LASSBio-1422: a new molecular scaffold with efficacy in animal models of schizophrenia and disorders of attention and cognition

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Aiming to identify new antipsychotic lead-compounds, our group has been working on the design and synthesis of new *N*-phenylpiperazine derivatives. Here, we characterized LASSBio-1422 as a pharmacological prototype of this chemical series. Adult male Wistar rats and CF1 mice were used for in-vitro and in-vivo assays, respectively. LASSBio-1422 [1 and 5 mg/kg, postoperatively (p.o.)] inhibited apomorphine-induced climbing as well as ketamine-induced hyperlocomotion (1 and 5 mg/kg, p.o.), animal models predictive of efficacy on positive symptoms. Furthermore, LASSBio-1422 (5 mg/kg, p.o.) prevented the prepulse impairment induced by apomorphine, (±)-2,5-dimethoxy-4-iodoamphetamine, and ketamine, as well as the memory impairment induced by ketamine in the novel object-recognition task at the acquisition, consolidation, and retrieval phases of memory formation. Potential extrapyramidal side-effects and sedation were assessed by catatonia, rota-rod, locomotion, and barbiturate sleeping time, and LASSBio-1422 (15 mg/kg, p.o.) did not affect any of the parameters observed. Binding assays showed that LASSBio-1422 has a binding profile different from the known atypical antipsychotic drugs: it does not bind to AMPA, kainate, *N*-methyl-D-aspartate, glicine, and $mGluR_2$ receptors and has low or negligible affinity for D_1, D_2, and 5-HT_{2A}/C receptors, but high affinity for D4 receptors ($K_i = 0.076$ μmol/l) and, to a lesser extent, for 5-HT_{1A} receptors ($K_i = 0.493$ μmol/l). The antagonist action of LASSBio-1422 at D_4 receptors was assessed through the classical GTP-shift assay. In conclusion, LASSBio-1422 is effective in rodent models of positive and cognitive symptoms of schizophrenia and its ability to bind to D_4 and 5-HT_{1A} receptors may at least in part explain its effects in these animal models. *Behavioural Pharmacology* 28:48–62

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Introduction

Antipsychotics remain the current standard of care for mental disorders including schizophrenia (~1% prevalence) and generate over 16 billion dollars worldwide in annual sales (Lieberman *et al.*, 2005). However, the large CATIE trial, sponsored by the US National Institutes of Health, found that 74% of patients discontinue the use of therapy within 18 months because of either poor tolerability or incomplete efficacy, indicating a need for novel therapies (Lieberman *et al.*, 2005).

Although the initial breakthrough with the discovery of chlorpromazine was a huge step forward, subsequent advances have been small, despite the introduction of many new antipsychotics (McEvoy, 2009). The first generation of antipsychotics, termed conventional or typical antipsychotics, such as haloperidol, inhibit dopamine D_2 receptors and are effective in treating positive symptoms of schizophrenia, but may cause extrapyramidal movement disorders. Second-generation or atypical antipsychotics, such as clozapine, olanzapine, and risperidone, inhibit D_2 receptors in conjunction with other receptors, notably 5-HT_{2A} receptors (Snyder and Murphy, 2008). Although the superior tolerability of the second-generation antipsychotics with respect to extrapyramidal movement disorders is beyond doubt, new detrimental side-effects, such as metabolic complications, including weight gain, hyperglycemia, and hyperlipidemia, have been associated with them (Newcomer, 2007).

Several pharmacological properties have been proposed to account for the atypicality of an antipsychotic drug. These include a high ratio of 5-HT_{2A}/D_2 antagonism (Meltzer *et al.*, 1989), a high ratio of noradrenaline/D_2

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antagonism (Wadenberg *et al.*, 2007), and fast dissociation of the antipsychotic from the D_2 receptor (Kapur and Seeman, 2001).

Previous studies from our group described the synthesis of a new *N*-phenylpiperazine derivative, LASSBio-579 [1-(1-(4-chlorophenyl)-1*H*-4-pyrazolylmethyl)-4-phenylh exahydropyperazine], designed by molecular hybridization between the prototypes clozapine and L-741, a D_2 antagonist ligand (Menegatti *et al.*, 2003). In-vitro assays indicated that LASSBio-579 could act as an agonist at presynaptic dopamine D_2-like receptors (Menegatti *et al.*, 2003) and binds to D_2-like, D_4, and 5-HT_{1A} receptors with moderate affinity (Neves *et al.*, 2010; Gomes *et al.*, 2013). In-vivo studies have shown that LASSBio-579 acts on dopaminergic and serotonergic neurotransmission; it prevents the apomorphine-induced climbing behavior, the ketamine-induced hyperlocomotion, the prepulse inhibition (PPI) deficits induced by apomorphine, (±)-2,5-dimethoxy-4-iodoamphetamine (DOI), and ketamine (Neves *et al.*, 2013) at doses devoid of cataleptogenic effects in mice (Neves *et al.*, 2010), as well as protecting against the long-term memory impairment induced by ketamine (Antonio *et al.*, 2016). However, high doses of LASSBio-579 (5 and 15 mg/kg, p.o.) induced a cataleptic behavior in mice and it impaired motor coordination on rota-rod test in a dose-dependent manner (Neves *et al.*, 2013).

