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Research report

Monoamine involvement in the antidepressant-like effect induced by P2 blockade

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ABSTRACT

Depression is a common mental disorder that affects millions of individuals worldwide. Available monoaminergic antidepressants are far from ideal since they show delayed onset of action and are ineffective in approximately 40% of patients, thus indicating the need of new and more effective drugs. ATP signaling through P2 receptors seems to play an important role in neuropathological mechanisms involved in depression, since their pharmacological or genetic inactivation induce antidepressant-like effects in the forced swimming test (FST). However, the mechanisms involved in these effects are not completely understood. The present work investigated monoamine involvement in the antidepressant-like effect induced by non-specific P2 receptor antagonist (PPADS) administration. First, the effects of combining sub-effective doses of PPADS with sub-effective doses of fluoxetine (FLX, selective serotonin reuptake inhibitor) or reboxetine (RBX, selective noradrenaline reuptake inhibitor) were investigated in mice submitted to FST. Significant antidepressant-like effect was observed when subeffective doses of PPADS was combined with subeffective doses of either FLX or RBX, with no significant locomotor changes. Next, the effects of depleting serotonin and noradrenaline levels, by means of PCPA (p-Chlorophenylalanine) or DSP-4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride) pretreatment, respectively, was investigated. Both, PCPA and DSP-4 pretreatment partially attenuated PPADS-induced effects in FST, without inducing relevant locomotor changes. Our results suggest that the antidepressant-like effect of PPADS involves modulation of serotonin and noradrenaline levels in the brain.

1. Introduction

World Health Organization (WHO) estimates 350 million of individuals are affected by depression globally, and it predicts depression will be the main cause of morbidity and loss of productivity among all health conditions by 2030 (World Health Organization, 2016). The current antidepressant drug therapy, based on increasing monoamines availability (Elhwuegi, 2004), is only effective in approximately 60% of the patients, and it takes 3–4 weeks to be clinically effective (Blier, 2003; Racagni and Popoli, 2008).

Great attention has been paid to the purinergic signaling in neuropathological disorders such as Alzheimer and Parkinson disease, anxiety, schizophrenia, drug addiction, and, of importance to the present work, depression, (Abbracchio et al., 2009; Burnstock, 2008). Purinergic neurotransmission emerged from the work of Prof. Burnstock describing adenosine 5'-triphosphate (ATP) as non-adrenergic, non-cholinergic inhibitory transmitter in the guinea-pigs (Abbracchio and Burnstock, 1994; Burnstock, 1972; Burnstock, 1976). Since then, ATP has been described as a cotransmitter in noradrenergic (Sperlagh et al., 1998), gabaergic (Jo and Role, 2002), glutamatergic (Mori et al., 2001) and cholinergic (Richardson and Brown, 1987) synaptic terminals, but vesicles containing exclusively ATP have also been reported (Pankratov et al., 2006). ATP released in synaptic cleft can be degraded by

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ectonucleotidases into various metabolites, such as adenosine, which also acts as a ligand for purinergic receptors (Zimmermann, 2006).

Purinergic neurotransmission comprises receptors for adenosine and ATP, respectively termed P1 and P2. Based on molecular cloning and pharmacological differences, P2 receptors can be divided into P2Y and P2X receptors (Abbracchio and Burnstock, 1994). P2Y are G-protein coupled receptors that modulate inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG) levels from membrane phosphoinositide metabolism (Pfeilschifter, 1990; Burnstock, 2007), while P2X are ion channels permeable to Na^+, K^+ and Ca^{2+} (Burnstock, 2007; Benham and Tsien, 1987; Bean, 1992). Currently, 8 subtypes of metabotropic P2Y receptors ($P2Y_{1,2,4,6,11,12,13,14}$) and 7 subtypes of ionotropic P2X receptors ($P2X_{1-7}$) have been described (Burnstock, 2007). P2 receptors are widely expressed in cerebral structures involved in emotional behavior such as hippocampus, cerebral cortex, ventral tegmental area and locus coeruleus (Norenberg and Illes, 2000), either in glial or neuronal cell types (Burnstock, 2008).

It has been recently reported that pharmacological blockade of P2 receptors induces antidepressant-like effects in preclinical models (Pereira et al., 2013; Csolle et al., 2013). Selective P2X7 antagonist also induced antidepressant-like effect in the chronic mild stress model (Iwata et al., 2016) and similar behavioral phenotype was also described in animals with genetic deletion of P2X7 receptors (Boucher et al., 2011; Basso et al., 2009). Despite evidences of an antidepressant-like effect induced by P2R antagonist treatment, the involvement of serotonergic and/or noradrenergic mechanisms has not yet been investigated. Monoamine and purinergic interplay is plausible given the fact that activation of P2R can modulate brain glutamate and NO levels (Florenzano et al., 2008; Pereira et al., 2013), which are both able to control serotonin synthesis, stability and release (Kuhn and Arthur, 1997; Fossier et al., 1999). In support of that, P2X7R knockout animals showed changes in cerebral levels of noradrenaline and serotonin (Csolle et al., 2013). Furthermore, the antidepressant-like effect of NOS inhibitors and NMDA antagonists (Diniz et al., 2016) are dependent of serotonin levels in the brain (Harkin et al., 2003; du Jardin et al., 2016; Ulak et al., 2010). Therefore, since P2R signaling modulate glutamate and NO release in the brain, which then affects monoamine levels, we hypothesized the antidepressant-like effect of the non-specific P2R antagonist PPADS would involve the modulation of brain serotonin and/or noradrenaline levels.

Therefore, the present aims were: 1. To investigate if the combination of sub-effective doses of P2R antagonist with subeffective doses of antidepressants of different pharmacological classes (serotonin or noradrenaline reuptake inhibitors) would be able to induce antidepressant-like effect in mice submitted to forced swimming test; 2. To investigate if depleting serotonin or noradrenalin levels, by means of pharmacological pretreatment with PCPA (tryptophan hydroxylase inhibitor; Koe and Weissman, 1966) or DSP4 (noradrenergic neurotoxin; Grzanna et al., 1989), could attenuate or even block the behavioral effect of P2R antagonist in the FST. Altogether, these data could help understanding if monoamines would be involved in the mechanisms underlining the antidepressant-like effect induced by P2R blockade.

2. Results

2.1. Dose-response curves for FLX, RBX and PPADS treatment on mice submitted to FST or OFT

One-way ANOVA indicates a significant effect of drug treatment on immobility time in the FST [$F_{9,60} = 3.453$. $p < 0.05$]. Treatment with PPADS (6.25 mg/kg), FLX (20 mg/kg) and RBX (5 mg/kg) significantly decreased immobility time on FST (Dunn's *post hoc* test, $p < 0.05$), as seen in Fig. 1A. Effective doses of PPADS (6.25 mg/kg), FLX (20 mg/kg) and RBX (5 mg/kg) were not able to change locomotor behavior of animals tested on OFT (Fig. 1B – $F3_{,20} = 1.715$, non-significant – NS).

