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INTERNATIONAL JOURNAL OF NEUROPSYCHOPHARMACOLOGY, NEW YORK, v. 15, n. 3, pp. 389-400, APR, 2012

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Serotonin-2C receptors in the basolateral nucleus of the amygdala mediate the anxiogenic effect of acute imipramine and fluoxetine administration

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Abstract

A growing body of evidence indicates that facilitation of serotonin-2C receptor (5-HT_{2C}R)-mediated neurotransmission in the basolateral nucleus of the amygdala (BLA) is involved in anxiety generation. We investigated here whether BLA 5-HT_{2C}Rs exert a differential role in the regulation of defensive behaviours related to generalized anxiety (inhibitory avoidance) and panic (escape) disorders. We also evaluated whether activation of BLA 5-HT_{2C}Rs accounts for the anxiogenic effect caused by acute systemic administration of the antidepressants imipramine and fluoxetine. Male Wistar rats were tested in the elevated T-maze after intra-BLA injection of the endogenous agonist 5-HT, the 5-HT_{2C}R agonist MK-212 or the 5-HT_{2C}R antagonist SB-242084. This test allows the measurement of inhibitory avoidance acquisition and escape expression. We also investigated whether intra-BLA administration of SB-242084 interferes with the acute anxiogenic effect caused by imipramine and fluoxetine in the Vogel conflict test, and imipramine in the elevated T-maze. While intra-BLA administration of 5-HT and MK-212 facilitated inhibitory avoidance acquisition, suggesting an anxiogenic effect, SB-242084 had the opposite effect. None of these drugs affected escape performance. Intra-BLA injection of a sub-effective dose of SB-242084 fully blocked the anxiogenic effect caused either by the local microinjection of 5-HT or the systemic administration of imipramine and fluoxetine. Our findings indicate that 5-HT_{2C}Rs in BLA are selectively involved in the regulation of defensive behaviours associated with generalized anxiety, but not panic. The results also provide the first direct evidence that activation of BLA 5-HT_{2C}Rs accounts for the short-term aversive effect of antidepressants.

Received 15 September 2010; Reviewed 3 November 2010; Revised 6 May 2011; Accepted 10 May 2011;

First published online 14 June 2011

Key words: Antidepressant, anxiety, basolateral amygdala, serotonin.

Introduction

The serotonin-2C receptor (5-HT_{2C}R) plays an important role in emotional processing (Berg *et al.* 2008; Giorgetti & Tecott, 2004; Landolt & Wehrle, 2009; Millan, 2005). This receptor is a member of the family of G-protein-coupled receptors and was first identified in the choroid plexus (Bockaert *et al.* 2010; Hoyer *et al.* 2002; Pazos *et al.* 1984). High levels of 5-HT_{2C}R-binding sites and 5-HT_{2C}R mRNA are also observed in the cortex and limbic areas such as the hippocampus,

amygdala and periaqueductal grey (Clemett *et al.* 2000; Mengod *et al.* 2010; Pompeiano *et al.* 1994).

Compelling evidence from clinical and animal studies shows that facilitation of 5-HT_{2C}R-mediated neurotransmission increases anxiety. This has been reported after the peripheral administration of both non-selective 5-HT_{2C}R agonists such as m-CPP and TMFPP as well as of more selective, recently developed agonists such as MK-212 and RO 60-0175 (Bagdy *et al.* 2001; Broocks *et al.* 1997; Jan *et al.* 2010; Kennett *et al.* 1989; Overstreet *et al.* 2003; Setem *et al.* 1999; Walker *et al.* 2005). On the other hand, treatment with 5-HT_{2C}R antagonists such as ritanserin (non-selective) or SB-242084 (selective) causes the opposite effect (Bressa *et al.* 1987; Ceulemans *et al.* 1985; Kennett *et al.* 1997; Martin *et al.* 2002). Clinical studies indicate that

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agomelatine, a melatonin receptor agonist and 5-HT_{2C}R antagonist that has recently been marked as an antidepressant, also has anxiolytic properties (de Bodinat *et al.* 2010; Stein *et al.* 2008). In animal models, the anxiolytic effect of agomelatine has been mainly attributed to its interaction with 5-HT_{2C}Rs (Millan *et al.* 2005).

The involvement of 5-HT_{2C}Rs in the genesis/regulation of anxiety has also been revealed by genetic approaches. It has been reported that whereas transgenic mice overexpressing 5-HT_{2C}Rs in the cerebral cortex and different limbic areas (e.g. amygdala, hippocampus) show increased anxiety in the elevated plus-maze (Kimura *et al.* 2009), 5-HT_{2C}R knockout mice display an anxiolytic-like phenotype as measured by different experimental models (Heisler *et al.* 2007).

Among the brain areas showing abundant distribution of 5-HT_{2C}Rs, the amygdala has been seen as the critical neuronal substrate for this receptor-mediated effect on anxiety. For instance, 5-HT_{2C}R mRNA is overexpressed in this area following a single session of prolonged exposure to stressors, a procedure shown to increase anxiety (Harada *et al.* 2008). Higher levels of anxiety are also observed in rats after exposure to unavoidable tail-shocks, systemic injection of MK-212 or repeated withdrawal from chronic ethanol diets, and in all cases this effect is fully blocked by the injection of 5-HT_{2C}R antagonists such as SB-242084 into the basolateral nucleus of the amygdala (BLA) (Christianson *et al.* 2010; de Mello Cruz *et al.* 2005; Overstreet *et al.* 2006). The BLA has long been associated with anxiety processing (Pesold & Treit, 1995, Rosen, 2004) and receives dense 5-HT innervation from the dorsal raphe nucleus (Vertes, 1991).