In the course of our ongoing project aiming to identify molecules as new antipsychotic lead-compounds, a new LASSBio-579 analog, LASSBio-1422, was synthesized on the basis of the following rational design: the introduction of two isosteric groups, that is, methyl and chlorine, attached to C-3 and C-5 of the pyrazole ring to promote a dissymmetric ortho effect on the *N*-phenylpiperazine side chain that could adopt a more fixed conformation and, as a consequence, present a differentiated molecular recognition profile by the target receptors (Barreiro *et al.*, 2011). Moreover, a fluorine atom was placed at C4′ of the *N*-phenylpiperazine ring of LASSBio-579 to protect the main oxidative metabolization site (Gomes *et al.*, 2013) (Fig. 1).

The aim of this study was to evaluate LASSBio-1422 in different animal models predictive of positive and especially cognitive symptoms of schizophrenia as the lack of efficacy in treating cognitive symptoms remains a major challenge in schizophrenia therapeutics, as well as its binding profile to the receptors classically considered as antipsychotic targets.

Methods

Chemistry

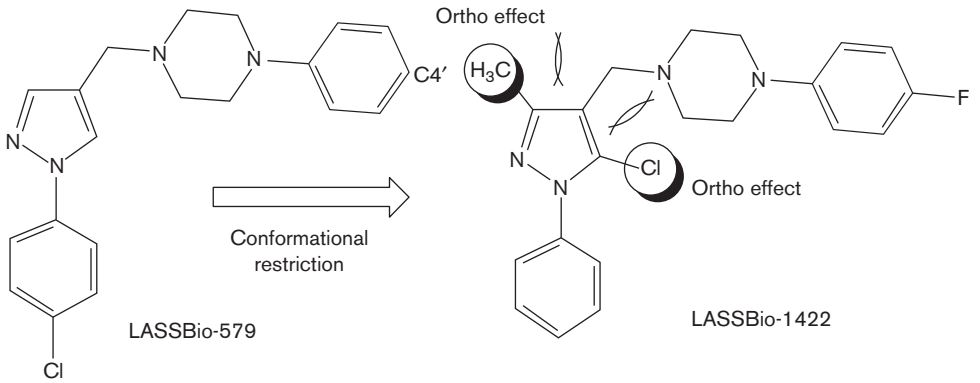
LASSBio-1422 [1-(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)methyl-4-(4-fluorophenyl)piperazine] was synthesized and purified following a protocol adapted from Kim *et al.* (1985). Instead of performing a reaction with anhydrous methanol under a nitrogen atmosphere, we used a reductive amination with $NaCNBH_3$ and $ZnCl_2$ (Fig. 2) (yield: 51%). The structure of the *N*-phenylpiperazine derivative LASSBio-1422 was fully characterized by ^{1}H-NMR, ^{13}C-NMR, IR spectroscopy, and mass spectrometry. Moreover, the analytical results for C, H, and N were within ± 0.4% of the theoretical values, assuring a purity superior to 99.5%. Microanalyses were carried out using a FlashEA 1112 Elemental analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and a Mettler Toledo MT-SICS MX5 balance (Mettler Toledo, Greifensee, Switzerland). Calculated: C (66.53%), H (5.76%), N (9.21%); determined: C (66.45%), H (5.77%), N (9.25%).

