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Research report

5-HT_2- and D_1-mechanisms of the basolateral nucleus of the amygdala enhance conditioned fear and impair unconditioned fear

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Abstract

The inferior colliculus (IC) is involved in processing of auditory information, but also integrates acoustic information of aversive nature. In fact, chemical stimulation of the IC with semicarbazide (SMC) – an inhibitor of the GABA synthesizing enzyme glutamic acid decarboxylase – has been found to cause defensive behavior in an open-field test and functions as an unconditioned stimulus in the place conditioned aversion test (PCA). A question has arisen regarding whether the basolateral nucleus of the amygdala (BLA) is involved in the acquisition of the aversive information ascending from the IC and whether dopaminergic and serotoninergic mechanisms of the BLA regulate this process. Recent evidence has shown that inactivation of the BLA with muscimol inhibits the PCA and causes an increase in the aversiveness of the chemical stimulation of the IC. Based on this, we examined the effects of ketanserin and SCH-23390, antagonists of the $5HT_2$ and D_1 receptors, respectively, on the conditioned and unconditioned fear elicited by IC stimulation with SMC. The results obtained confirm the crucial role of 5-HT_2- and D_1-mechanisms of the BLA on conditioned fear in that ketanserin and SCH-23390 injections into the BLA caused a reduction in the PCA. On the other hand, ketanserin and SCH-23390 injections into the BLA enhanced the aversiveness of the IC injections of SMC. These findings suggest that while 5-HT_2 and DA_1 mechanisms in the BLA appear to facilitate the conditioned fear they inhibit the unconditioned fear triggered by IC activation.

Keywords: 5-HT; Dopamine; Inferior colliculus; Basolateral amygdala; Unconditioned fear; Conditioned fear

1. Introduction

It has been proposed that the amygdala, the medial hypothalamus, the dorsal periaqueductal gray (dPAG) and the superior and inferior colliculi together constitute the brain aversion system, which has been related to the organization of fear [5–7,26]. The inferior colliculus (IC) is primarily involved in processing of auditory information, but it also integrates acoustic information of aversive nature [5,6,10,11]. Electrical stimulation of the IC induces defensive responses such as arousal, freezing, and escape that mimic fearful behavior elicited by environmental challenges [5,6,10,11,72]. In addition, much evidence has demonstrated that GABA has a regulatory function on the aversive state generated and elaborated in the IC [5,6,10,11]. In this context, semicarbazide (SMC) – an inhibitor of the glutamic acid decarboxilase [9,32] – has been used in behavioral studies on defensive behavior because it causes a slow and gradual reduction in the brain GABA levels so as to allow the study of the hierarchical expression of defensive reactions [1,9,56]. Chemical stimulation of the IC with SMC functions as an unconditioned stimulus in the place conditioned aversion test (PCA).

A differential involvement of the amygdaloid nuclei in the regulation of unconditioned and conditioned fear is well established. For instance, the BLA is crucial for the acquisition of conditioned fear while the CeA is crucial for the expression of innate and learned responses [20,23,36–38]. It has been found that inactivation of either the BLA or the CeA causes distinct changes in the aversiveness of the electrical stimulation of the

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IC; that is, electrolytic lesions of the BLA or CeA caused an increase or reduction, respectively, of the aversiveness of the electrical stimulation of the IC [49]. However, the regulatory mechanisms of conditioned and unconditioned fear may differ even within a given amygdaloid nucleus. Indeed, injections of muscimol into the BLA have been found to cause proaversive effects in rats placed in an open-field test and to reduce the conditioned place aversion when using injections of SMC into the IC as unconditioned stimulus [43]. In view of these results, it has been proposed that the filtering mechanisms in the BLA have special characteristics when the aversive states are generated at the level of the IC. Furthermore, such proposal opens the need for studies that extend our knowledge of the involvement of GABA regulation of BLA on conditioned and unconditioned fear also for the involvement of other neurotransmitters such as 5-HT and DA.

The dopamine (DA) and serotonin (5-HT) biogenic amine systems have been studied extensively in the neurobiology of fear and anxiety [27–29,47,51,60,63,64]. It has been shown that electrolytic or neurotoxic lesions of 5-HT neurons in the BLA, but not in the CeA, as well as blockade of the 5-HT_2 receptors in the BLA, increase the aversiveness of the electrical stimulation of the IC [44,48,49]. Based on these findings, we set out to determine whether injections of ketanserin (0.5 and 1.0 μg/0.2 μl) or SCH23390 (1.0 and 2.0 μg/0.2 μl) into the BLA would affect the conditioned fear (evaluated by the corral test) or the unconditioned fear (assessed by the open-field test) produced by chemical stimulation of the IC using SMC as unconditioned stimulus. In the PCA test, the conditioned fear was measured from the time rats spent in the quadrant in which they had previously experienced the aversive effects of SMC injected into the IC. The freezing behavior and behavioral activation (running) induced by injections of SMC into the IC were the unconditioned responses measured by means of an open field test. Ketanserin shows good selectivity for the 5-HT_2 receptor [2,39] and SCH 23390 for D_1 receptors [24,30]. We have selected to study the role of 5-HT_2 receptors because many of the drugs currently used to treat anxiety disorders affect this type of 5-HT receptor [53]. For instance, although the 5-HT_{2A} and 5-HT_{2C} subtypes have been mainly associated with anxiety, chronic administration of several antidepressant drugs that benefit patients with anxiety disorders downregulates 5-HT_{2A} receptors in the rat brain [27]. Moreover, the majority of binding studies showing 5-HT_2 receptor downreguation have used [H^3] ketanserin [75]. The rationale to evaluate the role of the D_1 receptor subtype in the conditioned and unconditioned fear is based on the fact that DA receptors in the BLA belongs almost exclusively to the D1-like category [41,68,74].