It has also been proposed (Burghardt *et al.* 2007; Millan, 2005) that activation of BLA 5-HT_{2C}Rs accounts for the anxiogenic effect found clinically during the early stages of treatment with antidepressants such as SSRIs (e.g. fluoxetine, sertraline) or tricyclic compounds (e.g. imipramine, clomipramine). While this hypothesis has been put forward based on evidence showing that systemic injection of 5-HT_{2C}R antagonists fully counteracts the acute anxiogenesis caused by antidepressants in different animal models of anxiety (Bagdy *et al.* 2001; Greenwood *et al.* 2008; Yamauchi *et al.* 2004), no study has directly investigated this proposal by showing that intra-BLA injection of 5-HT_{2C}R antagonists exerts the same inhibitory effect.

In the present study, we further addressed the role played by BLA 5-HT_{2C}Rs in anxiety. More specifically, we first investigated whether this receptor exerts a

differential role in the regulation of defensive behaviours that have been related to different subtypes of clinically recognized anxiety. To this end, the effect caused by intra-BLA microinjection of the endogenous agonist 5-HT, the selective 5-HT_{2C}R agonist MK-212 or the 5-HT_{2C}R antagonist SB-242084 was investigated in the elevated T-maze. This animal model, derived from the elevated plus-maze, allows measurement, in the same rat, of an anxiety- and a fear-related defensive response, respectively, inhibitory avoidance and escape (for a review of this test, see McNaughton & Zangrossi, 2008; Pinheiro *et al.* 2007; Zangrossi & Graeff, 1997). Based on a series of pharmacological studies (for a review see Blanchard *et al.* 2001; Graeff & Zangrossi, 2002; McNaughton & Gray, 2000), behavioural inhibition responses such as inhibitory avoidance have been linked with anxiety while escape has been associated with fear. In terms of psychopathology, these responses have been related to generalized anxiety and panic disorders, respectively (Blanchard *et al.* 2001, 2003; Graeff & Zangrossi, 2002; Gray & McNaughton, 2000; McNaughton & Corr, 2004).

In a previous study from our group, we reported that whereas a single systemic injection of imipramine facilitated inhibitory avoidance acquisition in the elevated T-maze, indicating an anxiogenic effect, a 21-d treatment caused the opposite effect. Chronic, but not acute, treatment with imipramine inhibited escape expression, suggesting a panicolytic-like effect (Teixeira *et al.* 2000). These results are in full agreement with clinical evidence showing that the beneficial effects of antidepressants on generalized anxiety and panic disorders are only seen after prolonged administration (Bakker *et al.* 2005; Millan, 2005).

In a second experiment performed, we investigated whether activation of 5-HT_{2C}Rs in the BLA accounts for the acute anxiogenic effect caused by imipramine in the elevated T-maze inhibitory avoidance task. For comparison, we verified whether these receptors were also implicated in the acute imipramine and fluoxetine anxiogenic effect detected in another generalized anxiety-associated model, the Vogel conflict test (for further details of this test see Graeff & Zangrossi, 2002; Millan & Brocco, 2003). Imipramine and fluoxetine were chosen as being the prototype drugs for the tricyclic antidepressants and SSRIs, respectively. These two classes of drugs are shown to be equally effective in treating depression and anxiety disorders such as generalized anxiety and panic, even though the use of the latter has supplanted the former due to its better safety, tolerability and cost (Dupuy *et al.* 2011; Ravindran & Stein, 2010).

Methods

Animals

Male Wistar rats weighing 290–310 g were group-housed ($n = 5$ per group) under a 12-h light/dark cycle (lights on 07:00 hours) at 22 ± 1 °C with free access to food throughout the experiment, except during testing. Water was also freely available, except in expts 2b and 2c where periods of deprivation were followed (see below). The experiments reported in this paper were performed in compliance with the recommendations of the SBNeC (Brazilian Society of Neuroscience and Behaviour), which are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals.

Apparatus

The elevated T-maze was made of wood and had three arms of equal dimensions (50 × 12 cm). One arm, enclosed by 40-cm-high walls, was perpendicular to two opposed open arms. To avoid falls, the open arms were surrounded by a 1 cm high Plexiglas rim. The whole apparatus was elevated 50 cm above the floor.

The open-field test was performed in a wooden square arena (60 × 60 cm), with 30-cm-high walls. Luminosity at the level of the T-maze arms or open field was 60 lx.

The Vogel conflict test was performed as described by Pelosi *et al.* (2009) in a Plexiglas box (length 42 cm, width 25 cm, height 20 cm) with a stainless-steel grid floor. A metallic spout of a drinking bottle containing water projected into the box. The contact of the animal with the spout and grid floor closed an electrical circuit controlled by a sensor (Insight Instruments, Brazil), which produced 7 pulses/s whenever the animal was in contact with both components. Each pulse was considered as a lick, and every 20 licks the animal received a 0.5 mA shock for 2 s. The sensor recorded the total number of licks and shocks delivered during the test period. The apparatus was located inside a sound-attenuated cage.

After each experimental session, the models were cleaned with a 10% ethanol solution.

Drugs

The following drugs were used: 5-hydroxytryptamine creatinine sulphate (5-HT; Sigma, USA), 6-chloro-2-(1-piperazinyl)pyrazine hydrochloride (MK-212; Tocris, USA), 6-chloro-2,3-dihydro-5-methyl-N-[6-(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1H-indole-1-carboxamide dihydrochloride (SB-242084; Tocris), imipramine hydrochloride (Sigma) and fluoxetine

hydrochloride (EMS, Brazil). All drugs were dissolved in sterile saline, except fluoxetine, which was dissolved in a solution containing sterile saline with 2% Tween-80.