In-vitro assays

Radioligands and drugs

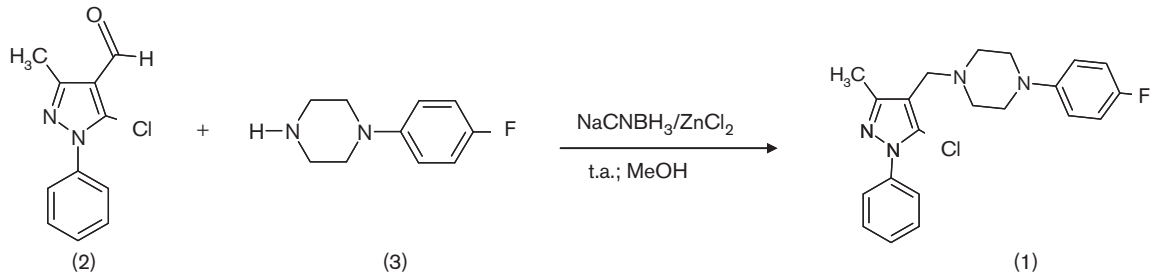
[^{3}H]-YM-09151-2 (82.7 Ci/mmol), [^{3}H]-8-OH-DPAT (154.2 Ci/mmol), [^{3}H]-ketanserin (67 Ci/mmol), [^{3}H]-SCH23390 (81.9 Ci/mmol), and [^{3}H]-glutamate (51.1 Ci/mmol) were purchased from New England Nuclear Life

Fig. 1



Design concept of *N*-phenylpiperazine derivative, LASSBio-1422, from the antipsychotic lead-compound, LASSBio-579.

Fig. 2



LASSBio-1422 (1) synthesis route; (2) 5-chloro-3-methyl-1-phenyl-1H-pyrazole-4-carbaldehyde; (3) 4-fluorophenyl piperazine.

Science Products (PerkinElmer, Waltham, Massachusetts, USA). Ketanserin tartrate, pargyline, serotonin creatinine sulfate, L-glutamic acid monosodium salt hydrate, and (−)-sulpiride were purchased from Sigma (São Paulo, Brazil).

The test substances and (−)-sulpiride were dissolved in dimethyl sulfoxide to obtain stock solutions. Subsequent dilutions were prepared in water. At the final concentration used (0.2% at most), dimethyl sulfoxide had no effect in our assays.

Tissue preparation

Adult male Wistar rats (200–300 g) were euthanized by decapitation. The brains were immediately removed on ice and the hippocampus, striatum, and total cortex were dissected, weighted, and stored in liquid nitrogen until use. This procedure was approved by the Institutional Ethical Committee for Animal Care from the Federal University of Rio de Janeiro (CEUA, protocol no. DFBCICB011).

Homogenates from the hippocampus, cortex, and striatum were prepared and centrifuged as described previously in detail (Pompeu *et al.*, 2013). The final pellets were resuspended in buffer, yielding a proportion of 1.5 ml/g tissue, and stored in liquid nitrogen until use.

The protein concentration was determined by the method of Lowry *et al.* (1951) using BSA as a standard.

Binding assays

We performed the receptor-binding assays in rat brain membranes, which are generally used for such studies. For a great range of receptors, mouse and rat receptors show similar affinities (Neylon and Summers, 1985; Maemoto *et al.*, 1997; Maina *et al.*, 2005), even when differences exist between rodent and human species (Liu *et al.*, 2001). In the case of the D_4 and 5-HT_{2C} receptors, because of the lack of a specific radioligand for these subtypes, we used cells transfected with human receptors.

For binding to 5-HT_{1A} receptors, rat hippocampal membranes (40 μg protein), test compound, and 1 nmol/l [^{3}H]-8-OH-DPAT were incubated at 37°C for 15 min in a solution containing 1 mmol/l $CaCl_2$, 1 mmol/l $MnCl_2$, 10 μmol/l pargyline, and Tris-HCl 50 mmol/l (pH 7.4) in a final volume of 500 μl. Nonspecific binding was estimated in the presence of 10 μmol/l serotonin.

For binding to 5-HT_{2A} receptors, rat cortical membranes (150 μg protein), test compound, and 1 nmol/l [^{3}H]-ketanserin were incubated at 37°C for 15 min in a solution containing 100 nmol/l prazosin and Tris-HCl 50 mmol/l (pH 7.4) in a final volume of 500 μl. Nonspecific binding was estimated in the presence of 1 μmol/l ketanserin.

For binding to D_1 receptors, rat striatal membranes (50 μg protein), test compound, and 0.2 nmol/l [^{3}H]-SCH23390 were incubated at 37°C for 60 min under yellow light in a solution containing 120 mmol/l NaCl, 5 mmol/l KCl, 5 mmol/l $MgCl_2$, 1.5 mmol/l $CaCl_2$, 1 mmol/l EDTA, and Tris-HCl 50 mmol/l (pH 7.4) in a final volume of 500 μl. Nonspecific binding was estimated in the presence of 1 mmol/l dopamine.