2. Methods

2.1. Animals

One hundred ninety-nine male Wistar rats weighing 250–300 g, obtained from the animal house of the campus of the University of São Paulo at Ribeirão Preto, were housed in a temperature-controlled (22 ± 1 °C) room and maintained on a 12 h light/12 h dark cycle with lights on at 7:00 a.m. They were housed in groups of five per cage and given free access to food and water. All testing in the experiments was conducted during the light phase of the light/dark cycle. The experiments reported in this paper were performed in compliance with the recommendations of the SBNeC (Brazilian Society of Neuroscience and Behavior), which are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Surgery

The animals were anesthetized with tribromoethanol (250 mg/kg, i.p.) and placed in a stereotaxic frame (David Kopf, USA) with the incisor bar at −3.3 mm below interaural zero. Each animal was implanted with unilateral stainless steel guide-cannulae (17 mm, 24 G) aimed at the IC and BLA of the same side of the brain. Taking bregma as the reference point for each plane, the coordinates for IC were: antero-posterior (AP) = −8.2 mm; medio-lateral (ML) = 1.6 mm; and dorso-ventral (DV) = −5.0 mm, and the coordinates for the BLA were: AP = −2.8 mm, ML = 5.0 mm, DV = −8.7 mm [61]. The basolateral complex of the amygdala (BLA) is considered here to be composed of the lateral, basal, and accessory basal nuclei. At the end of the surgery, the guide-cannulae were fixed with the aid of two stainless steel screws and dental cement, and were sealed with a stainless steel wire to protect it from obstruction.

2.3. Drugs

Ketanserin, SCH-23390 and semicarbazide (Sigma, USA) were dissolved and diluted to the desired concentration with saline (0.9%) shortly before use. Independent groups were tested with only one combined treatment BLA versus IC. Semicarbazide was injected at a dose of 6 μg/0.2 μl into the IC, and ketanserin at doses of 0.5 and 1 μg/0.2 μl, and SCH-23390 at doses of 1 μg and 2 μg/0.2 μl into the BLA. The efficacy of these doses has been reported in several other studies from this and other laboratories [1,34,56,57,73].

2.4. Microinjections of drugs

The animals were gently wrapped in a cloth, and a thin infusion-cannula (outer diameter 0.3 mm) was introduced through the guide-cannula until its lower end was 1 mm below the guide-cannula. The infusion-cannula was linked to a 5 μl Hamilton syringe by means of polyethylene tubing (PE-10; Becton-Dickinson, USA). The solutions were injected into the BLA or IC (0.2 μL/min) with the help of an infusion pump (Harvard Apparatus, USA). The displacement of an air bubble inside the polyethylene catheter connecting the syringe needle to the intracerebral infusion-cannula was used to monitor the microinjection. The infusion-cannula was held in place for an additional 1 min to maximize diffusion away from the tip. The displacement of an air bubble inside the PE-10 catheter connecting the syringe needle to the intracerebral needle was used to monitor the microinjection. This procedure has already been used successfully in this laboratory [44–46,56].

2.5. Procedure

In both tests, place-conditioned aversion and open-field tests, the rats were randomly assigned to one of the groups defined in Table 1.

The animals received two injections: one injection of ketanserin (0.5 or 1 μg/0.2 μl) or SCH-23390 (1 or 2 μg/0.2 μl) or saline into the BLA, followed 10 min later by one injection of semicarbazide or saline into the IC. Then, 5 min after the second injection the animals were placed in one quadrant of the corral for the conditioning sessions in the place aversion test, or in the middle of the arena for the unconditioned fear test. All intracerebral injections into the BLA or IC were done in a volume of 0.2 μl.

2.6. Apparatus

Conditioned testing (corral procedure) or unconditioned testing (open-field test) was conducted 1 week after surgery. In both procedures the rats were placed in an arena, a circular enclosure 60 cm in diameter and 50 cm high, in an isolated room and illuminated with an incandescent lamp (50 lux at the level of the arena floor). For the conditioned place aversion test, two crossed lines on the

Table 1
Groups with the respective number of rats allocated to the experiments to assess the effects of injections of ketanserin and SCH 23390 into the basolateral nucleus of the amygdala (BLA) in the place-conditioned aversion (corral test) and open field tests

Groups (BLA and IC injections)	Abbreviation	*n*	
		Corral test	Open field test
Saline + saline	Sal + Sal	10	9
Saline + semicarbazide	Sal + SMC	8	9
Ketanserin (0.5 μg) + saline	K0.5 + Sal	8	6
Ketanserin (1 μg) + saline	K1 + Sal	8	7
Ketanserin (0.5 μg) + semicarbazide	K0.5 + SMC	8	9
Ketanserin (1 μg) + semicarbazide	K1 + SMC	8	8
Saline + saline	Sal + Sal	9	9
Saline + semicarbazide	Sal + SMC	11	9
SCH-23390 (1 μg/0.2 μl) + saline	SCH1 + Sal	8	7
SCH-23390 (2 μg/0.2 μl) + saline	SCH2 + Sal	8	7
SCH-23390 (1 μg/0.2 μl) + semicarbazide	SCH1 + SMC	8	9
SCH-23390 (2 μg/0.2 μl) + semicarbazide	SCH2 + SMC	8	8

Semicarbazide injections into the IC were used as the unconditioned stimulus.

floor marked four quadrants of equal size and identical floor and wall textures. External visual cues were provided by the position of a video camera, pictures and stripes on the wall, an arrangement of cages on one side, and the uneven structure of the ceiling. Wide-spectrum masking noise (70 dB) was provided by a noise generator. The behavior of the animal was recorded by the video camera and registered outside the experimental chamber with the aid of a computer. In the corral test, transparent Plexiglas barriers were inserted into the field over the perpendicular lines drawn on the floor during the conditioning sessions as described below. The open-field test was conducted under the same experimental conditions in just one session, in which the animal had free access to the entire enclosure, the floor of which was divided into eight sections instead of four as in the corral test. The apparatus was thoroughly cleaned after each session with damp and dry cloths. The experimental sessions were conducted during the same time of the day.

