a: Antagonism of the anti-escape effect of 8-OHDPAT by CTOP

Figure 4 (panel a) shows that intra-DMH injection of 8-OHDPAT significantly increased the escape threshold, and this panicolytic-like effect was blocked by pretreatment with CTOP. The two-way ANOVA revealed significant effects of pretreatment with CTOP ($F_{(1,19)} = 22.49$, $p < 0.01$) and treatment with 8-OHDPAT ($F_{(1,19)} = 39.78$, $p < 0.01$), as well as an interaction between pretreatment and treatment ($F_{(1,19)} = 34.76$, $p < 0.01$).

Experiment 3b: Antagonism of the anti-escape effect of DAMGO by WAY-100635

Figure 4 (panel b) shows that DAMGO injection into the DMH significantly increased the escape threshold, and this panicolytic-like effect was counteracted by pretreatment with WAY-100635. The two-way ANOVA revealed significant effects of pretreatment with WAY ($F_{(1,17)} = 35.92$, $p < 0.01$) and treatment with DAMGO ($F_{(1,17)} = 35.92$, $p < 0.01$), as well as a pretreatment × treatment interaction ($F_{(1,17)} = 21.73$, $p < 0.01$).

Discussion

Replicating the strategy previously used in the DPAG, we presently explored the interaction between serotonergic and opioidergic mechanisms in the DMH by measuring the effect of intra-DMH injection of agonists and antagonists of 5-HT and opioid receptors on the escape response elicited by electrical stimulation of the DMH.

The obtained results show that 5-HT increased the threshold intensity of the electrical current needed to elicit escape, considered a panicolytic-like effect. This effect was counteracted by previous administration of the non-selective opioid antagonist naloxone. These findings are a first indication of interaction between 5-HT and opioids in the DMH. Similar results have been

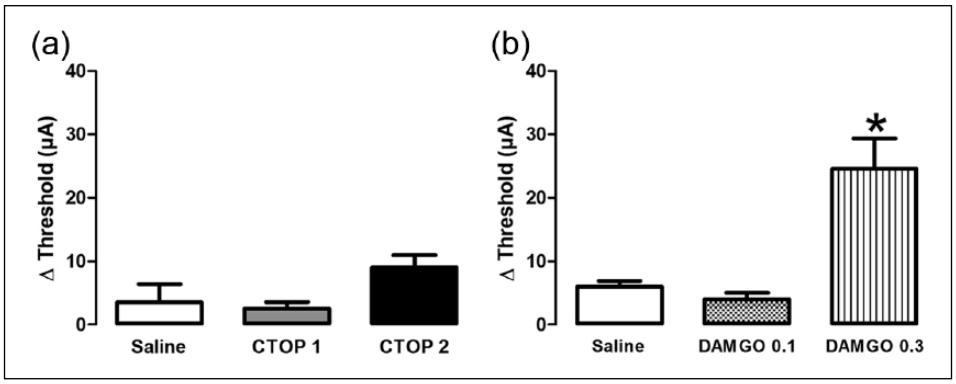

Figure 3. (a) No effect of the MOR antagonist CTOP (1.0 and 2.0 nmol) and (b) anti-escape effect of the MOR agonist DAMGO (0.3 nmol) injected into the DMH. For further details, see Figure 2 legend. *$p < 0.05$, compared with the saline-injected group.
DMH: dorsomedial hypothalamus; MOR: μ-opioid receptor.

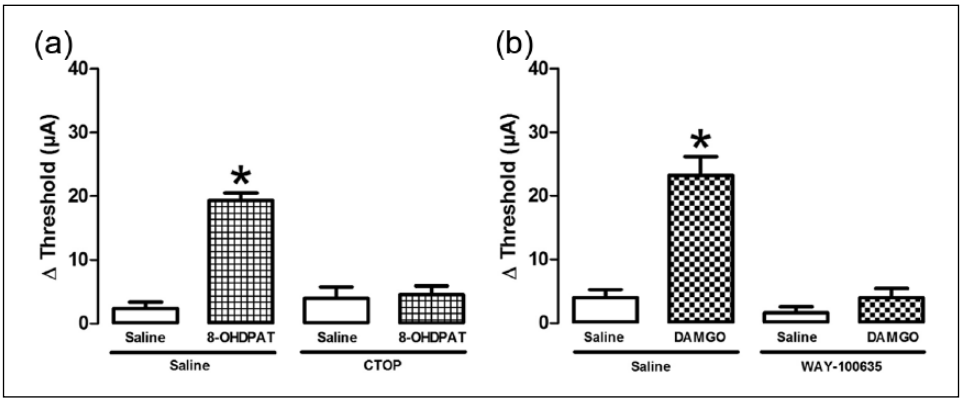

Figure 4. (a) Previous administration of CTOP (1.0 nmol) blocked the anti-escape effect of 8-OHDPAT (8.0 nmol) evoked by electrical stimulation of the DMH. (b) Pre-administration of WAY-100635 (0.37 nmol) antagonized the anti-escape effect of DAMGO (0.3 nmol). For further details, see Figure 2 legend. *$p < 0.01$, compared with all groups.
DMH: dorsomedial hypothalamus.

reported in the DPAG with escape performed in the elevated T-maze test (Roncon et al., 2012).