For binding to the D_2, D_3, and D_4 receptors, we used commercially available (ChemiscreenTM; Millipore, Billerica, Massachusetts, USA) crude membrane preparations of recombinant Chem-1 cells, an adherent mammalian cell line without any endogenous D_2 and D_3 dopamine receptor expression or detectable endogenous dopamine receptor expression, which have been transfected through a process using human DRD2 encoding D_2 dopamine receptor long isoform (D_{2L}; accession number NM_000795), human D_3 receptor (accession number NM_000796), or full-length human DRD4.4 cDNA encoding the D_4 isoform with four tandem repeats in the third cytoplasmic loop (accession number NM_0000797). A volume of 5 μg protein, test compound, and [^{3}H]-YM-09151-2 (0.1 nmol/l) were incubated at 37°C for 60 min under yellow light in a solution containing 120 mmol/l NaCl, 5 mmol/l KCl, 5 mmol/l $MgCl_2$, 1.5 mmol/l $CaCl_2$, 1 mmol/l EDTA, and Tris-HCl 50 mmol/l (pH 7.4) in a final volume of 500 μl.

Nonspecific binding was estimated in the presence of 3 μmol/l sulpiride (D_2 and D_3) or 10 μmol/l clozapine (D_4).

The binding assay to $mGluR_2$ receptors was performed using commercially available (ChemiscreenTM) crude membrane preparations of recombinant cells that have been transfected using human GRM2 cDNA encoding mGlu2 (accession number NM_000839). A volume of 20 μg protein, test compound, and 40 nmol/l [^{3}H]-glutamate were incubated at 30°C for 30 min in a solution containing 50 mmol/l Tris-HCl (pH 7.4) in a final volume of 500 μl. Nonspecific binding was estimated in the presence of 30 μmol/l glutamate.

After incubation, samples were diluted rapidly with 3 × 4 ml Tris-HCl 5 mmol/l (pH 7.4) and immediately filtered under vacuum on glass fiber filters (GMF 3; Filtrak Brandt GmbH, Wiesenbad, Germany) previously soaked in 0.5% polyethyleneimine in the case of binding to the 5-HT receptors. Filters were then dried and immersed in a scintillation mixture [POPOP (1,4-bis-[2-(5-phenyloxazolyl)]-benzene) 0.1 g/l and POP (2,5-diphenyloxazole) 4.0 g/l in toluene]. The radioactivity retained in the filters was counted using a Packard Tri-Carb 1600 TR liquid scintillation analyzer (PerkinElmer).

Behavioral experiments

Subjects

Adult male CF1 mice (25–35 g) from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS-RS) breeding colony were used. Animals were housed in plastic cages in groups of eight mice (17 × 28 × 13 cm) with free access to food (Nuvital; Nuvital Nutrientes, Colombo, Parana, Brazil) and water. Mice were kept at constant room temperature (22 ± 2°C) under a 12 h light–dark cycle (lights off at 7.00 p.m.) and were adapted to local conditions for at least 72 h before the experiments. Mice were only tested once and the number of animals per group varied from 8 to 10. All experiments were conducted by observers blinded to treatment. The study was approved by the Local Ethical Committee (CEP), which is linked to the National Commission of Research Ethics (CONEP) (Protocol 2007975), and was carried out according to guidelines of The National Research Ethical Committee (published by National Heath Council – MS, 1998) and Brazilian law (Ministério Público, 2008), which are in compliance with the International Guiding Principles for Biomedical Research Involving Animals [Council for International Organizations of Medical Sciences (CIOMS), 1985].

Apomorphine-induced climbing

Mice were treated with LASSBio-1422 (1 and 5 mg/kg, p.o.), haloperidol (0.5 mg/kg, p.o.), clozapine (5 mg/kg, p.o.), or vehicle (first treatment) and immediately placed in cages (29 × 23 × 19 cm) with the floor, walls, and top consisting of metal bars (2 mm diameter). Animals were allowed to freely explore the cages for 30 min. Immediately afterwards, they were treated with apomorphine 4 mg/kg or vehicle subcutaneously (s.c.) (second treatment). The climbing behavior score was evaluated as follows: normal behavior (zero point), increased activity and sniffing (one point), occasional clinging to sides of cage with forepaws (two points), intermittent clinging to sides or top of cage with all four paws (three points), and uninterrupted climbing with all four paws (four points). Climbing behavior was scored at 5, 10, 15, 20, 25, and 30 min after the administration of the second drug. The period of observation in each interval was 1 min. The climbing index was calculated as the sum of all scores obtained by the same animal at each time interval.