2.2. Add on effects of PPADS sub-effective dose to FLX or RBX sub-effective doses on FST and OFT

Two-way ANOVA indicates a significant treatment effect of first injection with FLX or RBX [$F_{2,38} = 7.831$; $p < 0.01$], of second injection with PPADS [$F_{1,38} = 4.534$; $p < 0.05$] and an interaction between factors [$F_{2,38} = 4.727$; $p < 0.05$]. In column comparison, combination of sub-effective doses of PPADS (3 mg/kg) and FLX (10 mg/kg) decreased immobility time compared to VEH/VEH ($p < 0.05$) and VEH/PPADS ($p < 0.01$), whereas combination of sub-effective doses of PPADS and RBX (2.5 mg/kg) decreased immobility time compared to VEH/VEH ($p < 0.01$), VEH/PPADS ($p < 0.001$) and RBX/VEH ($p < 0.05$) groups (Fig. 2A – $F5_{,38} = 5.915$). Combination of sub-effective doses of PPADS (3 mg/kg) and FLX (10 mg/kg) or RBX (2.5 mg/kg) did not change locomotor activity of animals on OFT (Fig. 2B – $F2_{,14} = 0.6730$, NS).

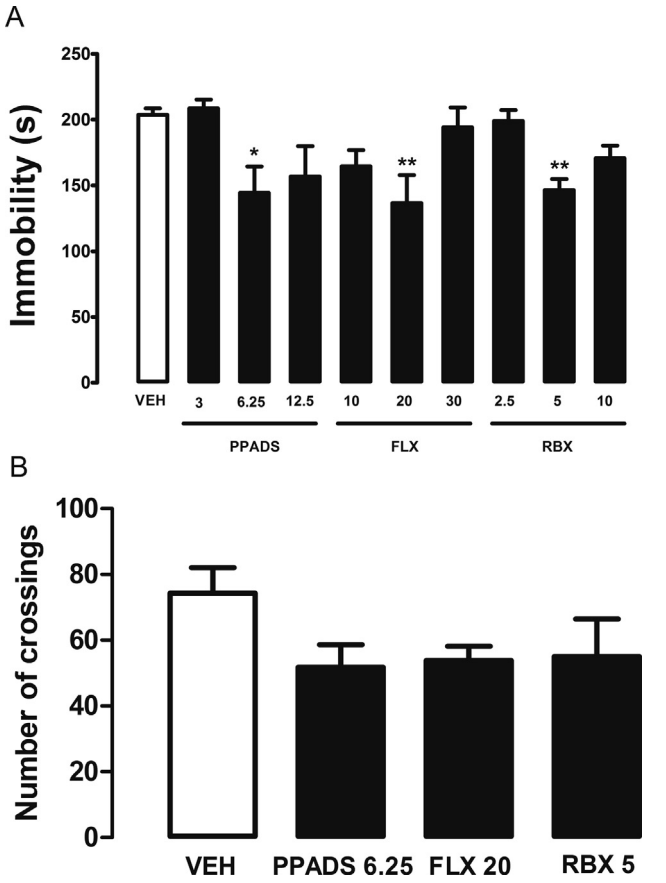

Fig. 1. Dose-response curves for FLX, RBX and PPADS treatment on mice FST and OFT. (A) PPADS (3, 6.25, 12.5), FLX (10, 20, 30), RBX (2.5, 5, 10) or vehicle (10 mL/kg) were administered 30 min before FST (n = 6, 7, 7, 7, 7, 5, 5, 7, 10, 9, respectively). Data are expressed as mean ± SEM of immobility time (s); $^{*}p < 0.05$, $^{**}p < 0.01$ from control group. (B) PPADS (6.25 mg/kg, n = 6), FLX (20 mg/kg, n = 6), RBX (5 mg/kg, n = 6) or VEH (n = 6) was administered 30 min before being submitted to OFT. Data are expressed as Mean ± SEM of total quadrant traveled in the OFT.

2.3. Effect of 5-HT depletion on PPADS effects on FST and OFT behavior

Two-way ANOVA indicated a significant treatment effect of PPADS [$F_{1,27} = 8.64$; $p < 0.01$] but not of PCPA [$F_{1,27} = 0.25$; NS], neither interaction between both factors [$F_{1,27} = 2.115$; NS]. In column comparison, immobility time of group VEH/PPADS is observed lower than VEH/VEH group [$F_{3,27} = 3.631$, $p < 0.05$ – Fig. 3A; Dunn's *post hoc* test]. Moreover, PCPA+VEH, as well as PCPA+PPADS combination did not significantly differ from VEH+VEH group (Fig. 3A; $p > 0.05$ vs control, Dunn's *post hoc* test). This indicates PCPA did not induce effect *per se*, but it attenuated PPADS-induced effects. Two-way ANOVA show no locomotor change