2.7. Corral test

The procedure was divided into three main phases and lasted 3 consecutive days in accordance with the original procedure [31]. It consisted of a baseline trial (on day 1), a treatment trial (on day 2) and a test trial (on day 3). Each baseline and testing trial lasted for 10 min, while each conditioning test (day 2) lasted for 30 min. During the baseline trial, the rat was placed in the center of the open field facing away from the experimenter and had free access to all parts of the apparatus (open corral). The time it spent in each of the four quadrants was scored manually with the aid of a computer. A rat was considered to be in a quadrant when the two forepaws were inside. For each rat, the treatment corral was determined to be one of the four quadrants in which it had spent neither the most, nor the least time during baseline (there was no evidence for significant preferences before drug treatment; see Section 3). The treatment quadrants were counterbalanced within each group and the animals were randomly assigned to the treatment. On day 2, transparent Plexiglas barriers were inserted into the field over the perpendicular lines drawn on the floor. The barriers forced the animals to remain in one quadrant (closed corral). On the treatment day, the rats were given ketanserin or SCH-23390 or saline in the BLA and SMC, or saline injections into the IC, and soon afterwards they were placed in the treatment corral, as previously determined. During the test period, acquired place preference or aversion was assessed. For this purpose, the Plexiglas barriers were removed, and the animal was placed in the center of the arena, where it had free access to the four quadrants. The time spent in each of the quadrants was scored as during the baseline trial. As a measure of gross locomotor activity, the number of entries into each of the four quadrants was recorded. Each animal was tested just once.

2.8. Open-field test

After the BLA and IC injections according to the procedure described above, the animals were immediately placed in the middle of the enclosure and their behavior was recorded. The following behavioral responses were analyzed during the 30 min testing sessions: number of crossings (into an adjacent floor section with all four paws) and the duration of freezing. Freezing was defined as the absence of movement of the body and vibrissa, except that required for respiration, for at least 6 s. All experiments were monitored live by a trained investigator, and also through a video camera positioned in front of the arena. The apparatus was thoroughly cleaned after each session using damp and dry cloths. At the end of the experiments the corral and open field were also cleaned with a 5% solution of ammonium.

2.9. Histology

Upon completion of the experiments, 0.2 μl of 2% Evans blue was microinjected into the BLA and the IC in order to mark the positions of the microinjection sites. A similar volume has been used successfully in our laboratory with good selectivity of drug action in several brain structures (Macedo et al., 2002, 2005a,b; Nobre et al., 2004). Afterwards, the animals were given a lethal dose of chloral hydrate (500 mg/kg, i.p.) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4). The brains were removed from the skulls and maintained in PBS solution for 2 h, and then cryoprotected in 30% sucrose in 0.1 M PBS until soaked. Serial 60 μm brain coronal sections were cut using a freezing microtome. They were mounted on gelatin-coated slides and stained with 5% cresyl violet (Sigma Aldrich, USA) in order to localize the positions of the microinjection sites according to the Paxinos and Watson atlas (2005). The microinjection sites were evaluated by microscopy. Data from rats with injection cannula tips located at sites outside the BLA or IC were not included in the present study.

2.10. Data analysis

In the place-conditioned aversion test, the time spent and number of entries into the treatment quadrants before (baseline) and after (test) BLA/IC microinjections (Sal + Sal; Sal + SMC; K0.5 + Sal; K1 + Sal; K0.5 + SMC; K1 + SMC; SCH1 + Sal; SCH2 + Sal; SCH1 + SMC; SCH2 + SMC) were expressed as mean ± S.E.M. Analysis of data was performed using the two-way repeated measures ANOVA, with treatments as between-group factor and trials (baseline and test) as within-group factor. In the open field test, time of freezing and number of crossings after the four treatments were analyzed using one-way ANOVA. Data from both behavioral tests were also analyzed with Newman–Keuls *post hoc* comparisons whenever significant overall *p*-values were obtained with ANOVA.

3. Results

The tips of the injection-cannulae were located within the BLA and the central and external nuclei of the IC. Representative sites of drug injections into the BLA and IC are shown in Fig. 1 (Panels A and B, respectively). Analysis of the spread of stain in all slides documenting this work revealed the good anatomical selectivity of our injection procedure. Panel C of Fig. 1 shows sites of injection of drugs into the BLA and IC.

3.1. Serotonin

During the baseline trial (day 1), the animals spent a comparable amount of time in each of the four quadrants (119.30 ± 14.32; 123.10 ± 11.23; 149.55 ± 24.44; 131.12 ± 15.79). There was no significant preference for any quadrant [$F(5, 44) = 1.08$; $p > 0.05$].