Surgery

The animals were anaesthetized with 2,2,2-tribromoethanol (250 mg/kg *i.p.*) and placed in a stereotaxic frame. In all experiments, two guide cannulae made of stainless steel (0.6 mm outer diameter, 0.4 mm inner diameter) were bilaterally implanted in the brain aimed at the BLA. The following coordinates from bregma were used (Paxinos & Watson, 2007), BLA: AP -2.5 mm, lateral ± 5.1 mm, ventral -6.2 mm. The guide cannulae were fixed to the skull with acrylic resin and two stainless-steel screws. Stylets, the same length as the guide cannulae, were introduced inside them to prevent obstruction.

At the end of the surgery, all animals were injected intramuscularly with 0.3 ml of antibiotic preparation (benzylpenicillin and streptomycin, Pentabiotico Veterinário Pequeno Porte, Brazil) to prevent possible infections. In addition, flunixin meglumine (Schering-Plough, Brazil; 3 mg/kg), a drug with analgesic, antipyretic and anti-inflammatory properties, was administered subcutaneously for post-surgery analgesia. The animals were left undisturbed for 5–7 d after the surgery, except for normal handling during cage cleaning.

Procedure

Elevated T-maze and open-field tests

One day before the test, the rats were exposed to one of the open arms of the T-maze for 30 min. A wooden barrier mounted on the border between the central area of the maze and the proximal end of the open arm isolated this arm from the rest of the maze. It has been shown that this pre-exposure to the open arm renders the escape task more sensitive to the effects of anti-panic drugs, because it shortens the latencies of withdrawal from the open arm during the test (Poltronieri *et al.* 2003; Teixeira *et al.* 2000).

On the test day, in expt 1a, independent groups of test-naive rats received an intra-BLA injection of MK-212 (0, 0.01, 0.1 or 1 nmol; $n = 8-9$), 5-HT (0 or 16 nmol; $n = 10-11$) or SB-242084 (0, 0.1, 1, 10 nmol; $n = 7-9$). Ten minutes after the microinjections, the animals were submitted to the behavioural tests.

In order to investigate if SB-242084 was able to block the effects of 5-HT, in expt 1b the animals were microinjected in the BLA with SB-242084 (0.01 nmol) or saline, 5 min before microinjection of 5-HT (16 nmol)

or saline. Thus, the following groups ($n=6-7$) were formed: Sal/Sal, SB/Sal, Sal/5-HT and SB/5-HT. Ten minutes after the last microinjection, the animals were submitted to the behavioural tests.

In expt 2a, the animals were microinjected in the BLA with SB-242084 (0.01 nmol) or saline, 10 min before the systemic injection (i.p.) of imipramine (10 mg/kg, 1 ml/kg) or saline. Thus, the following groups ($n=7-10$) were formed: Sal/Sal, SB/Sal, Sal/Imi and SB/Imi. The animals were tested in the elevated T-maze and open-field tests 30 min after the systemic injections.

The test in the elevated T-maze was initiated by inhibitory avoidance measurement. Each animal was placed at the distal end of the enclosed arm of the elevated T-maze facing the intersection of the arms. The time taken by the rat to leave this arm with the four paws was recorded (baseline latency). The same measurement was repeated in two subsequent trials (avoidance 1 and 2) at 30-s intervals. Following avoidance training (30 s), rats were placed at the end of the same previously experienced open arm and the latency to leave this arm with the four paws was recorded for three consecutive times (escape 1, 2 and 3) with 30-s inter-trial intervals. A cut-off time of 300 s was established for the avoidance and escape latencies.

In order to assess possible drug effects on locomotor activity, immediately after testing in the elevated T-maze, the total distance travelled by each rat in an open field was evaluated by a video-tracking system (Ethovision, The Netherlands) for 5 min.

Vogel conflict test

Animals were water-deprived for 48 h prior to the test. After the first 24 h of deprivation, they were allowed to drink freely for 3 min in the test cage in order to find the drinking bottle spout. Some animals that failed to find the spout were not included in the experiment. Twenty-four hours later, animals were injected in the BLA with SB-242084 (0.01 nmol) or saline, 10 min before the systemic injection (i.p.) of imipramine (10 mg/kg, 1 ml/kg) or saline (expt 2b, $n=8-9$) or fluoxetine (15 mg/kg, 1 ml/kg) or vehicle solution (expt 2c, $n=8-11$). Thirty minutes later, they were returned to the test cage. The test period lasted for 3 min, and the animals received a 0.5-mA shock for 2 s through the bottle spout every 20 licks.

In an independent group of non-operated rats ($n=4-6$), we evaluated the effect of a single injection (i.p.) of imipramine (10 mg/kg) or fluoxetine (15 mg/kg) on water consumption. The procedure adopted was the same used in the punished licking

test described above, except that the electric-shock delivery system was inoperative.

The doses of the drugs were chosen on the basis of previously published studies (Alves *et al.* 2004; Christianson *et al.* 2010; Navailles *et al.* 2006; Teixeira *et al.* 2000; Zanoveli *et al.* 2003).