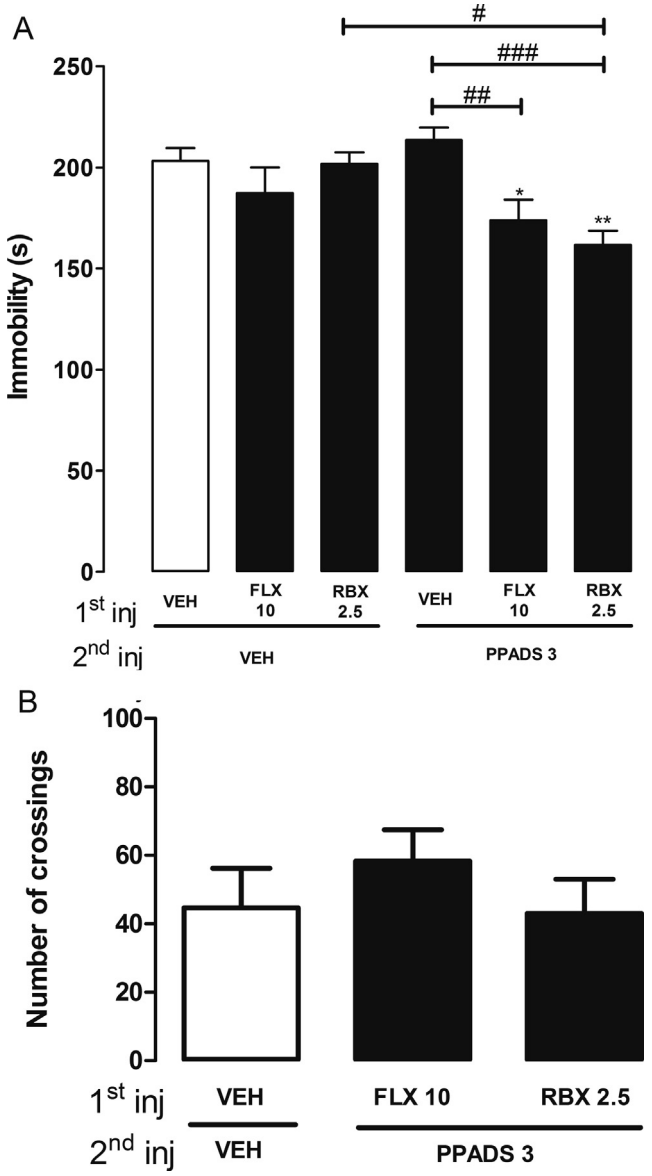

Fig. 2. Add on effects of PPADS sub-effective dose with FLX or RBX sub-effective doses on FST and OFT. (A) Sub-effective dose of FLX (10 mg/kg) or RBX (2.5 mg/kg) was carried out after PPADS sub-effective dose (3 mg/kg) administration, 30 min before FST. Six groups were obtained: VEH + VEH (n = 9), FLX + VEH (n = 6), RBX + VEH (n = 6), VEH + PPADS (n = 8), FLX + PPADS (n = 8), RBX + PPADS (n = 7). Data are expressed as mean ± SEM of immobility time (s); $^{*}p < 0.05$, $^{**}p < 0.01$ from control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ from respective groups outlined. (B) Sub-effective dose of FLX (10 mg/kg) or RBX (2.5 mg/kg) was carried after PPADS sub-effective dose (3 mg/kg) administration, 30 min before OFT. Three groups were obtained: VEH + VEH (n = 6), FLX + PPADS (n = 6), RBX + PPADS (n = 5). Data are expressed as Mean ± SEM of total quadrant traveled in the OFT.

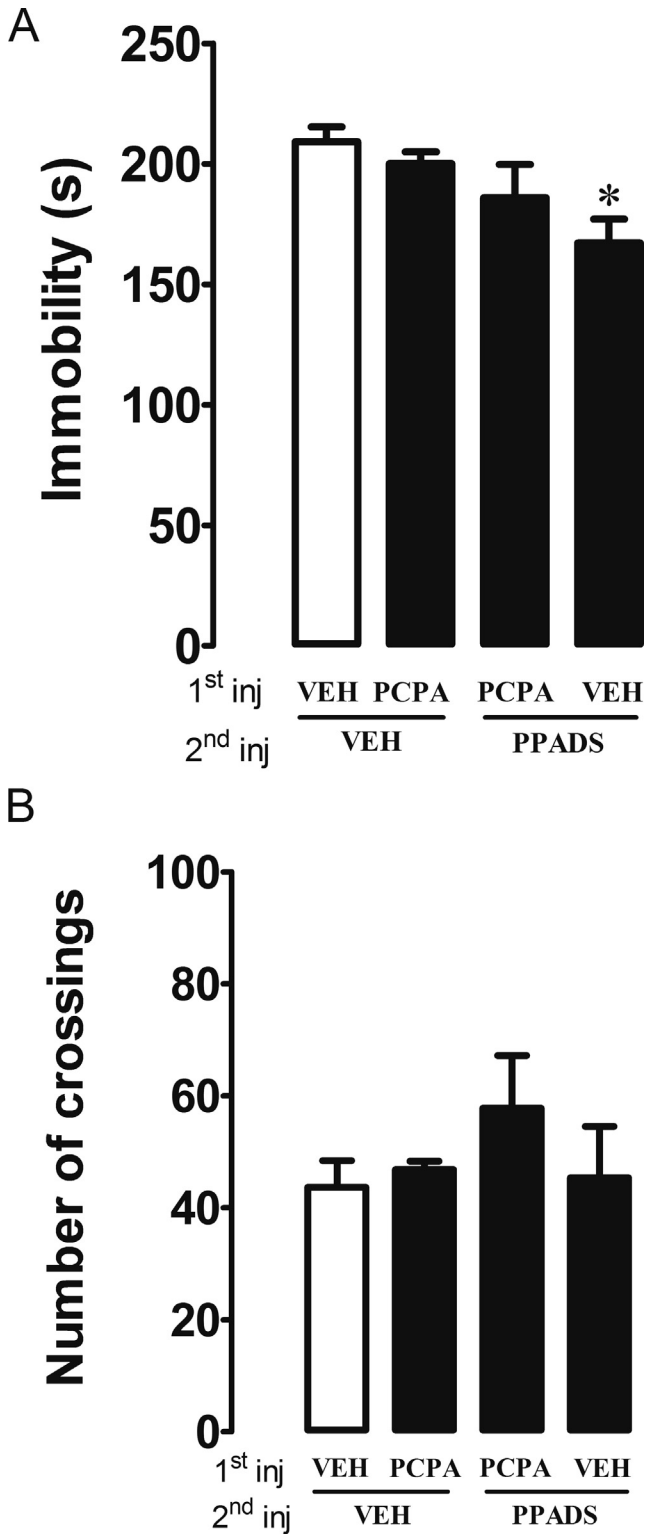

Fig. 3. Effect of 5-HT depletion on PPADS effects on FST and OFT behavior. (A) PCPA (150 mg/kg) or VEH was administered once daily for 4 days. On the fourth day, the last dose of PCPA or VEH infusion were done 30 min before the second treatment with VEH or PPADS (6.25 mg/kg). Animals were submitted to the FST 30 min after the second injection. Thus, four groups were obtained: VEH + VEH (n = 7), PCPA + VEH (n = 8), PCPA + PPADS (n = 8), VEH + PPADS (n = 8). Data are expressed as mean ± SEM of immobility time (s). (B) The same treatment protocol was established as described above, but animals were submitted to the OFT instead of the FST. Four groups were obtained: VEH + VEH (n = 6), PCPA + VEH (n = 6), PCPA + PPADS (n = 5), VEH + PPADS (n = 6). Data are expressed as Mean ± SEM of total quadrant traveled in the OFT. $^{*}p < 0.05$ from control group.

induced by any of the treatments (PCPA: $F_{1,19} = 0.859$; NS; PPADS: $F_{1,19} = 1.316$; NS; interaction: $F_{1,19} = 0.466$; NS – Fig. 3B).