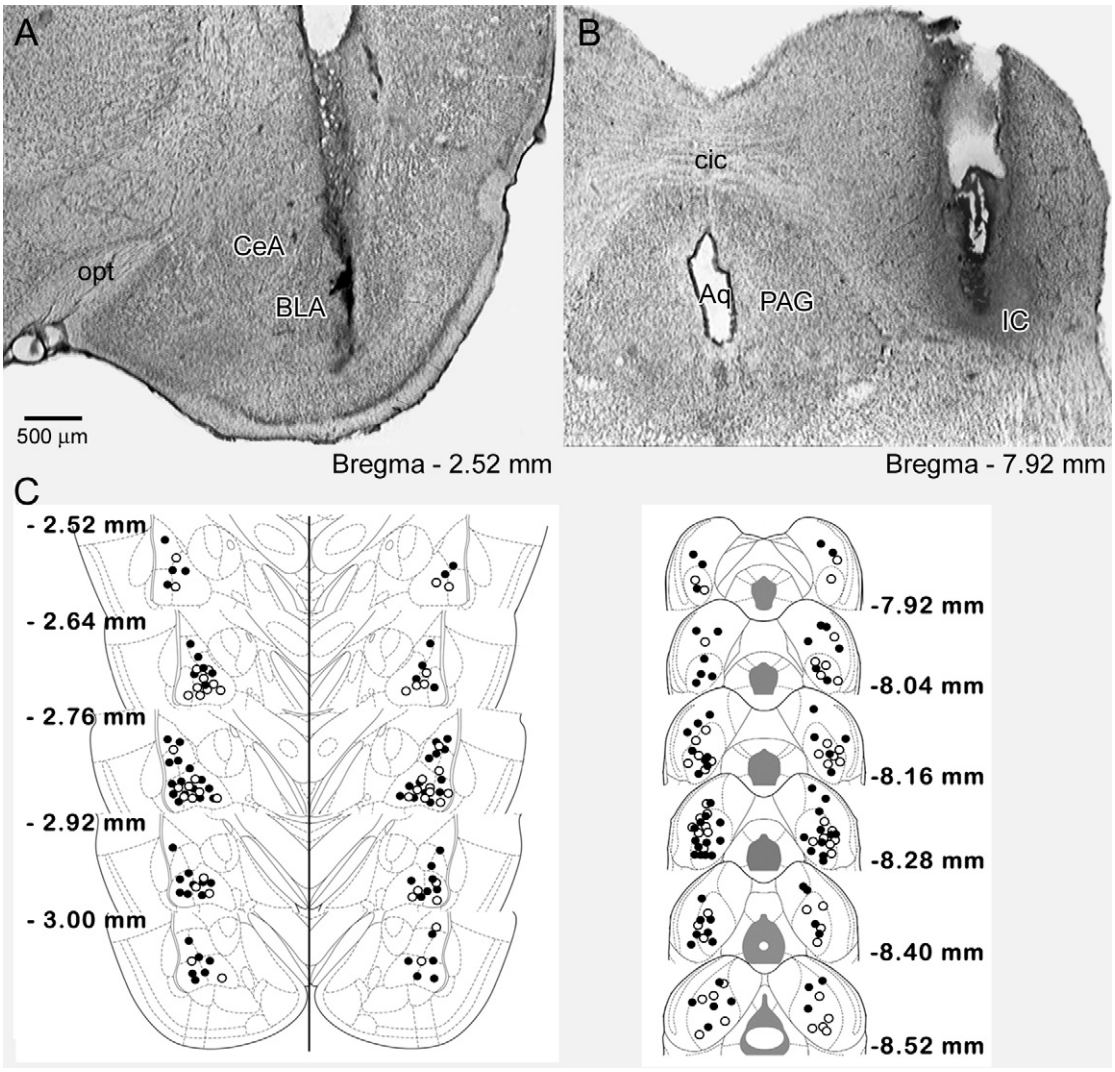

Fig. 1. Representative photomicrographs of microinjections (A) into the basolateral nucleus of the amygdala, and (B) into the inferior colliculus. Scale bar represents 500 μm. (C) Sites of microinjections into the BLA and IC. Open and closed symbols indicate injection sites for saline and drug injections, respectively. The injection sites for the ketanserin and SCH23390 experiments are represented on the left and right, respectively. The number of sites indicated in the figure is less than the number of injected animals because of several overlaps. Aq, aqueduct; BLA, basolateral complex of the amygdala; CeA, central nucleus of the amygdala; cic, commissure of the inferior colliculus; IC, inferior colliculus; opt, optic tract; PAG, periaqueductal gray.

As described later, during the treatment period of the corral test it was observed the same pattern of effects as the open-field test, with freezing being the only response observed for 8–10 min after injection of SMC into the IC. After this period of time, bouts of running interspersed with freezing behavior occurred in the SMC groups. An aversive effect of drug treatment was characterized by the occurrence of place avoidance, as reflected by a significant reduction in the amount of time spent in the treatment quadrant during the testing sessions. Fig. 2 (Panel A) depicts the mean amount of time spent in the treatment quadrant during baseline and test trials for the different treatment groups. Two-way repeated-measures ANOVA applied to the data obtained with the combined drug injections into the BLA and IC showed a statistically significant effect for treatments [$F(5, 44) = 7.67$; $p < 0.05$], trials [$F(1, 44) = 9.58$; $p < 0.05$] and interaction between treatments and trials [$F(5, 44) = 8.27$; $p < 0.05$]. *Post hoc* analysis revealed a reduction in the time spent in the treatment quadrant on the testing day for the Sal + SMC and K0.5 + SMC groups relative to the other treatment groups ($p < 0.05$). Within-group comparisons indicated that the animals in the Sal + SMC and K0.5 + SMC groups showed aversion to the treatment quadrant; that is, the time spent in the treatment quadrant was reduced in test trials ($p < 0.05$). *Post hoc* analysis also revealed that the groups Sal + Sal, K0.5 + Sal, K1 + Sal, and K1 + SMC were not statistically different ($p > 0.05$). Gross locomotor activity expressed as the mean number of entries into the four quadrants was not influenced by treatments [$F(5, 44) = 0.04$; $p > 0.05$].