For drug injection, needles (0.3 mm outer diameter) were introduced through guide cannulae until their tips were 2 mm below the end of the cannulae. The drugs were microinjected in the BLA in a volume of 0.2 μ l (for each side) over a period of 120 s using a 10 μ l microsyringe (Hamilton 701-RN, USA) attached to a microinfusion pump (KD Scientific, USA). The displacement of an air bubble inside the polyethylene catheters connecting the syringe needles to the intracerebral needles was used to monitor the microinjection. The intracerebral needles were removed 60 s after the end of the injections.

Histology

After the experiments, animals were sacrificed under deep urethane anaesthesia. The brain was perfused intracardially with saline solution (0.9%) followed by 10% formalin solution before being removed and fixed in 10% formalin. Brain slices of 60 μ m were made by means of a microtome in order to localize the site of the drug injection, according to the atlas of Paxinos & Watson (2007).

Statistical analysis

Repeated-measures analysis of variance (ANOVA) was used to analyse both avoidance and escape data. In expt 1a, treatment (saline, MK-212, 5-HT, SB-242084) was considered the independent factor and trial (baseline, avoidance 1 and 2 latencies, escape 1, 2 and 3 latencies), the dependent, repeated measure. In expt 1b, besides the dependent measure (trial), two independent factors were considered: pre-treatment with SB-242084 and treatment with 5-HT. In expt 2a, the two independent factors were pre-treatment with SB-242084 and treatment with imipramine. Locomotion data in the open-field were analysed by one-way ANOVA (expt 1a) or two-way ANOVA (expts 1b, 2a). The number of punished licks in the Vogel conflict test was analysed by two-way ANOVA (expts 2b, 2c). Multiple comparisons were performed by Duncan's *post-hoc* test.

Results

Figure 1 depicts the sites of drug injections into the BLA of animals tested in the study.

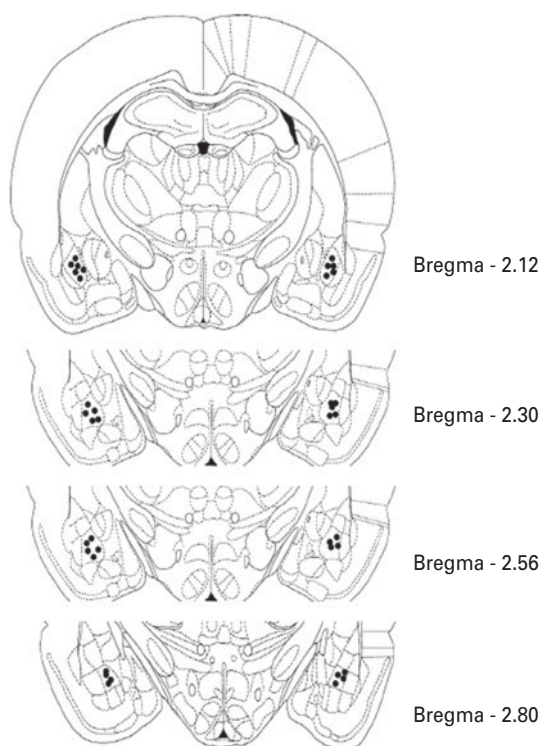


Fig. 1. Diagrammatic representation of coronal sections of the rat brain showing the location of injection sites in the basolateral nucleus of the amygdala (●). Figures represent coordinates from the rat brain atlas of Paxinos & Watson (2007), with respect to bregma. The number of points in the figures is fewer than the total number of rats used because of several overlaps.

Expt 1a: MK-212 effects

Figure 2a shows that MK-212 facilitated inhibitory avoidance acquisition (treatment effect: $F_{3,31}=3.31$, $p<0.05$), suggesting an anxiogenic effect. Repeated-measures ANOVA revealed a trial effect ($F_{2,62}=33.95$, $p<0.05$), but not a trial \times treatment interaction. The *post-hoc* test showed that MK-212 (0.01 and 0.1 nmol) significantly increased ($p<0.05$) avoidance 2 latency compared to the saline group. This agonist did not affect escape performance (Fig. 2b) or locomotion in the open field (Table 1).

5-HT effects

Figure 3a shows that, similarly to MK-212, 5-HT facilitated inhibitory avoidance acquisition. There were significant effects of treatment ($F_{1,19}=4.57$, $p<0.05$), trial ($F_{2,38}=18.96$, $p<0.05$), and trial \times treatment interaction ($F_{2,38}=3.99$, $p<0.05$). Student's *t* test revealed that 5-HT significantly increased ($p<0.05$) avoidance 2 latency. 5-HT did not affect

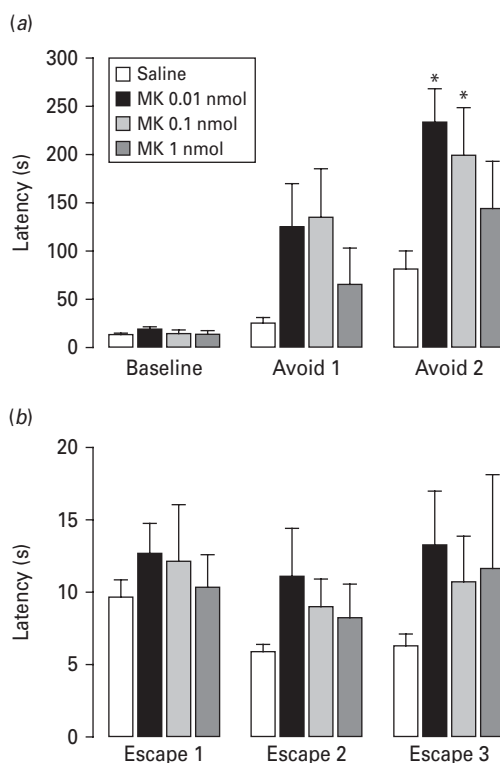


Fig. 2. MK-212 injection in the basolateral nucleus of the amygdala facilitated (a) inhibitory avoidance acquisition, without interfering with (b) escape expression in the elevated T-maze ($n=8-10$). Values are mean \pm S.E.M. * $p<0.05$ compared to the saline-injected group.

escape expression (Fig. 3b) or locomotion in the open field (Table 1).