2.4. Effect of NA depletion on PPADS effects on FST and OFT

Two-way ANOVA indicates a significant interaction between DSP4 and PPADS [$F_{1,31} = 8.624$; $p < 0.01$], however no effect of the isolated treatments [DSP4: $F_{1,31} = 1.016$; PPADS: $F_{1,31} = 1.198$, NS for both]. In column comparison, both groups DSP4/VEH and VEH/PPADS showed decreased immobility time on FST compared to VEH/VEH group [$F_{3,31} = 3.423$, $p < 0.05$ – Fig. 4A]. In addition, two-way ANOVA indicated a significant effect of DSP4 [$F_{1,24} = 9.58$; $p < 0.005$] but not of PPADS [$F_{1,24} = 1.91$; NS] or interaction between factors [$F_{1,24} = 1.11$; NS] on OFT. In column comparison, a significant effect of DSP4 infusion on OFT was observed [Fig. 4B – $F1_{,24} = 9.580$; $p < 0.01$].

2.5. 5HT, NA and 5HIAA levels on PFC and HPC of PCPA and DSP4 treated animals

According to student's *t* test there was a significant decrease of NA ($t_8 = 2.451$), 5HIAA ($t_8 = 2.491$) and 5HT ($t_8 = 3.885$) hippocampal levels in DSP4 treated mice. No difference was observed with DSP4 treatment in frontal cortex (NA $t_{10} = 0.018$; 5HIAA $t_{10} = 1.178$ and 5HT $t_{10} = 1.672$). Student's *t* test identified a reduction in NA ($t_7 = 2.488$) frontal cortex levels from PCPA treatment but no change was observed in 5HIAA ($t_6 = 1.100$) and 5HT ($t_7 = 1.096$) levels. In hippocampus, 5HIAA ($t_7 = 2.647$) levels were significantly reduced by PCPA treatment, but no change was observed in NA ($t_7 = 0.159$) and 5HT ($t_7 = 0.490$) levels. All data are found in Table 1.

3. Discussion

This work was designed to investigate the involvement of monoamines in PPADS induced antidepressant-like effects in FST. We found that: 1. Combination of subeffective doses of PPADS with subeffective doses of serotonergic (FLX) or noradrenergic (RBX) antidepressants induced significant effects in mice FST; 2. Pretreatment with PCPA (serotonin synthesis inhibitor) or DSP-4 (noradrenergic toxin) decreased serotonin and noradrenaline levels in the brain and attenuated PPADS induced antidepressant-like effects. None of these effects were associated to locomotor changes in the OFT.

Stressful events, a key factor for susceptibility to depression, would be expected to affect behavior in the FST. In fact, immobility time of rats exposed to swim session is increased after uncontrollable stress and decreased by antidepressant treatment (Porsolt et al., 1977; Cryan et al., 2002a; Nestler and Hyman, 2010). However, no consensus is established about what immobility in the FST represents, whether it is a behavioral despair or a passive-coping (Cryan et al., 2002a). Either way, the antidepressant efficacy of decreasing immobility time confers to FST a presumable predictive validity and allows the study of mechanism of action for putative new drugs. In this work, systemic treatment with P2R antagonist reduced immobility time in the FST, further supporting the modulation of the purinergic system as a new target for putative antidepressant drugs.

The dose-dependent antidepressant effect induced by PPADS in the present work is in accordance with our previous published paper (Pereira et al., 2013). Further, PPADS co-treatment with FLX and RBX, in subeffective doses, induced a significant antidepressant-like effect, thus suggesting that both serotonergic and noradrenergic mechanisms might participate in PPADS-induced antidepressant-like effects. When two drugs are combined

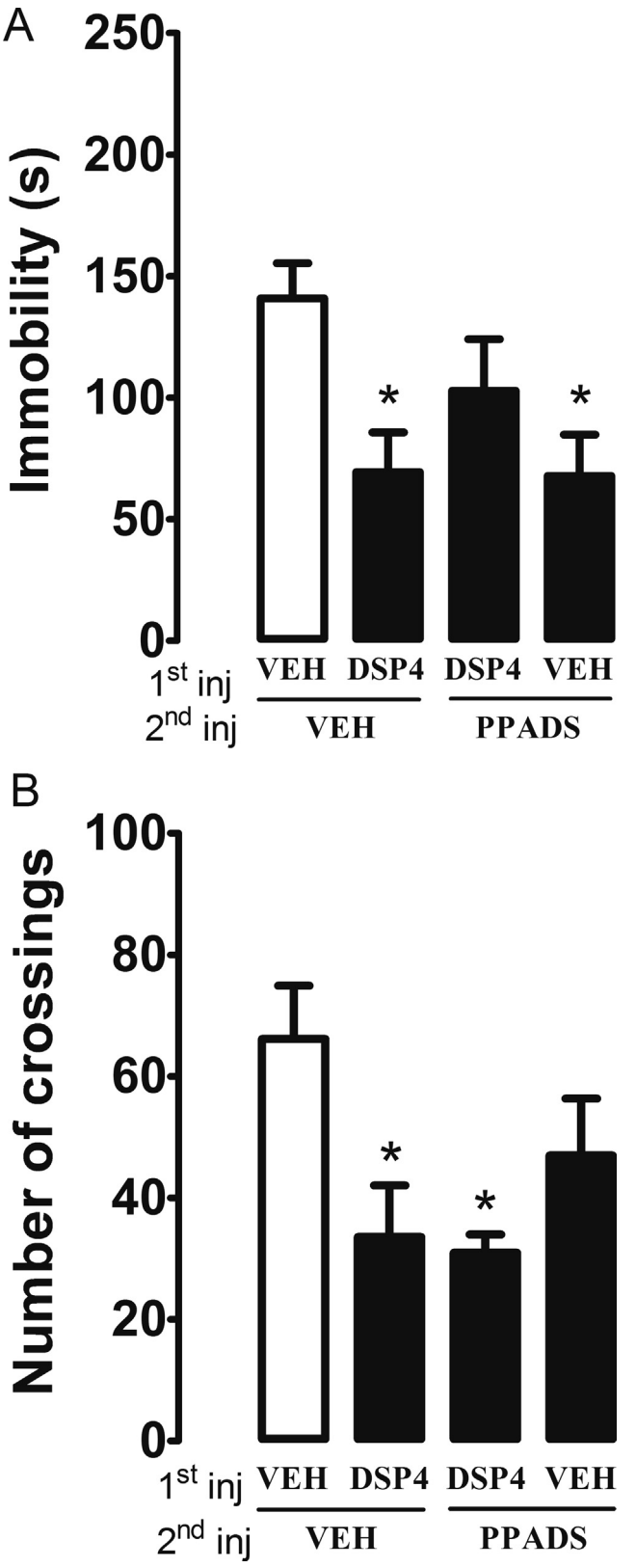

Fig. 4. Effect of NA depletion on PPADS effects on FST and OFT. (A) DSP4 (20 μg) was infused i.c.v. 24 h before FST. Injection of PPADS (6.25 mg/kg) was performed 30 min before FST. Four groups were obtained: VEH + VEH (n = 7), DSP4 + VEH (n = 8), DSP4 + PPADS (n = 9), VEH + PPADS (n = 8). Data are expressed as mean ± SEM of immobility time (s). (B) The same treatment protocol was established as described above, but animals were submitted to the OFT instead of the FST. Four groups were obtained: VEH + VEH (n = 7), DSP4 + VEH (n = 7), DSP4 + PPADS (n = 7), VEH + PPADS (n = 7). Data are expressed as Mean ± SEM of total quadrant traveled in the OFT. *p < 0.05 from control group.