Panels A and B of Fig. 3 illustrate the effects of the same six BLA/IC treatments on the unconditioned behavior (freezing and crossings) measured in the open-field test. One-way ANOVA showed significant changes in the duration of freezing [$F(7, 42) = 8.04$; $p < 0.05$] among treatments (Panel A). *Post hoc* comparisons showed that the most noticeable effects in this

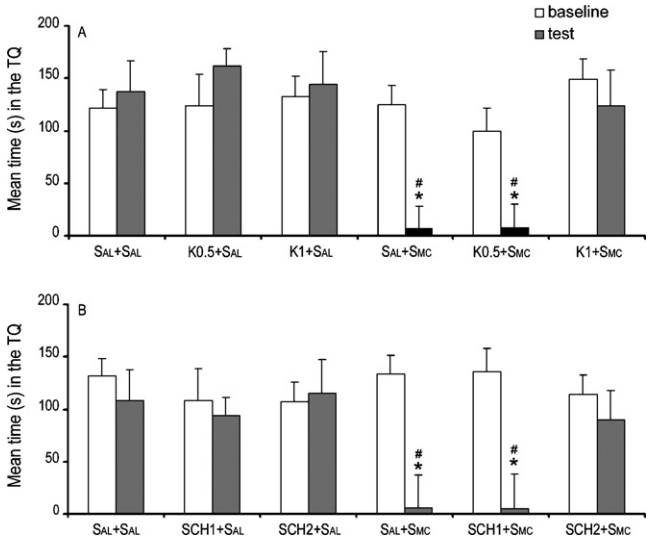

Fig. 2. Mean (±S.E.M.) time in seconds spent in the treatment quadrant of the corral during baseline trials (open columns) and test trials (closed columns). During the conditioning sessions, the animals were injected with ketanserin (K) at 0.5 μg/0.2 μl or 1.0 μg/0.2 μl (Panel A), SCH-23390 (SCH) at 1 μg/0.2 μl or 2 μg/0.2 μl (Panel B), or 0.2 μl saline (SAL) into the basolateral nucleus of the amygdala and semicarbazide (SMC) at 6 μg/0.2 μl or saline into the inferior colliculus. All animals received two injections before being confined in one of the quadrants of the corral (for 30 min) in the conditioning sessions. (Panel A) Saline + saline (SAL + SAL, $n = 10$); ketanserin + saline (K0.5 + SAL, $n = 8$; K1.0 + SAL, $n = 8$); saline + semicarbazide (SAL + SMC, $n = 8$); ketanserin + semicarbazide (K0.5 + SMC, $n = 8$; K1.0 + SMC, $n = 8$). (Panel B) Saline + saline (SAL + SAL, $n = 9$); SCH-23390 + saline (SCH1 + SAL, $n = 8$; SCH2 + SAL, $n = 8$); saline + semicarbazide (SAL + SMC, $n = 11$); SCH-23390 + semicarbazide (SCH1 + SMC, $n = 8$; SCH2 + SMC, $n = 8$). In the baseline and test trials, animals were allowed to access the entire arena for 10 min. $^{*}p < 0.05$ vs. corresponding test sessions of the control group. $^{\#}p < 0.05$ vs. baseline sessions of the same group. Two-way ANOVA followed by the Newman–Keuls test.

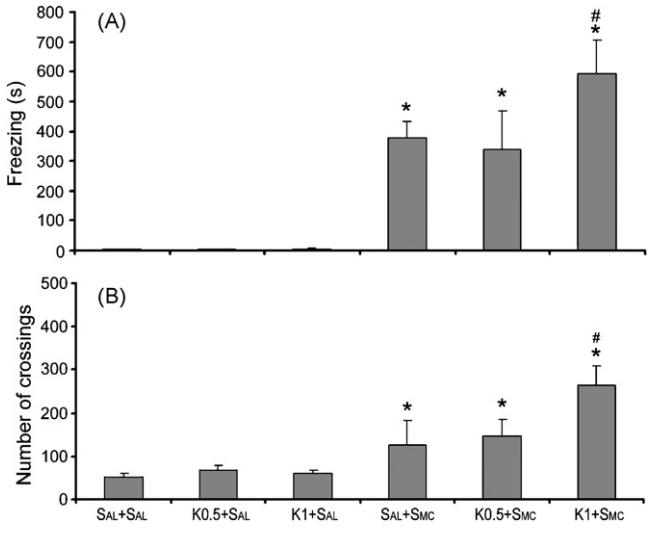

Fig. 3. Mean (± S.E.M.) time in seconds of (A) freezing and (B) number of crossings in the open-field test. The animals were injected with ketanserin (K) at 0.5 μg/0.2 μl or 1.0 μg/0.2 μl, or 0.2 μl saline (SAL) into the basolateral nucleus of the amygdala, and semicarbazide (SMC) at 6 μg/0.2 μl or saline into the inferior colliculus. All animals received two injections before being submitted to the 30 min sessions of the open-field test: saline + saline (SAL + SAL, $n = 9$); ketanserin + saline (K0.5 + SAL, $n = 6$; K1.0 + SAL, $n = 7$); saline + semicarbazide (SAL + SMC, $n = 9$); ketanserin + semicarbazide (K0.5 + SMC, $n = 9$; K1.0 + SMC, $n = 8$). The first injection into the BLA was done 15 min before the test, and the second into the IC was done 5 min before the test. $^{*}p < 0.05$ vs. SAL + SAL. $^{\#}p < 0.05$ vs. SAL + SMC. One-way ANOVA followed by the Newman–Keuls test.