SB-242084 effects

Figure 4a shows that SB-242084 impaired inhibitory avoidance acquisition, indicating an anxiolytic effect. There was a significant effect of trial ($F_{2,48}=13.03$, $p<0.05$), and trial \times treatment interaction ($F_{6,48}=2.81$, $p<0.05$). The effect of treatment was marginal for statistical significance ($F_{3,24}=2.71$, $p<0.07$). SB-242084 at doses of 0.1 and 10 nmol significantly decreased ($p<0.05$) avoidance 2 latency compared to the saline group. This antagonist did not affect escape expression (Fig. 4b) or locomotion in the open field (Table 1).

Expt 1b: previous intra-BLA administration of SB-242084 and 5-HT effects

As illustrated in Fig. 5a intra-BLA injection of 5-HT facilitated inhibitory avoidance acquisition. Previous microinjection of SB-242084 counteracted this effect. Repeated-measures ANOVA showed significant main

Table 1. Effect of treatment with intra-BLA or systemic injection of drugs on the distance travelled in the open-field test

Treatment (nmol)	Distance travelled (m)
Expt 1a	
Saline	21.87 ± 1.51
MK-212 (0.01)	21.00 ± 1.69
MK-212 (0.1)	21.35 ± 1.11
MK-212 (1)	26.66 ± 1.92
Saline	27.87 ± 3.22
5-HT (16)	23.53 ± 1.40
Saline	24.57 ± 3.34
SB-242084 (0.1)	28.89 ± 4.16
SB-242084 (1)	32.28 ± 4.83
SB-242084 (10)	22.13 ± 3.85
Expt 1b	
Saline/saline	24.50 ± 1.78
SB-242084/saline	23.56 ± 2.01
Saline/5-HT	22.27 ± 2.36
SB-242084/5-HT	23.96 ± 1.82
Expt 2a	
Saline/saline	24.07 ± 1.74
SB-242084/saline	22.31 ± 1.72
Saline/imipramine	25.36 ± 1.24
SB-242084/imipramine	22.71 ± 1.29

Values are mean ± S.E.M.

effects of trial ($F_{2,46}=33.40$, $p<0.05$) and treatment with the agonist ($F_{1,23}=9.00$, $p<0.05$). There was also a significant interaction among the three factors analysed (agonist × antagonist × trial: $F_{2,46}=7.12$, $p<0.05$). The *post-hoc* test showed that 5-HT significantly increased ($p<0.05$) avoidance 1 and 2 latencies. Prior intra-BLA administration of SB-242084 fully blocked this anxiogenic effect.

None of the treatments employed altered escape expression (Fig. 5b) or locomotion in the open field (Table 1).

Expt 2a: previous intra-BLA administration of SB-242084 and systemic imipramine effect in the elevated T-maze

Figure 6a shows that imipramine significantly facilitated inhibitory avoidance acquisition and that this anxiogenic effect was blocked by prior intra-BLA administration of SB-242084. Repeated-measures ANOVA showed significant main effects of trial ($F_{2,60}=37.21$, $p<0.05$) and antagonist treatment ($F_{1,30}=10.09$, $p<0.05$). There was also a significant interaction among the three factors analysed (systemic

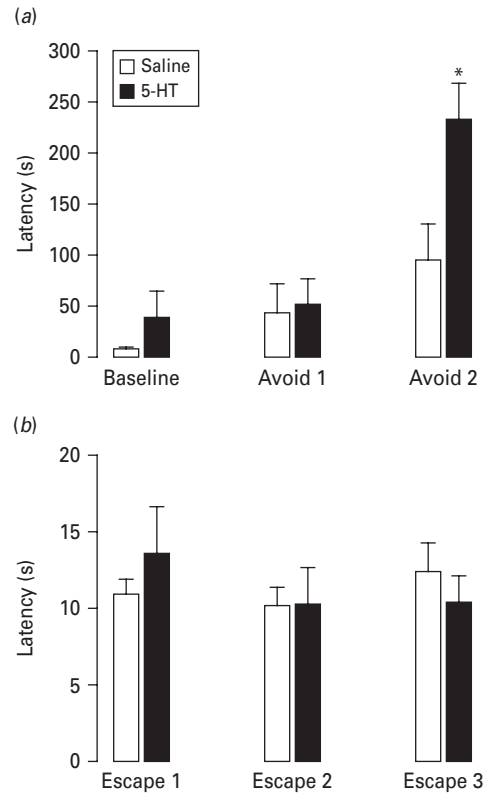


Fig. 3. 5-HT injection (16 nmol) in the basolateral nucleus of the amygdala facilitated (a) inhibitory avoidance acquisition without interfering with (b) escape expression in the elevated T-maze ($n=10-11$). Values are mean ± S.E.M. * $p<0.05$ compared to the saline-injected group.

drug × antagonist × trial: $F_{2,60}=3.42$, $p<0.05$). The *post-hoc* test showed that imipramine significantly increased ($p<0.05$) avoidance 1 and 2 latencies. Prior intra-BLA administration of SB-242084 fully blocked this anxiogenic effect.

None of the treatments employed altered escape expression (Fig. 6b) or locomotion in the open field (Table 1).