Because evidence obtained in the DPAG pointed to the 5-HT1AR and the MOR as the receptor types involved in this interaction (Rangel et al., 2014), we used selective ligands of these receptors to carry on our exploration. In this way, we found that both the 5-HT1AR agonist 8-OHDPAT and the MOR agonist DAMGO had a panicolytic-like effect, and that there was a crossed antagonism by the respective receptor blockers. Thus, pretreatment with the MOR antagonist CTOP, which is reported to block electrophysiological and behavioral effects of DAMGO in different brain areas (e.g. Gear and Levine, 2011; Kishimoto et al., 2001), prevented the panicolytic-like effect of 8-OHDPAT in the present study. Conversely, previous treatment with WAY-100635 blocked the panicolytic-like effect induced by intra-DMH injection of DAMGO. It is noteworthy that previous intra-DMH injection of this 5-HT1AR antagonist also counteracted the anti-escape effect caused by local injection of 8-OHDPAT in rats tested in another experimental model that associates escape with panic, the elevated T-maze (Nascimento et al., 2014).

Again, these results are similar to those previously reported in the DPAG with either escape induced by electrical stimulation or escape performed in the elevated T-maze (Rangel et al., 2014; Roncon et al., 2013). Therefore, in both brain structures an interaction between 5-HT1AR and MOR for the regulation of panic-like defensive behavior seems to occur.

Reinforcing the parallelism between the regulation of panic-like behavior in the DPAG and in the DMH, former results have shown that stimulation of either the 5-HT1AR or the 5-HT2AR within the DMH has an anti-escape effect in the electrical stimulation of this area (De Bortoli et al., 2013), and that intra-DMH administration of 8-OHDPAT inhibits escape responses in the elevated T-maze (Nascimento et al., 2014). Similar results have been reported in the DPAG with the electrical stimulation of this area (De Bortoli et al., 2006, 2008) and in the elevated T-maze test (De Paula Soares and Zangrossi, 2004). It is unlikely that the effects caused by the injection of the current drugs in the DMH are due to diffusion to the DPAG, since previous work from our group has shown that the escape threshold evoked by electrical stimulation of the DMH was not affected by intra-DPAG injection of either 5-HT1AR or 5-HT2AR agonists (De Bortoli et al., 2013).

Rats chronically infused with a GABA synthesis inhibitor in the DMH become vulnerable to panic-like manifestations induced by systemic injection of sodium lactate (Johnson and Shekhar, 2012), seemingly because these animals fail to activate

5-HT-containing neurons localized in the lateral wings of the dorsal raphe nucleus (DRN) and neighboring ventrolateral PAG that project rostrally to the DPAG and caudally to sympathomotor control areas in the rostral ventrolateral medulla (Johnson et al., 2004). These projections are supposed to inhibit the behavioral and neurovegetative manifestations of the panic attack, respectively (Johnson et al., 2008). Interestingly, a recent study from our research group has shown that 5-HT1AR-containing neurons in the MH receive serotonergic projections from the DRN, and that stimulation of 5-HT1ARs with 8-OHDPAT inhibited panic-like defense reactions induced by intra-DMH injection of the GABAA receptor-blocker bicuculline (Biagioni et al., 2016). Therefore, ascending 5-HT pathways from the DRN to the DMH may also modulate panic-like defensive behaviors, in addition to the DPAG.

There is reported evidence indicating that formation of heterodimers underpins the cooperative interaction between 5-HT1AR and MOR, suggested also by our results. In this regard, an in vitro study by Cussac et al. (2012) used tagged 5-HT1A and MORs and their respective anti-tag antibodies in cell lines. Their results showed that the immune-precipitated receptor complexes contained each other's receptors when both were co-expressed. In addition, the MOR agonist DAMGO was not capable of activating 5-HT1AR by itself, but only when the serotonergic receptor was co-expressed with MOR, suggesting that DAMGO cannot activate 5-HT1AR directly. Such evidence, if shown to occur in the DPAG and DMH neurons, would provide a molecular basis for the above pharmacological results with animal models of panic.

Summing up the last results with the experimental evidence obtained in laboratory animals mentioned in the introduction (Freitas et al., 2009; Milani and Graeff, 1987; Silveira and Graeff, 1992; Ullah et al., 2015, 2017), and taking into account the existence of anatomical interconnections between the dorsomedial, the posterior hypothalamus and the DPAG (Canteras, 2002; Canteras and Graeff, 2014; Falconi-Sobrinho et al., 2017; Ullah et al., 2017), it may be suggested that these brain structures work together to regulate the behavioral manifestations of a panic attack. If so, the present results indicating an interplay between serotonergic and opioidergic mechanisms controlling defensive behavior in the DMH, which in the DPAG has been shown to be affected by drugs that have actual or potential usefulness for the treatment of panic disorder (De Bortoli et al., 2006, 2008; Jacob et al., 2002; Maraschin et al., 2015; Roncon et al., 2012), may have clinical implications that deserve further exploration.

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