Ketamine-induced hyperlocomotion

Mice were treated with vehicle, haloperidol (0.01 mg/kg, p.o.), clozapine (1 mg/kg, p.o.), or LASSBio-1422 (1 and 5 mg/kg, p.o.) and allowed 30 min to explore the locomotor cage, which was made of acrylic (transparent walls and black floor, 30 × 30 × 45 cm) divided into 24 squares of equal area. Then, ketamine was injected at 10 mg/kg s.c. Immediately after the ketamine injection, locomotor activity was recorded for 20 min.

Prepulse inhibition of startle reflex

The PPI test was performed in a startle chamber (Insight, São Paulo, Brazil), in which a loudspeaker produced a continuous background noise of 65 dB of sound as well as the acoustic startle pulses. A white noise pulse was used as the startle stimulus, which had an intensity of 115 dB and a duration of 50 ms; three different noise intensities (80, 85, and 90 dB, duration 20 ms) were used as prepulses. An acclimatization time of 5 min, during which the mice received no stimulus except the background noise, was followed by five initial startle stimuli. After this habituation program, the test program was started, with five different trial types presented in a random order: (a) pulse alone, (b) control (no stimulus), (c) pulse with preceding prepulse of 80 dB, (d) pulse with preceding prepulse of 85 dB, and (e) pulse with preceding prepulse of 90 dB. A total of 10 presentations of each trial type were provided with an inter-stimulus interval randomized between 10 and 30 s. Percent PPI of the startle response was calculated as follows: 100 – [(response to acoustic prepulse plus startle stimulus trials/startle response alone trials) × 100]. Mice were treated with vehicle, LASSBio-1422 (5 mg/kg, p.o.), clozapine (15 mg/kg, p.o.), or haloperidol (0.5 mg/kg, p.o.) and 30 min later they were injected s.c. with either saline or the PPI disrupting drugs: apomorphine (3 mg/kg), a dopaminergic agonist; ketamine (30 mg/kg), an *N*-methyl-D-aspartate (NMDA) receptor antagonist; or (±)-DOI (0.5 mg/kg), a 5-$HT_{2A/2C}$ agonist. The animals treated with apomorphine or ketamine were immediately placed in the startle chambers and the PPI measurement was started. The

animals that received (±)-DOI were placed in the startle chambers 15 min after treatment and the PPI measurement was started.

Novel object-recognition task

The animal model used in this study is based on the observation that rodents preferentially explore unfamiliar (novel) objects (Ennaceur and Delacour, 1988). The test was performed in an acrylic box with three closed walls and one transparent wall (45 × 30 × 30 cm). Before starting the experiment (day 1), mice were gently handled once a day for 5 days.

Effect on short-term memory: On day 1 (habituation), the animals were placed in the box for 10 min and allowed to freely explore the environment. On day 2, the animals were treated by gavage with vehicle, LASSBio-1422 (5 mg/kg), clozapine (1 mg/kg), haloperidol (0.01 mg/kg), or intraperitoneally with ketamine (10 mg/kg) 1 h before the training session. In the training session, mice were placed in the box for 10 min in the presence of two identical objects (A, A). Ninety minutes later, mice were placed once again in the box containing the familiar object (A) and the unfamiliar (B) object. The time exploring each object was measured during 10 min. Object exploration was recorded when the animal spent the time sniffing the object or in direct contact with the object. Time spent on the top of the object was not considered object exploration. All objects had the same color, texture, and size, but different shapes.

Effect on long-term memory: Habituation, training session, and treatments were carried out as described above using another group of animals. Twenty-four hours after the training session, mice were placed again in the box in the presence of a familiar object (A) and a new object (C). The time exploration of each object was recorded for 10 min.

Effect on long-term memory impairment induced by ketamine: On day 1, the animals were placed in the box for 10 min and allowed to freely explore the environment. On day 2, the animals were treated intraperitoneally with vehicle or ketamine (10 mg/kg) 1 h before the training session. In the training session, the animals were placed in the box during 10 min in the presence of two identical objects (A, A). Twenty-four hours later, the animals were placed again in the box containing the familiar object (A) and a different object (C).

LASSBio-1422 (5 mg/kg), clozapine (1 mg/kg), and haloperidol (0.01 mg/kg) were administered by gavage at three different time points as follows.

Experiment 1: the drugs were administered 1 h before training (at the same time as ketamine or vehicle administration).
Experiment 2: the drugs were administered immediately after training.
Experiment 3: the drugs were administered 1 h before long-term memory testing.

In all experiments, the object exploration index was calculated using the following formula: Exploration index = time observing each object (A, B, or C)/time observing both objects.

Models of potential antipsychotic adverse effect

The potential of LASSBio-1422 to induce three classical antipsychotic adverse effects was evaluated: catalepsy, motor coordination impairment, and sedation/hypnosis. LASSBio-1422 was tested at a dose three times higher than the dose that was effective in behavioral models predictive of positive and cognitive symptoms.