Expt 2b: previous intra-BLA administration of SB-242084 and systemic imipramine effect in the Vogel conflict test

Figure 7 shows that imipramine significantly decreased the number of punished licks in the Vogel conflict test and that this anxiogenic effect was counteracted by previous microinjection of SB-242084. Two-way ANOVA showed a significant effect of treatment with the antagonist ($F_{1,33}=4.65$, $p<0.05$) and a marginal effect of the systemic treatment ($F_{1,33}=3.70$, $p=0.06$). There was also a significant interaction

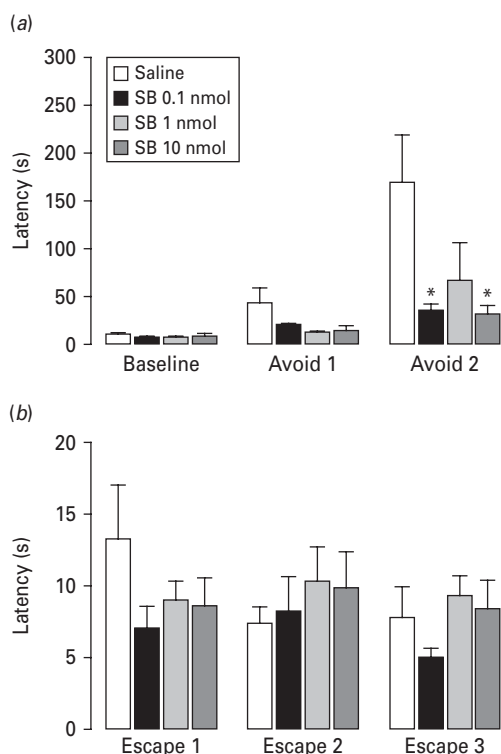


Fig. 4. Intra-basolateral nucleus of the amygdala injection of SB-242084 impaired (a) inhibitory avoidance acquisition without affecting (b) escape expression in the elevated T-maze ($n=6-8$). Values are mean \pm S.E.M. * $p < 0.05$ compared to the saline-injected group.

between the two factors (systemic drug \times antagonist: $F_{1,33}=10.5$, $p < 0.05$).

Expt 2c: previous intra-BLA administration of SB-242084 and systemic fluoxetine effect in the Vogel conflict test

As observed with imipramine, fluoxetine also decreased the number of punished licks in the Vogel conflict test and this anxiogenic effect was blocked by previous microinjection of SB-242084 (see Fig. 8). Two-way ANOVA showed a significant effect of treatment with the antagonist ($F_{1,34}=21.85$, $p < 0.05$) and the systemic injection of fluoxetine ($F_{1,34}=4.12$, $p < 0.05$). There was also a significant interaction between the two factors (systemic drug \times antagonist: $F_{1,34}=5.14$, $p < 0.05$).

As revealed by the additional control experiment performed (values are mean \pm S.E.M.), neither imipramine (Sal = 1136.7 ± 64.7 , Imi = 1152.7 ± 62.9) nor fluoxetine (vehicle = 1169.3 ± 68.6 , Flx = 1200.2 ± 79.7) affected the number of unpunished licks on the test day.

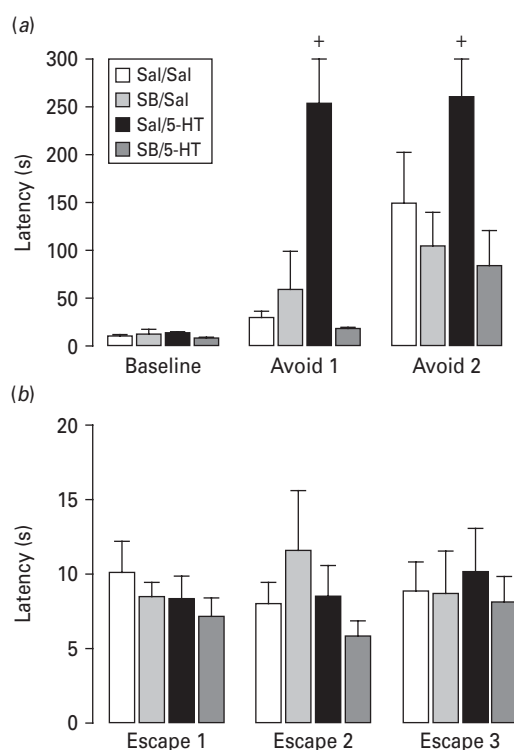


Fig. 5. Intra-basolateral nucleus of the amygdala administration of SB-242084 (0.01 nmol) blocked the facilitatory effect on (a) inhibitory avoidance acquisition caused by local microinjection of 5-HT (16 nmol) ($n=6-7$). (b) Lack of SB-242084 and 5-HT effect on escape performance ($n=6-7$). Values are mean \pm S.E.M. + $p < 0.05$ compared to all other groups.

Discussion

The objective of the present study was to investigate the role played by 5-HT_{2C}R of the BLA in the regulation of anxiety- and panic-related defensive behaviours and their involvement in the mediation of the acute anxiogenic effect caused by the antidepressant drugs imipramine and fluoxetine.

The results showed that intra-BLA injection of the 5-HT_{2C}R agonist MK-212 or the endogenous agonist 5-HT facilitated inhibitory avoidance acquisition, suggesting an anxiogenic effect, without affecting escape performance. On the other hand, microinjection of the selective 5-HT_{2C}R antagonist SB-242084 caused the opposite effect, i.e. anxiolysis, also without changing escape expression. The rise in anxiety observed after 5-HT microinjection was fully blocked by previous intra-BLA injection of SB-242084, confirming the recruitment of 5-HT_{2C}R for this effect.