Table 1
5HT, NA and 5HIAA levels on PFC and HPC of PCPA and DSP4 treated animals. (a) DSP4 was infused i.c.v. 24 h before euthanasia. Two groups were obtained: VEH (n = 6) and DSP4 (n = 6). (b) PCPA (150 mg/kg) was administered once a day for 4 days. On the fourth day PCPA was administered 1 h before euthanasia. Serotonin, noradrenaline and serotonin metabolite levels were analyzed on frontal cortex and hippocampus with HPCL method. All data are expressed as Mean ± SEM of ng/mg tissue, $^{*}p < 0.05$ from control group; $^{**}p < 0.01$ from control group. Number of animals per group here is based in number of samples which HPCL MS determination was possible (in the graphic), although number of animals euthanized is higher (VEH n = 6/DSP4 n = 6; VEH n = 5/PCPA n = 5).

Neurotransmitter levels								
	Vehicle	DSP4	*t*-test	p	Vehicle	PCPA	*t*-test	p
Frontal cortex								
Noradrenaline	0.127 ± 0.018 (n = 6)	0.127 ± 0.010 (n = 6)	t10 = 0.02	0.98	0.049 ± 0.004 (n = 4)	0.039 ± 0.003 (n = 5)	t7 = 2.49	0.04*
5HT	0.260 ± 0.025 (n = 6)	0.314 ± 0.020 (n = 6)	t10 = 1.67	0.12	0.087 ± 0.006 (n = 4)	0.077 ± 0.006 (n = 5)	t7 = 1.10	0.31
5HIAA	0.099 ± 0.017 (n = 6)	0.121 ± 0.007 (n = 6)	t10 = 1.18	0.27	0.035 ± 0.014 (n = 3)	0.023 ± 0.002 (n = 5)	t6 = 1.10	0.31
Hippocampus								
Noradrenaline	0.237 ± 0.028 (n = 5)	0.156 ± 0.017 (n = 5)	t8 = 2.45	0.04*	0.080 ± 0.009 (n = 4)	0.082 ± 0.006 (n = 5)	t7 = 0.16	0.88
5HT	0.324 ± 0.032 (n = 5)	0.192 ± 0.012 (n = 5)	t8 = 3.88	0.004**	0.082 ± 0.012 (n = 4)	0.076 ± 0.006 (n = 5)	t7 = 0.49	0.64
5HIAA	0.334 ± 0.048 (N = 5)	0.207 ± 0.017 (n = 5)	t8 = 2.49	0.04*	0.066 ± 0.007 (n = 4)	0.048 ± 0.003 (n = 5)	t7 = 2.65	0.03*

in the same organ, they may promote no interference with each other effects, reduce or block each other's action (antagonism), or facilitate each other's action (synergistic or additive effects). Therefore, the combination of subeffective doses of different drugs allows the investigation of "agonistic" drug interactions amongst them (Chou, 2006). Based on that, researchers have used this pharmacological approach as a first step in identifying possible pharmacological interactions that could guide to further investigations on a given drug mechanism of action (Chou, 2006). For instance, Harkin, et al. (2004) demonstrated that the combination of subeffective doses of nNOS inhibitor with subeffective doses of fluoxetine, but not with desipramine, induced effect in the FST, suggesting the participation of serotonergic, but not noradrenergic, mechanisms in the evaluated antidepressant-like effects. This was further confirmed in that study when PCPA pretreatment blocked drug-induced antidepressant effect. Thus, a similar approach was employed in the present study as an attempt to investigate serotonin and noradrenaline involvement on PPADS effects in the FST. Data obtained with depletion studies partially supported the findings with subeffective doses, since both PCPA and DSP-4 attenuated PPADS-induced antidepressant-like effects in the FST.

Absence of *per se* effect of PCPA on immobility is in agreement with animals and human research regarding monoamine hypothesis of depression. In fact, our data are in agreement with other papers that failed to find effects of PCPA on immobility in the FST and other animal models (O'Leary et al., 2007; Page et al., 1999). Accordingly, serotonin depletion does not precipitate depressive symptoms in health individuals, but blocks the antidepressant effect of serotonergic drugs (Delgado, 2006; Moreno et al., 2010). Similarly, PCPA treatment protocols and results have been described by others where PCPA blocked antidepressant effects in the FST (Harkin et al., 2003; O'Leary et al., 2007).

PCPA is considered a potent depletor of brain serotonin in mice, rats and dogs (Koe and Weissman, 1966). It is well documented that PCPA does not inhibit monoamine oxidase or 5HTP-decarboxylase and it has no effect on monoamine oxidase or 5HTP decarboxylase activity of rat tissues in vivo, thus inducing 5-HT depletion by inhibiting the biosynthesis of this monoamine, possibly by blocking tryptophan hydroxylation (Koe and Weissman, 1966). Other studies have confirmed PCPA acts as a serotonin synthesis inhibitor leading to decreased serotonin levels in different brain regions, depending on dose and treatment schedule (Datla and Curzon, 1996; Kornum et al., 2006).

Regarding DSP4 experiments, a decreased locomotor activity was verified in both DSP4 + VEH and DSP4 + PPADS groups, which might originate from DSP4 effect *per se* on locomotor activity. In fact, it cannot be excluded that partial reversal of PPADS antidepressive-like effect by DSP4 might be related to a non-specific DSP4 effect on locomotion. However, that is unlikely, provided DSP4's effect in decreasing locomotor activity did not prevent its own antidepressant-like effect.

DSP-4 mechanism of action is less understood. However, it is known it depletes noradrenaline content in dose-dependent fashion, in different brain regions (Dailly et al., 2006) and rodent species (Fornai et al., 1996). It is reported that after a single i.p. injection of DSP-4, this neurotoxin is uptaken by noradrenergic neurons (Ross and Renyl, 1976; Hallman and Jonsson, 1984), which start to degenerate in the LC target structures (Fritschy and Grzanna, 1989).

Taken together, drugs that inhibit monoamine synthesis or release, such as PCPA and DSP-4 represent important tools that can help characterizing the mechanisms underlying an antidepressant-like effects of a new drug or reveal underlying vulnerability to stress (Cryan et al., 2002b; O'Leary et al., 2007).