experiment were the increase in the time of freezing in the groups injected with Sal + SMC and K0.5 + SMC and the enhancement of this behavior in the animals injected with K1 into the BLA ($p < 0.05$). Panel B of Fig. 3 presents the data related to the locomotor activity of the six treatment groups of rats submitted to the open-field test. One-way ANOVA showed a significant effect of treatments [$F(5, 42) = 3.14$; $p < 0.05$]. *Post hoc* analysis revealed an increase in locomotor activity for the Sal + SMC and K0.5 + SMC in comparison with the other treatment groups ($p < 0.05$) and enhancement of this behavior in the animals injected with K1 into the BLA ($p < 0.05$). This behavioral activation appeared in bursts, which gave way to a freezing response soon afterwards. Four rats in the Sal + SMC group and 10 in the K0.5 + SMC and K1 + SMC groups presented a complete sequence of escape behaviors (running–galloping–jumping). A simple inspection of the Fig. 3 shows that there was no difference between the groups Sal + Sal, K0.5 + Sal and K1 + Sal ($p > 0.05$).

3.2. Dopamine

During the baseline trial (day 1), the animals spent a comparable amount of time in each of the four quadrants (106.83 ± 27.33; 112.55 ± 12.11; 99.55 ± 13.27; 97.22 ± 13.33). There was no significant preference for any quadrant [$F(5, 46) = 0.86$; $p > 0.05$].

Panel B of Fig. 2 depicts the mean amount of time spent in the treatment quadrant during baseline and test trials for the different treatment groups. Two-way repeated-measures ANOVA applied to the data obtained with the combined drug injections into the BLA and IC showed a statistically significant effect for treatments [$F(5, 46) = 3.25$; $p < 0.05$], trials [$F(5, 46) = 18.15$; $p < 0.05$] and interaction between treatments and trials [$F(5, 46) = 16.75$; $p < 0.05$]. *Post hoc* analysis revealed a reduction in the time spent in the treatment quadrant on the testing day for the Sal + SMC and SCH1 + SMC groups relative to the other treatment groups ($p < 0.05$). Within-group comparisons indicated that the animals of the Sal + SMC and SCH1 + SMC groups showed aversion to the treatment quadrant; that is, the time spent in the treatment quadrant was reduced in the test trials ($p < 0.05$). *Post hoc* analysis also revealed that there was no statistically significant difference between the groups Sal + Sal, SCH1 + Sal, SCH2 + Sal and SCH2 + SMC ($p > 0.05$). There was no influence of treatment on gross locomotor activity, expressed as the mean number of entries into the four quadrants [$F(5, 46) = 0.09$; $p > 0.05$].

Panels A and B of Fig. 4 illustrate the effects of the same six BLA/IC treatments on the unconditioned behavior (freezing and crossings) measured in the open-field test. One-way ANOVA showed significant changes in the duration of freezing [$F(5, 43) = 6.00$; $p < 0.05$] among treatments (Panel A). *Post hoc* comparisons showed that the most noticeable effects in this

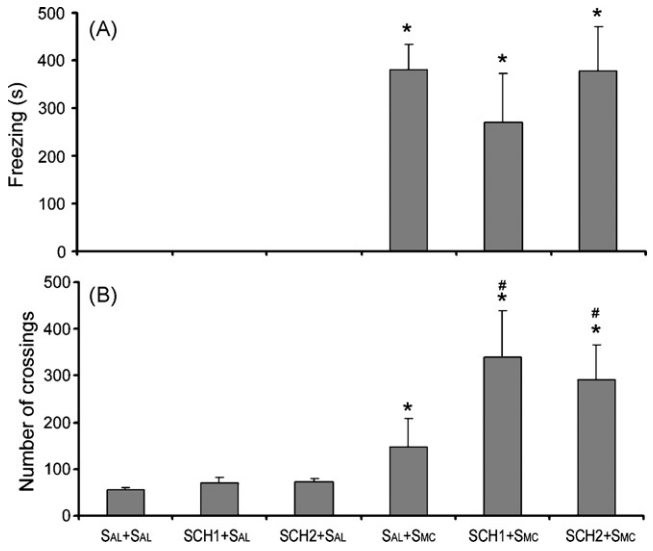

Fig. 4. Mean (±S.E.M.) time in seconds of (A) freezing and (B) number of crossings in the open-field test. The animals were injected with SCH-23390 (SCH) at 1 μg/0.2 μl or 2 μg/0.2 μl, or 0.2 μl saline (SAL) into the basolateral nucleus of the amygdala and semicarbazide (SMC) at 6 μg/0.2 μl or saline into the inferior colliculus. All animals received two injections before being submitted to the 30 min sessions of the open field test: saline + saline (SAL + SAL, $n = 9$); SCH-23390 + saline (SCH1 + SAL, $n = 7$; SCH2 + SAL, $n = 7$); saline + semicarbazide (SAL + SMC, $n = 9$); SCH-23390 + semicarbazide (SCH1 + SMC, $n = 9$; SCH2 + SMC, $n = 8$). The first injection into the BLA was done 15 min before the test, and the second into the IC was done 5 min before the test. $^{*}p < 0.05$ vs. SAL + SAL. $^{\#}p < 0.05$ vs. SAL + SMC. One-way ANOVA followed by the Newman–Keuls test.