Regarding the results of MK-212, it is of note that the highest dose tested did not share the anxiogenic effect observed with lower doses. This lack of effect

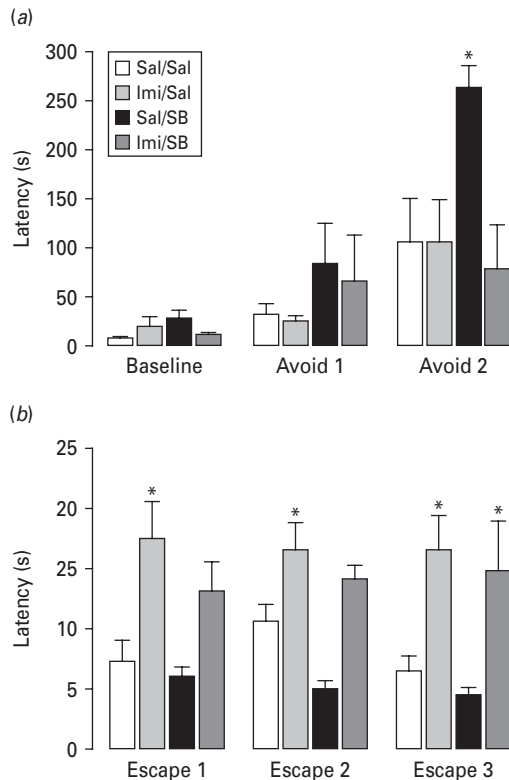


Fig. 6. Intra-basolateral nucleus of the amygdala administration of SB-242084 (0.01 nmol) blocked the facilitatory effect on (a) inhibitory avoidance acquisition caused by an acute systemic injection of imipramine (10 mg/kg) ($n=7-10$). (b) Lack of SB-242084 effect on the anti-escape consequence of imipramine administration ($n=7-10$). Values are mean \pm s.e.m. * $p < 0.05$ compared to all other groups.

may conceivably reflect the interaction of this drug with other sites besides 5-HT_{2C}Rs. Although MK-212 displays high affinity for 5-HT_{2C}Rs it also binds with moderate affinity to 5-HT_{2A}Rs (Knight *et al.* 2004). It has been shown in our laboratory that intra-BLA administration of the preferential 5-HT_{2A}R agonist DOI causes anxiolysis in the elevated T-maze (C. A. Strauss, M. A. Vicente, H. Zangrossi Jr., unpublished results). It is therefore possible that interaction of MK-212 with 5-HT₂Rs, that exert an opposed role in anxiety modulation, may have contributed to the lack of effect observed with the highest dose.

None of the compounds tested in the present study interfered with the distance travelled in the open field, thereby excluding non-specific motor interference as the main factor accounting for the effects observed upon inhibitory avoidance acquisition.

Taken together these results are indicative that 5-HT_{2C}Rs in the BLA are selectively involved in the

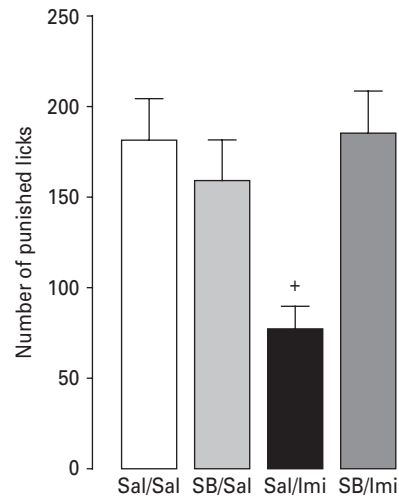


Fig. 7. Intra-basolateral nucleus of the amygdala administration of SB-242084 (0.01 nmol) blocked the decrease in the number of punished drinking caused by an acute systemic injection of imipramine (10 mg/kg) ($n=8-9$). Values are mean \pm s.e.m. + $p < 0.05$ compared to all other groups.

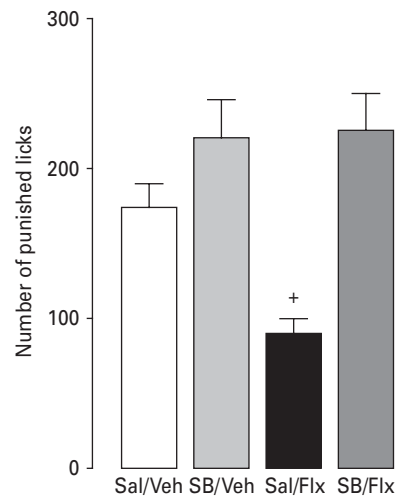


Fig. 8. Intra-basolateral nucleus of the amygdala administration of SB-242084 (0.01 nmol) blocked the decrease in the number of punished drinking caused by an acute systemic injection of fluoxetine (15 mg/kg) ($n=8-11$). Values are mean \pm s.e.m. + $p < 0.05$ compared to all other groups.

regulation of defensive behaviours associated with generalized anxiety (inhibitory avoidance), but not panic (escape).

The facilitatory role of BLA 5-HT_{2C}Rs in anxiety has also been demonstrated in other experimental models. Campbell & Merchant (2003) reported that bilateral injection of m-CPP or IL-639, non-selective and selective 5-HT_{2C}R agonists, respectively, had anxiogenic consequences in the ultrasonic vocalization and

open-field tests. This effect was blocked by the systemic injection of SB-242084. In the elevated plus-maze, systemic injection of MK-212 increased anxiety and intra-BLA injection of the non-selective 5-HT_{2C}R antagonist ritanserin counteracted this effect (de Mello Cruz *et al.* 2005).