Spontaneous locomotor activity: Locomotor activity was monitored in an arena made of acrylic (45 × 30 × 30 cm) divided into 24 squares of equal area. Mice were treated with LASSBio-1422 (15 mg/kg, p.o.), haloperidol (0.5 and 4 mg/kg, p.o.), clozapine (5 and 15 mg/kg, p.o.), or vehicle and 60 min later positioned at the center of the apparatus. After a 5-min habituation period, mice were observed during 15 min. The following parameters were recorded: number of squares crossings, rearings, and groomings.

Catalepsy test: Mice were treated with vehicle, LASSBio-1422 (15 mg/kg, p.o.), haloperidol (0.5 mg/kg, p.o.), or clozapine (5 and 15 mg/kg, p.o.). After 30, 60, and 90 min, mice were gently placed by the forepaws on a wood bar elevated 6.5 cm from the floor. The time spent by the animals in this position (up to 30 s) was recorded.

Rota-rod test: Motor impairment was evaluated using the rota-rod test. The apparatus consisted of a cylinder 3 cm in diameter rotating at 5 rpm. One day before the test, the animals were trained during 5 min. On the test day, the mice that could balance on the rotating rod for at least 90 s were selected for testing. Performance was measured by human observers before and 60 min after drug administration. Mice were treated with vehicle, LASSBio-1422 (15 mg/kg, p.o.), haloperidol (0.5 mg/kg, p.o.), or clozapine (5 and 15 mg/kg, p.o.). The integrity of motor coordination was assessed on the basis of the longest time of permanence and the number of falls in a 5-min period.

Barbiturate sleeping time: The hypnotic–sedative effect of LASSBio-1422 was evaluated using the barbiturate sleeping time test. Sixty minutes after gavage with vehicle, LASSBio-1422 (15 mg/kg, p.o.), haloperidol (0.5 and 4 mg/kg, p.o.), or clozapine (5 and 15 mg/kg, p.o.), the animals received pentobarbital sodium (40 mg/kg, intraperitoneally). Sleep latency and sleeping time (time elapsed between the loss and voluntary recovery of the righting reflex) were recorded. We assumed a 240 min cutoff for sleeping time, that is, sleeping time over 240 min was counted as 240 min.

Drugs and treatments

Apomorphine hydrochloride hemihydrate (Sigma), ketamine (Cristália, São Paulo, Brazil), (±)-DOI hydrochloride (Sigma), clozapine (Novartis, São Paulo, Brazil), haloperidol (Galena, São Paulo, Brazil), and sodium pentobarbital (Cristália) were used as reference drugs.

LASSBio-1422 and haloperidol were suspended in saline with the addition of 1% (v/v) of polissorbate 80 (Tween). Sodium pentobarbital was directly dissolved in saline. Apomorphine was dissolved in saline with addition of 0.1% ascorbic acid and clozapine was dissolved in saline with addition of 0.1% acetic acid 0.1 mol/l. Vehicle groups received saline with 1% (v/v) polissorbate 80. The doses (expressed as free base) were chosen on the basis of previous results published by our group (Neves *et al.*, 2010; 2013). The drugs were administered by the intraperitoneal and oral (gavage) routes (10 ml/kg body weight) or s.c. (5 ml/kg body weight). All experiments were conducted according to a randomized protocol.

Statistical analysis

Median inhibitory concentrations (IC_{50}) were estimated from the competition binding curves using a computerized nonlinear regression analysis of the untransformed data (Prism 5.0; GraphPad Software Inc., La Jolla, California, USA), assuming a single population of binding sites. K_i values were calculated from the Cheng–Prusoff equation, $K_i = IC_{50}/[1 + (\text{radioligand})/K_d]$. The K_d values used were obtained from two to three saturation experiments conducted in our preparations as follows: D_1-like receptors: 0.57 ± 0.08 nmol/l; D_2 receptors: 0.64 ± 0.13 nmol/l; D_3 receptors: 4.18 ± 0.32 nmol/l, D_4 receptors: 1.25 ± 0.074 nmol/l; 5-HT_{1A} receptors: 0.70 ± 0.10 nmol/l; and 5-HT_{2A} receptors: 2.69 ± 0.50 nmol/l.