In the present work, HPLC analysis of monoamine levels in brain regions of animals treated with PCPA revealed decreased noradrenaline levels in the frontal cortex, while it decreased serotonin main metabolite 5-hydroxyindoleacetic acid (5HIAA) levels in hippocampus. Changes in brain catecholamines after PCPA systemic administration, although subtle, were observed in the original work of Koe and Weissman (1966). Indeed, catecholamine levels can be slightly decreased after PCPA administration, especially in high doses (Koe and Weissman, 1966; Datla and Curzon, 1996; Kornum et al., 2006), as we observed in the present work. Tyrosine hydroxylase was also slightly inhibited *in vitro* with PCPA infusion and plasma tyrosine levels were slightly reduced after systemic PCPA injection (Koe and Weissman, 1966). Curiously, 5HT levels did not change in either frontal cortex or hippocampus, which is in disagreement with other works (Shutoh et al., 2000; Fletcher et al., 2001; Page et al., 1999). Differences in methodological analysis of serotonin content or in drug administration and brain dissection protocols might explain such contradictory results. Besides, other structures involved with modulation of behavioral consequences of stress such as hypothalamus, bed nucleus of the stria terminalis, nucleus accumbens and amygdala could present serotonin levels changes, but this possibility was not verified in the present work. Although no change was observed on serotonin levels after PCPA treatment, 5HIAA hippocampal levels were decreased. Concentration of 5HIAA has been used to estimate the activity of serotonergic neurons (Shannon et al., 1986). In fact, electrical stimulation of the dorsal raphe nucleus increased 5HIAA levels and 5HIAA/5-HT concentration ratio in the nucleus accumbens, amygdala, suprachiasmatic nucleus and dorsomedial nucleus (Shannon et al., 1986). Therefore, PCPA treatment used in the present work was able to induce changes in both noradrenaline and serotonin neurotransmission in the brain. Based on that, PCPA effects cannot be considered selective to serotonin neurotransmission and its effects on other monoamines should be taken into con-

sideration when discussing PCPA-induced effects. In addition, even that other have described that similar treatment with PCPA blocked the effect of antidepressant drugs in the FST (Harkin et al., 2003; O'Leary et al., 2007), we observed only partial reversion of PPADS effects in the FST (Fig. 3). This indicates that PPDAS effects might involve monoaminergic mechanisms, although the specific participation of noradrenalin and/or serotonin in PPADS effects is not clear.

DSP4 treatment also decreased not only noradrenaline levels, but also serotonin (5HT) and 5HIAA levels, in the hippocampus. In the frontal cortex, no change was observed on noradrenaline, serotonin or 5HIAA. Although the main neurotoxic effect of DSP-4 on noradrenergic neurotransmission (Fornai et al., 1996), other works have described decreased serotonin levels in the cerebellum and in the spinal cord (Jonsson et al., 1981), which is in agreement with our results. It might also seem surprising that DSP-4 decreased neurotransmitter levels in a specific brain region, instead of all over the brain. However, this has been described by others that noradrenergic terminals originated in the *locus coeruleus* are more susceptible to DSP-4 depleting and neurotoxic effects than those in ventral forebrain and hypothalamus, which are supplied primarily by non-coerulean NE cells (Grzanna et al., 1989). The mechanisms responsible for those differences are not clear, but could rely on differences in the pharmacological properties of NE axon terminals, such as neuronal density and transporter availability (Grzanna et al., 1989). Another reasonable explication is that hippocampus is located close to the ventricular wall, then is possible that i.c.v. drug infusion reached hippocampal region easier than distant areas such as the frontal cortex.

Several reports describe noradrenaline levels are decreased in the brain after systemic injections of DSP4, whereas microdialysis studies indicate extracellular noradrenaline levels are actually increased (for review see Ross and Stenfors, 2015). Other in vivo microdialysis studies (Hughes and Stanford, 1998; Kask et al., 1997) have shown that pretreatment with DSP-4 (40 mg/ kg) actually leads to an increase in the basal extracellular concentration of norepinephrine, in rat frontal cortex, despite causing a 75% lesion of cortical norepinephrine content. That increase is further potentiated following challenge with the norepinephrine reuptake inhibitor desipramine. Accordingly, augmented behavioral response was seen in reboxetine treated groups after DSP-4 treatment (Cryan et al., 2002b), although an underlying mechanism for that effect remains to be elucidated. Therefore, increased extracellular levels of noradrenaline in some particular brain regions, despite decrements in other brain regions, might help explaining the antidepressant-like effect induced by DSP4 treatment *per se*. In this sense, DSP4 attenuating effect of PPADS could be the result of a complex interplay between both drugs acting through different brain regions in the CNS.

ATP is recognized as a neurotransmitter accessible to perisynaptic glia and neurons, in addition to being released from glial and neuronal cells (Pascual et al., 2005; Araque et al., 2014; Halassa and Haydon, 2010; Lalo et al., 2014; Kato et al., 2004; Koizumi et al., 2005). This dual functional role of purinergic system contributes to signal integration between glial cells and neuronal synapses, as a tripartite synapse (Pascual et al., 2005). Besides, ATP could evoke astrocyte calcium waves leading to the release of other transmitters such as glutamate, GABA and glycine (Guthrie et al., 1999). In fact, interaction between glutamatergic and purinergic neurotransmission has been the most studied so far, with ATP facilitating neuronal and astrocyte glutamate release (Burnstock, 2002; Burnstock, 2011; Shen et al., 2005).

Glutamate, as well as NO, which arises from NMDAR activation, can control serotonin availability (Kuhn and Arthur, 1997; Fossier et al., 1999). Evidences indicate that ATP itself is also able to attenuate serotonin release in rat brain cortex via P2R activation (Von Kügelgen et al., 1997). Conversely, serotonin is also able to modulate ATP levels in the brain (Koren-Schwartzer et al., 1994). In this context, the add-on effect observed with concomitant PPADS and FLX administration could derive from P2R blockade favoring serotonergic availability. In addition, a previous work from our group showed PPADS attenuated NO levels in the prefrontal cortex at the same dose that induced antidepressant-like effects in the FST (Pereira et al., 2013). In line with data of the present work, antidepressant-like effect induced by nNOS inhibitors is also dependent on brain serotonin levels (Harkin et al., 2003). In fact, it is known nNOS inhibition can increase the release of 5-TH in the brain (Karolewicz et al., 2001). Altogether, this evidence suggests serotonergic mechanisms might be involved in PPADS-induced antidepressant-like effects. However, pretreatment with PCPA only partially reversed PPADS effect on FST, which suggests that serotonin might be involved in PPADS mechanism, although other neurotransmitters may also play an important role.