The finding that SB-242084 has an anxiolytic effect when injected in the BLA raises two, possibly concurrent, interpretations: one is that 5-HT in this nucleus exerts a tonic facilitatory influence on anxiety-related behaviours (i.e. 5-HT is continuously released in the area) and the other is that BLA 5-HT_{2C}Rs are spontaneously active (constitutively active) in the regulation of these behaviours. Regarding the latter, a wealth of evidence indicates that 5-HT_{2C}Rs possess high levels of constitutive activity, i.e. they can activate intracellular signalling without the presence of the endogenous agonist (for a review see Aloyo *et al.* 2009). If this is the case in the present study, SB-242084 could be acting as an inverse agonist, a mode of action already reported for this drug (Aloyo *et al.* 2009). Further studies assessing, for instance, the behavioural effect of intra-BLA injection of SB-242084 in the brain of 5-HT-depleted animals (e.g. by intra-dorsal raphe nucleus injection of the neurotoxin 5,7-dihydroxytryptamine), will certainly contribute to the clarification of this question.

The effect of 5-HT_{2C}R activation in the BLA in the elevated T-maze was opposite to that caused by the stimulation of 5-HT_{1A}Rs in this subnucleus. In test-naive rats, bilateral microinjection of 8-OH-DPAT, a 5-HT_{1A}R agonist, or the benzodiazepine anxiolytic midazolam impaired inhibitory avoidance acquisition, without changing escape expression (Zangrossi *et al.* 1999). More recently, we observed that in animals previously exposed to one of the elevated T-maze open arms, as performed in the present study, intra-BLA injection of 8-OH-DPAT retained its anxiolytic effect, but also inhibited escape expression, indicating a panicolytic-like effect (C. A. Strauss, M. A. Vicente, H. Zangrossi Jr., unpublished results). As mentioned before, the same pattern of results was observed after the intra-BLA injection of the preferential 5-HT_{2A}R agonist DOI (C. A. Strauss, M. A. Vicente, H. Zangrossi Jr., unpublished results). Therefore, among those 5-HT receptor subtypes more consistently implicated in defence regulation, 5-HT_{2C}Rs in the BLA seem to be unique in their selective involvement with anxiety- but not panic-related behaviours.

The most important finding of this study is that intra-BLA administration of a sub-effective dose of SB-242084 fully blocked the acute anxiogenic effect caused by imipramine and fluoxetine in the Vogel

conflict test and imipramine in the elevated T-maze. This observation adds to a series of evidence showing that 5-HT_{2C}R in the BLA mediates the anxiogenic effect generated by different conditions/situations such as exposure to stressful stimuli (Christianson *et al.* 2010) and discontinuation of chronic ethanol ingestion (Overstreet *et al.* 2006). It also extends previous evidence showing that systemic injection of 5-HT_{2C}R antagonists fully counteracted the acute anxiogenesis caused by antidepressants in different animal models of anxiety (Bagdy *et al.* 2001; Burghardt *et al.* 2007; Greenwood *et al.* 2008; Yamauchi *et al.* 2004).

Although no study has directly investigated the effect of imipramine or fluoxetine, *in-vivo* microdialysis studies have shown that acute systemic injection of the SSRI citalopram enhances 5-HT levels in the amygdala (Bosker *et al.* 2001; Gobert *et al.* 2009). The same effect on 5-HT concentration was observed when the SSRI fluvoxamine was injected directly into the BLA (van der Stelt *et al.* 2005). In keeping with the present result with 5-HT, intra-BLA microinjection of fluoxetine enhanced anxiety in a contextual fear paradigm (Martinez *et al.* 2007). Therefore, it is conceivable that facilitation of 5-HT neurotransmission in the BLA, through mediation of 5-HT_{2C}Rs, may account for the acute anxiogenic effect caused by different classes of antidepressants known to interfere with the extracellular availability of 5-HT. In the case of imipramine and fluoxetine, evidence in the literature shows that these antidepressants may interact directly with 5-HT_{2C}Rs, although with weak affinity (Ni & Miledi, 1997; Pälvimäki *et al.* 1996). However, given evidence that these drugs exert antagonistic and not agonistic activity on these receptors (Ni & Miledi, 1997; Pälvimäki *et al.* 1996) and that injection of SB-242084 in the BLA is anxiolytic (expt 1a), it is unlikely that this mechanism of action mediates their acute anxiogenic effect.

We are currently investigating whether prolonged treatment with imipramine or fluoxetine affects the reactivity of 5-HT_{2C}Rs in the BLA. According to Millan (2003, 2005), the shift from anxiogenesis to anxiolysis caused by these drugs over time involves both the desensitization of 5-HT_{2C}Rs and the sensitization of 5-HT_{1A}Rs located in limbic areas. In line with this hypothesis, as mentioned above, facilitation of 5-HT_{1A}R-mediated neurotransmission in the BLA is anxiolytic in the elevated T-maze (Zangrossi *et al.* 1999). It also remains to be investigated whether this effect is potentiated after chronic treatment with antidepressants.

In conclusion, our findings highlight the selective involvement of BLA 5-HT_{2C}Rs in the regulation of

anxiety-related behaviours and provide the first direct evidence of their role in mediation of the short-term aversive effect of antidepressants.

Acknowledgements

The authors thank Afonso Paulo Padovan for helpful technical support. The experiments described in this paper were funded by FAPESP and CNPq, Brazil.

Statement of Interest

None.

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