experiment were an increase in the time of freezing in the groups injected with Sal + SMC, SCH1 + SMC, SCH2 + SMC relative to the other treatment groups ($p < 0.05$). Panel B of Fig. 4 shows the data relating to the locomotor activity of the six treatment groups of rats submitted to the open-field test. One-way ANOVA showed a significant effect of treatments [$F(5, 43) = 3.07$; $p < 0.05$]. *Post hoc* analysis revealed an increase in locomotor activity for the Sal + SMC, SCH1 + SMC and SCH2 + SMC groups relative to the other treatment groups ($p < 0.05$) and the enhancement of this behavior in the animals injected with SCH1 and SCH2 into the BLA in comparison with the Sal + SMC group ($p < 0.05$). This behavioral activation appeared in bursts, which gave way to a freezing response soon afterwards. Three rats in the Sal + SMC group and nine in the SCH1 + SMC and SCH2 + SMC groups showed a complete sequence of escape behaviors (running–galloping–jumping). A simple inspection of the Fig. 4 shows that the groups Sal + Sal, SCH1 + Sal and SCH2 + Sal had comparable performance in the test.

4. Discussion

Evidence for the involvement of the IC in the generation and elaboration of defensive behavior has been extensively reported from behavioral, immunohistochemical and electrophysiological data [see 6, 7 for reviews]. Behavioral studies have shown that electrical or chemical stimulation of the IC produces fear-like responses such as freezing and flight behavior [13,14,57]. GABA-mediated processes may well be important for this proposed function of the IC since immunohistochemical studies have shown that this structure contains high concentrations of GABA and its synthesizing enzyme GAD [58,66,70]. In this regard, it was shown a long time ago that chemical stimulation of the IC with GAD blockers causes fearful behavioral reactions together with autonomic responses such as tachycardia, high blood pressure, piloerection, exophtalmus, micturition and defecation, as well as supports fear conditioning as assessed by place conditioned aversion test [1,10]. In appropriate doses, injections of semicarbazide into the IC cause freezing behavior, with simultaneous enhancement of the magnitude of the auditory evoked potentials, indicating that GABA-mediated mechanisms are involved in the sensorimotor gating activated by emotional stimuli at this midbrain level [8,56]. The present study brings further support for these findings, in so far as injections of semicarbazide into the IC also caused freezing, escape and served as unconditioned stimulus in the corral test. This supports associative learning, as revealed by the aversion to the quadrant in which the animals were previously injected in the conditioning sessions [1,12,71,72].

Based on the evidence showing that the amygdala is critically involved in the regulation of innate and conditioned reactions to threatening stimuli [3,18,20,36–38,65], the question that arises is to what extent do telencephalic structures regulate the ascending information coming from the IC. Indeed, conjoint Fos expression in the BLA and central amygdaloid nuclei after activation of the neural substrates of aversion in the IC supports the existence of a IC-amygdala loop [35]. In this respect, we have been investigating the BLA as a probable regulator of the unconditioned and conditioned responses organized at this midbrain tectum structure. To find an answer to this question, we have examined the effects of the inactivation of the BLA (by enhancing its GABAergic inhibitory tone with local injections of the GABA agonist muscimol) on unconditioned and conditioned fear elicited by injections of SMC into the IC as the unconditioned stimulus. We have found that intra-BLA muscimol decreased the acquisition of fear conditioning and caused an increase in the aversiveness of the chemical stimulation of the BLA [43]. Based on this evidence, we suggested that distinct modulatory mechanisms in the BLA are recruited during the conditioned and unconditioned fear triggered by activation of the IC. Thus, disruption of the modulatory mechanisms of the BLA appears to amplify or facilitate the occurrence of defensive behaviors induced by stimulation of the IC. Interestingly, electrolytic or local injections of muscimol into the BLA do not change the freezing and escape thresholds determined by stepwise increases in the current of the electrical stimulation of the dPAG [59,67]. The latter findings thus suggest a different functional role for BLA mechanisms in the regulation of unconditioned fear generated either in the dPAG or IC.

Once established that GABAergic mechanisms of BLA regulate unconditioned fear generated by stimulation of the IC we were also interested to find out whether or not the proposed connection IC-amygdala would also be modulated by 5-HT- and/or DA-mediated mechanisms since these biogenic amine systems have also been extensively studied in the neurobiology of fear and anxiety [27–29,47,51].

4.1. 5-HT modulation

A number of studies have demonstrated that the 5-HT-mediated mechanisms of the amygdala are involved in the processing of information in the BLA during conditioned fear [19,20,37]. The present data lend further support to this assumption, in so far as ketanserin injections into the BLA clearly inhibit the conditioned aversion to the quadrant in which rats have experienced the aversive effects of injections of semicarbazide into the IC. This finding adds to our current knowledge that the 5-HT-mediated mechanisms of the amygdala have a regulatory role in the processing of information in the BLA during conditioned fear [20,37]. The proposal of an increased functioning of 5-HT networks within the BLA may strengthen the acquisition of conditioned aversive information is supported by results of other studies [15,19,20,37]. The fact that the 5-HT_2 antagonist ketanserin reversed the conditioned fear responses elicited by SMC injections into the IC suggests that 5-HT acting on 5-HT_2 receptors could facilitate the acquisition of conditioned fear in a paradigm that uses the stimulation of the IC as an unconditioned stimulus. This assumption is in agreement with the general idea that 5-HT enhances anxiety in the amygdala by acting on 5-HT_2 receptors. In this regard, intra-BLA injection of ketanserin releases responding suppressed by punishment [62]. Moreover, a more recent study using systemic injections of serotonergic drugs in rats submitted to an elevated T-maze test showed that 5-HT_2-mediated mechanisms have a facilitatory role in the expression of learned fear [53].