Regarding noradrenaline involvement on PPADS effects, it is shown that activation of presynaptic P2R increase the release of noradrenaline in the locus coeruleus (Tschopl et al., 1992), whereas it decreases noradrenaline availability in the rat cortex (von Kügelgen et al., 1994) and hippocampus (Koch et al., 1997). These data reveal a complex role for ATP in regulating noradrenaline levels in the brain. Our results showing that combination of subeffective doses of PPADS with RBX induced antidepressant-like effect suggests the involvement of central noradrenergic mechanisms in PPADS effects on FST. Moreover, the fact that DSP4 was able to reduce PPADS effects, although without blocking it, gives further support to that possibility.

Previous work by Iwata et al. (2016) proposes an alternative mechanism for P2X7-antagonist induced antidepressant-like effects. Their work showed immobilization stress increased glutamate, ATP and inflammatory cytokines levels in hippocampus microdialysates, which was blocked by systemic treatment with P2X7 antagonist. Authors suggested that modulation of inflammasome complex after stress exposure would be involved in the antidepressant-like effect induced by P2X7 blockade. In fact, P2X7 receptors are also expressed in glia and they can trigger inflammasome activation and depressive-like behaviors (Yue et al., 2017). On the other hand, inhibition of inflammasome response seems to be a common mechanism of different antidepressant drugs (Alcocer-Gómez et al., 2017). However, the activation of inflammasome by stress has been shown only after chronic stress exposure (Yue et al., 2017; Iwata et al., 2016; Kaufmann et al., 2017). Since we employed acute stress in mice, it is unlikely this would have played an important role in our experiments. It is, however, possible that blockade of glutamate release from astrocytes by P2X blockade might have contributed to the observed antidepressant effect. However, since neuronal release of ATP is also evidenced (North, 2002) and P2R are expressed either in glial or neuronal cell types (Burnstock, 2008), it is not possible to presume which cell types might have contributed to PPADS effects in the present work. Besides that, the complex and close relationship between glial and neuronal cells mediating purinergic signaling (tripartite synapse) makes cell types discernment harder (Pascual et al., 2005). Nevertheless, it is possible to speculate that different mechanisms and cell types could be involved in acute and chronic effects on P2X7-mediated signaling. Accordingly, the antidepressant-like effect of PPADS, acquired after acute stress exposure, most probably would be provided by direct regulation of ATP activity on P2X7R present in neurons. However, in chronic stress conditions, P2X7 blockade might attenuate depressive-like behaviors by mixed effects, either by blocking ATP/P2X7R binding in astrocytes and thus attenuating neuroinflammatory responses or glutamate release, or by direct regulation of neuronal firing rate. Therefore, blockade of P2X7

receptors could induce antidepressant-like effects through different mechanisms, depending on the model under investigation (acute vs. chronic stress).

Finally, the results of the present study about serotonin and noradrenalin involvement on PPADS effects should be considered in the light of its limitations. For instance, a parsimonious interpretation must be done regarding the add-on effect of subeffective doses of PPADS plus RBX or FLX, since the combination of subthresholding doses does not necessarily imply a convincing interaction or synergistic effect among two drugs with different mechanism of action (Foucquier and Guedj, 2015). In fact, to assert a reliable synergistic or interaction of mechanism of action between the drugs, an isobologram analysis is required (Tallarida, 2006). Moreover, although PPADS + VEH group was not different from PPADS + PCPA or PPADS + DSP-4, significantly differed from VEH + VEH control group, indicating only partial reversion of PPADS effects without complete blockade. In addition, the complex effect of PCPA and DSP-4 on monoamine levels observed herein is an important limitation of the present study that should also be taken into consideration for the interpretation of the results. Nevertheless, in conclusion, the present study adds further evidence to previous studies, suggesting blockade of P2R induces antidepressant-like effects. Moreover, it suggests the involvement of serotonin and/or noradrenaline in such effects, although further studies regarding purinergic/monoaminergic interplay are still necessary to better understand the pharmacological mechanisms involved on that.

4. Materials and methods

4.1. Animals

A total of 352 male Swiss mice weighing 30–40 g (6–7 weeks) were used. Animals were housed in groups of 6–8 per cage (570 cm^2) in a temperature controlled room (24 ± 1 °C) under standard laboratory conditions with free access to food and water and a 12 h light/12 h dark cycle (light on at 6:30 a.m.). Animals were randomly assigned for experimental groups described bellow and procedures were performed in conformity with the Brazilian Council for the Control of Animals under Experiment (CONCEA), which comply with international laws and politics. The local Ethical Committee approved experimental protocol (146/2009), and all efforts to minimize animal suffering were made.

4.2. Drugs

Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium salt (PPADS, a non-selective P2R antagonist, Tocris, USA, #0625), fluoxetine hydrochloride (FLX, selective serotonin reuptake inhibitor, Prati-Donaduzzi, Brazil, #713198), reboxetine mesylate (RBX, selective noradrenaline reuptake inhibitor, Ascent Scientific, USA, #157), p-Chlorophenylalanine (PCPA, tryptophan hydroxylase inhibitor, Sigma-Aldrich, EUA, #C6506), N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP4, noradrenergic neurotoxin, TOCRIS, USA, #2958) were used. All drugs were prepared administered intraperitoneally (i.p.) at 10 mL/kg, except for DSP4, which was infused intracerobroventricularly in 1μl volume. DSP4 and FLX were dissolved in Tween 80 2% in sterile saline, while all other drugs were dissolved in sterile isotonic saline. 2,2,2-tribromoethanol (Aldrich Chemical, USA, #T48402) was used for stereotaxic surgery and chloral hydrate (Sigma Aldrich, USA, #V000554) was used to euthanize the animals for tissue collection.

4.3. Forced swimming test (FST)

Animals were individually submitted to 6 min of forced swimming in glass cylinders (height 25 cm, diameter 17 cm) containing 10 cm of water at 24–26 °C. The test was videotaped and immobility time (characterized by limb movements necessary for floating) was measured during last 4 min of the test by a trained observer blinded to the treatment condition. All behavioral procedures are in accordance to established protocols (Porsolt et al., 1977). The water was changed after each trial (Abel and Bilitzke, 1990).

4.4. Open field test (OFT)

The OFT was used to measure locomotor activity of animals according to Zanelati et al. (2010). Mice were placed individually in a circular open field arena (40 cm in diameter and 50 cm high Plexiglas wall) for 6 min. The exploratory activity was videotaped and number of crossings between quadrants was measured during last 4 min by an observer blinded to the treatment condition. After each trial, arena was cleaned with 70% alcohol solution.