Catalepsy and rota-rod results were analyzed by two-way repeated-measures analysis of variance (ANOVA), with treatment as the first factor and time interval or session as the repeated measure. Apomorphine-induced climbing, ketamine-induced hyperlocomotion, PPI, locomotor activity, and barbiturate sleeping time were subjected to a one-way ANOVA. Memory was assessed by comparing the relative exploration times for the familiar and novel object, two-way ANOVA, to enable a direct within-subjects comparison between treatments and object preferences . The total object exploration time was compared using one-way ANOVA. When appropriate, the post-hoc Student–Neumann–Keuls test was performed. The analyses were carried out using Sigma Stat 2.03 software (Jandel Scientific Corporation; Jandel Scientific, San Rafael, California, USA). Differences were considered statistically significant at P value less than 0.05.

Results

Binding assays

Competition curves showed that LASSBio-1422 has an affinity ($K_i = 0.076$ μmol/l) for the D_4 receptors and, to a lesser extent, for the 5-HT_{1A} receptor (Table 1). However, unlike clozapine, LASSBio-1422 has a much lower affinity for the D_2, D_3, and 5-HT_{2A} receptors (Table 1). The affinity for the D_1 subtype of dopamine receptors was negligible (estimated $K_i > 30$ μmol/l, the highest concentration tested).

The intrinsic efficacy of LASSBio-1422 for the D_4 receptor was investigated using a functional binding assay, the classical GTP-shift assay, which is based on the ternary complex model for GPCRs and has been validated for various metabotropic receptors (Kenakin, 2009; Noël *et al.*, 2014), including the D_4 receptor (Lawson *et al.*, 1994). This assay is based on the difference in affinity measured for agonists (but not antagonists) in the absence and presence of a large concentration of GTP that can destabilize the high-affinity state of the receptor formed by the agonist, receptor, and G protein. Figure 3 shows the profiles of the competition curves for [^{3}H]-YM-09151-2 binding to D_4 receptors using either a full agonist, dopamine (Fig. 3a), or LASSBio-1422 (Fig. 3b). The control curve for dopamine has a clear shallow aspect characterized by a Hill slope significantly lower than 1 ($n_H = -0.61 \pm 0.03$). In the presence of GTP, the competition curve was shifted to the right, indicating a loss of affinity for the receptor (IC_{50} from 19 to 74 nmol/l) and a nearly normal slope, with a Hill coefficient not different from 1 ($n_H = -0.80 \pm 0.12$). In contrast, the addition of GTP had no effect on LASSBio-1422 as the competition curves in the absence and presence of 1 mmol/l GTP were superimposed and with the Hill coefficient equal to 1, indicating that this compound is an antagonist of the D_4 receptors.

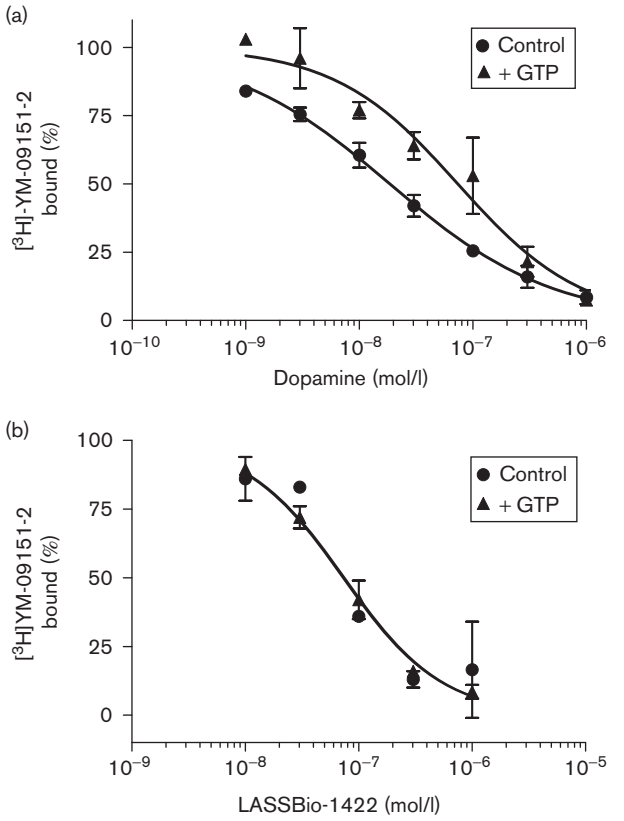
LASSBio-1422 did not bind (< 25% inhibition at 10 μmol/l) to AMPA, kainate, NMDA, glycine (CEREP Study number 20 593), and $mGluR_2$ receptors, all investigated as possible targets.