To study the functional role of the BLA in the unconditioned fear generated by activation of the neural substrates of aversion in the IC, similar injections of ketanserin into this nucleus were also performed in rats injected with SMC into the IC and submitted to the open field test. The injections of this 5-HT_2 antagonist into the BLA increased the aversiveness of IC stimulation with SMC, since K1 + SMC group exhibited higher level of freezing. These findings confirm previous reports using electrolytic or neurotoxic lesions showing that the BLA regulates the defensive behavior generated at the IC level [44,49]. Similarly, blockade of the 5-HT_2 receptors in the BLA has been found to increase the aversiveness of chemical stimulation of the IC with NMDA [48]. It has been proposed that 5-HT_2 mechanisms activate GABA interneurons in structures of the brain aversion system [8,27,69]. In other words, ketanserin injections into the BLA, would reduce the inhibitory mechanisms mediated by GABA, and thus enhance the unconditioned aversive effects elicited by IC stimulation. These effects seem to be mediated by 5-HT_{2A} receptors and not by 5-HT_{2C} receptors on which ketanserin also acts. In fact, it has been shown that ketanserin showed an anxiogenic-like effect in animal models of anxiety, while 5-HT_{2C} receptor antagonists did not [52]. Moreover, the anxiolytic-like effects induced by 5-HT_{2A} receptor agonist α-methyl-serotonin infused into the BLA on tonic immobility of guinea pigs was reversed by pretreatment of ketanserin [40]. In this study we report an opposite mediation by 5-HT mechanisms on learning process taking place in the BLA measured by a place aversion conditioning test and on the expression of emotional behavior induced by stimulation of the IC. In contrast with the 5-HT-mediation of conditioned fear, blockade of 5-HT signal in the BLA results in an increase of unconditioned fear. Thus, the present data go one step further in the neurobiology of fear and anxiety showing that 5-HT_2-mediated mechanisms of the BLA enhance the processing of information in the BLA during conditioned fear. On the other hand, 5-HT_2-mediated mechanisms of the BLA inhibit the unconditioned fear triggered by activation of the IC.

4.2. DA modulation

Our main goal in the current study was to find out whether the proposed connection IC-amygdala would also be modulated by DA acting on a D1 receptor in the amygdala since this neurotransmitter has been extensively postulated to participate in the neurobiology of fear and anxiety [51,60,63,64]. Several studies have suggested that DA-mediated mechanisms are also involved in the processing of information in the BLA during conditioned fear [30,33,51,54,55]. The present data support this assumption, since injections of SCH 23390 into the BLA reduced the place conditioned aversion measured by the corral test. This is not unexpected, in fact injections of a DA-antagonist into the BLA would cause anti-aversive effects since this structure is part of the mesocorticolimbic system, in which the amygdala is a main projection site and DA levels are highly sensitive to exposure to a wide variety of acute threatening stimuli [16,17,23,25,42]. In support of the notion that dopamine release in the mesolimbic structures is associated with the aversive properties of such stimuli comes from evidence indicating that this release is reduced following diazepam administration [23]. Several lines of evidence indicate that D_1-receptors, rather than D2-receptors, within the BLA are mainly involved in the acquisition and retrieval of fear conditioning using foot shocks as unconditioned stimuli [54,55]. These authors have found that infusion of the D_2-receptor agonist quinpirole into the ventral tegmental area (VTA) or of SCH 23390 into the BLA prior to a second-order fear conditioning caused a decrease in conditioned freezing in the drug-free testing session. These same treatments also blocked the expression of first-order conditioned fear responses [4,34]. The fact that D1 antagonist reversed the conditioned fear response elicited by IC stimulation suggest that DA acting on D1 sites could facilitate the acquisition of conditioned fear using this stimulation as an unconditioned stimulus. Systemic injections of quinpirole at doses supposed to act pre-synaptically (D_2-like receptors) at the VTA and thus reducing the DA transmission post-synaptically in the mesoamygdala dopaminergic pathway also reduced the fear-potentiated startle [60]. The involvement of D1-receptor mechanisms in BLA in fear conditioning is consistent with the notion that DA receptors in this region are almost exclusively of the D1-like category [41,68,74].

Concerning the unconditioned reactions, opposite effects were obtained with the D_1-receptor blockade on the behavioral activation induced by injections of SMC into the IC. In fact, SCH 23390 into the BLA increased the behavioral activity produced by the IC stimulation since animals administered with the D1 antagonist performed an enhanced number of crossing

in the open field test. These findings confirm previous reports on electrolytic or neurotoxic lesions showing that the BLA regulates the defensive behavior generated at the IC level [44,49]. Recently, we showed that electrical stimulation of the IC causes a concomitant increase in the levels of DA in the BLA and prefrontal cortex [45,46]. One possibility that has been put forward to explain these findings is that dopaminergic mechanisms of the mesocorticolimbic systems may be called into play in the setting up of adaptive responses aimed at coping with or signaling the presence of stimuli of aversive nature. Thus, the signal of the modulatory dopaminergic mechanism on defensive behavior will depend on the type of emotional stimulus triggering the coping reaction. Consonant with such view, malfunctioning of the BLA has been related to general anxiety disorders and the anxiolytic effects of benzodiazepines are thought to be the result of their depressive action on the activity of this nucleus [15,18,20–22,37].

Altogether, the present data indicate that the 5-HT_2- and D_1-mediated mechanisms of the BLA appear to have opposite influences on the conditioned and unconditioned fear. While these mechanisms in the BLA appear to facilitate the conditioned fear, they inhibit the unconditioned fear triggered by activation of the IC. These data support the two-dimensional view of defense in that anxiety and fear are represented in parallel systems in the brain, which are probably modulated by opposing neurochemical mechanisms [50].

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