4.5. Sample collection and High-performance liquid chromatography (HPLC)

Animals were deeply anesthetized with chloral hydrate 5% (0.75 g/kg, ip) for brain removal. The frontal cortex and hippocampus were dissected on ice and processed for determination of serotonin, noradrenaline and serotonin metabolite (5HIAA) levels as described by Patel et al. (2005). Samples were homogenized in 0.1 M perchloric acid, afterward centrifuged at 13000 rpm for 10 min at 4 °C. Supernatant was automatically injected into the chromatographic system to quantify neurotransmitters and metabolites by electrochemical detection in high-performance liquid chromatography (HPLC). The chromatograph (Waters® Alliance) consisted of a stripping column Symmetry® C18, 5 μm (150 × 4.6 mm) and mobile phase flow 1ml/min with the following composition: buffer (88.2%; citric acid 0.05 M, sodium octyl sulfate 250 mg/L, EDTA 0.1 mM, potassium chloride 2 mM and pH adjusted to 3.2 with NaOH), methanol (9.3%) and acetonitrile (2.5%). Mobile phase was vacuum filtered and degassed ultrasonically. Calibration curve was constructed with standard solutions of 2.5, 5, 10, 25, 50, 100 and 200 ng/ml of norepinephrine, serotonin and 5HIAA, which were injected into the chromatograph in triplicate. Detection and limit of quantification were 0.64 and 2.13 ng/ml for serotonin; 1.72 and 5.73 ng/ml for 5HIAA and 0.87 and 2.88 ng/ml for noradrenaline. Samples with concentrations found below limit of quantitation were discarded. Finally, concentrations of the substances were corrected by the mass of dissected tissue samples being expressed in ng of substance per mg of tissue.

4.6. Stereotaxic surgery and intracerebral administration

Mice were anesthetized with 2,2,2-tribromoethanol 2.5% (10 ml/kg intraperitoneal, ip) and fixed in a stereotaxic frame. One Stainless steel guide cannula (0.7 mm OD) was implanted and aimed at the lateral ventricle (coordinates: anteroposterior = −0.3 mm from lambda, lateral = 1.2 mm, dorsoventral = 2.5 mm) according to the Paxinos and Franklin's atlas (Paxinos and Franklin, 1997). The cannula tip was placed 1mm above site of injection and attached to the skull bone with stainless steel screws and acrylic cement. A stylet inside guide cannula prevented obstruction.

Five to seven days after surgery intra-cerebroventricular (i.c.v) injections were performed with a thin dental needle (0.3 mm OD). A volume of 1 μl per animal was injected in 30 s using a micro-syringe (Hamilton) controlled by an infusion pump (Insight

Equipamentos Científicos, Brazil). A polyethylene catheter (PE10) was interposed between upper end of the dental needle and the micro-syringe. Movement of an air bubble inside the polyethylene catheter confirmed drug flow.

4.7. Histology

After the behavioral tests, mice were deeply anesthetized with chloral hydrate 5% (0.75 g/kg, ip) and a dental needle was inserted through the guide cannulae to infuse 0.5 μl of methylene blue. Immediately after, animals were euthanized for brain removal. The injection sites were confirmed as correct, on fresh brain, after the dye has spread to the whole brain through cerebroventricular way. Results from injections outside target area were discarded from statistical analysis.

4.8. Experimental protocols

4.8.1. Dose-response curves for FLX, RBX and PPADS treatment on mice FST and OFT

Experimentally naïve mice received a single ip injection of PPADS (3, 6.25, 12.5 mg/kg), FLX (10, 20, 30 mg/kg), RBX (2.5, 5, 10 mg/kg) or vehicle and, 30 min later, submitted to FST. An independent cohort of animals received single ip injections of effective doses of PPADS (6.25 mg/kg), FLX (20 mg/kg), RBX (5 mg/kg) or vehicle and submitted to OFT 30 min later.

4.8.2. Add on effects of PPADS sub-effective dose to FLX or RBX sub-effective doses on FST and OFT

Independent group of animals received a first injection of saline, FLX 10 mg/kg or RBX 2.5 mg/kg, immediately followed by a second injection of saline or PPADS 3 mg/kg. Thus, six groups were obtained: VEH+VEH, FLX + VEH, RBX + VEH, PPADS + VEH, FLX + PPADS, RBX + PPADS. Animals were submitted to FST 30 min after last administration. An independent cohort of animals received ip injections of FLX (10 mg/kg), RBX (2.5 mg/kg) or vehicle, immediately followed by PPADS (3 mg/kg), and submitted to OFT 30 min after last administration.

4.8.3. Effect of 5-HT depletion on PPADS effects on FST and OFT behavior

Experimentally naïve mice received daily ip injections of PCPA (150 mg/kg for 4 days) or VEH. On the fourth day, the last PCPA dose or VEH infusion were done 30 min before the second injection with VEH or PPADS (6.25 mg/kg). Animals were submitted to FST 30 min after second injection. PCPA dose and infusion schedule were chosen based on a previous work (Haring et al., 2013). An independent cohort of animals was submitted to the same treatment regimen described above and submitted to the OFT 30 min after second injection.

4.8.4. Effect of NA depletion on PPADS effects on FST and OFT

Experimentally naïve mice, with surgically implanted cannula, received i.c.v. infusion of VEH or DSP4 (20 μg) followed, 24 h later, by ip injections of PPADS (6.25 mg/kg) or VEH, and submitted to FST 30 min after ip administrations. DSP4 dose was chosen based on a previous work (Choi et al., 2003). An independent cohort of animals was submitted to the same treatment regimen described above and submitted to the OFT 30 min after last injection.

4.8.5. 5HT, NA and 5HIAA levels on PFC and HPC of PCPA and DSP4 treated animals

Experimentally naïve mice received daily ip injections of PCPA (150 mg/kg for 4 days) or VEH and euthanized 30min after last administration. An independent cohort of mice, received i.c.v infusion of DSP4 (20 ug) or VEH 24 h before euthanasia. The levels of 5HT, NA and 5HIAA levels were analyzed on PFC and HPC through HPCL as described.

4.9. Statistical analysis

Data from experiment 1 were analyzed in column comparison by one-way ANOVA, followed by Dunnett's *post hoc* test. Experiments 2 (FST), 3 and 4 were firstly analyzed by two-way ANOVA and injections was described as factors (except for OFT concerning experiment 2 that only One-way ANOVA was used). Thereafter, consecutive any significant difference, a column comparison was performed followed by Newman-Keuls's Multiple comparisons *post hoc* test. Regarding column comparison, non-parametric Dunn's *post hoc* test was used when appropriate. Data from experiment 5 was analyzed by Student's *t* test. Statistical differences were considered significant when $p < 0.05$. SPSS 20.0 software for windows (IBM SPSS statistics®, Chicago, IL, USA) was used to the analysis.

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Conflict of interest

The authors declare no conflict of interest.

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