Table 1 Affinities of LASSBio-1422 for D_2, D_3, D_4, 5-HT_{1A}, and 5-HT_{2A} receptors

	K_i (μmol/l) (95% confidence interval)				
	D_2	D_3	D_4	5-HT_{1A}	5-HT_{2A}
LASSBio-1422	5.28 (3.94–7.09)	3.70 (2.76–4.98)	0.076 (0.055–0.105)	0.493 (0.395–0.616)	4.64 (3.95–5.46)
Clozapine	0.683 (0.497–0.938)	0.903 (0.715–1.14)	0.035 (0.026–0.048)	0.259 (0.223–0.300)	0.020 (0.016–0.025)

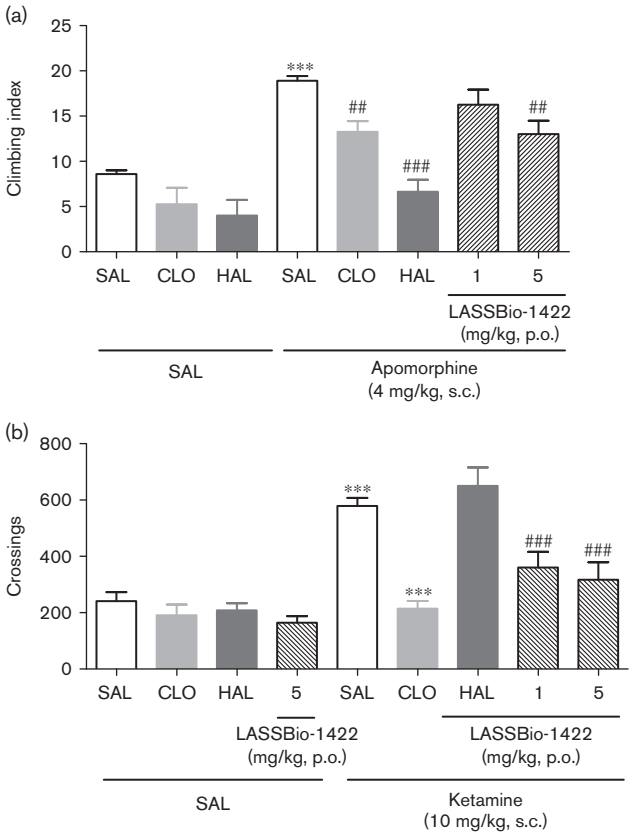
Clozapine was used as a reference drug.
The K_i values were calculated from the mean curves obtained from two to four experiments, conducted in triplicate.
The 95% confidence interval corresponds to the goodness of fit of the parameter calculated by nonlinear regression analysis of the mean curve.

Fig. 3



Effect of 1 mmol/l GTP on dopamine (a) and LASSBio-1422 (b) competition for [^{3}H]-YM-09151-2 binding to human D_4 receptors (GTP-shift assay). The data were fitted assuming the model of a sigmoidal dose–effect curve with variable slope (see the text for the IC_{50} and Hill coefficient values). In this assay, the competition curves of agonists (such as dopamine), but not antagonists (such as LASSBio-1422), are shifted to the right in the presence of GTP. The curves represent the mean curves from two independent experiments conducted in triplicate.

Fig. 4



(a) Effect of LASSBio-1422 (1 and 5 mg/kg, p.o.) on apomorphine-induced climbing behavior. Vehicle (SAL). Clozapine (CLO 5 mg/kg, p.o.) and haloperidol (HAL 0.5 mg/kg, p.o.) were used as reference drugs. Different from SAL + SAL: $^{***}P < 0.001$; different from SAL + apomorphine: $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. (b) Effect of LASSBio-1422 (1 and 5 mg/kg, p.o.) on ketamine-induced hyperlocomotion (number of crossings). CLO (1 mg/kg, p.o.) and HAL (0.01 mg/kg, p.o.) were used as reference drugs. Different from SAL + SAL: $^{***}P < 0.001$; different from SAL + ketamine: $^{\#\#\#}P < 0.001$. Data are expressed as mean ± SEM. p.o., postoperatively; SAL, saline (vehicle); s.c., subcutaneously.

Behavioral experiments

Apomorphine-induced climbing

Statistical analysis indicated a significant main effect of LASSBio-1422 [$F(7,67) = 17.83$, $P < 0.001$] on apomorphine-induced climbing (Fig. 4a). Post-hoc analysis indicated a significant decrease in apomorphine-induced climbing following the administration of LASSBio-1422 at the dose of 5 mg/kg ($P < 0.05$) and the positive controls haloperidol 0.5 mg/kg ($P < 0.001$) and clozapine 5 mg/kg ($P < 0.05$) compared with vehicle–apomorphine group. LASSBio-1422 did not prevent the climbing behavior effect at the lowest dose tested (1 mg/kg).

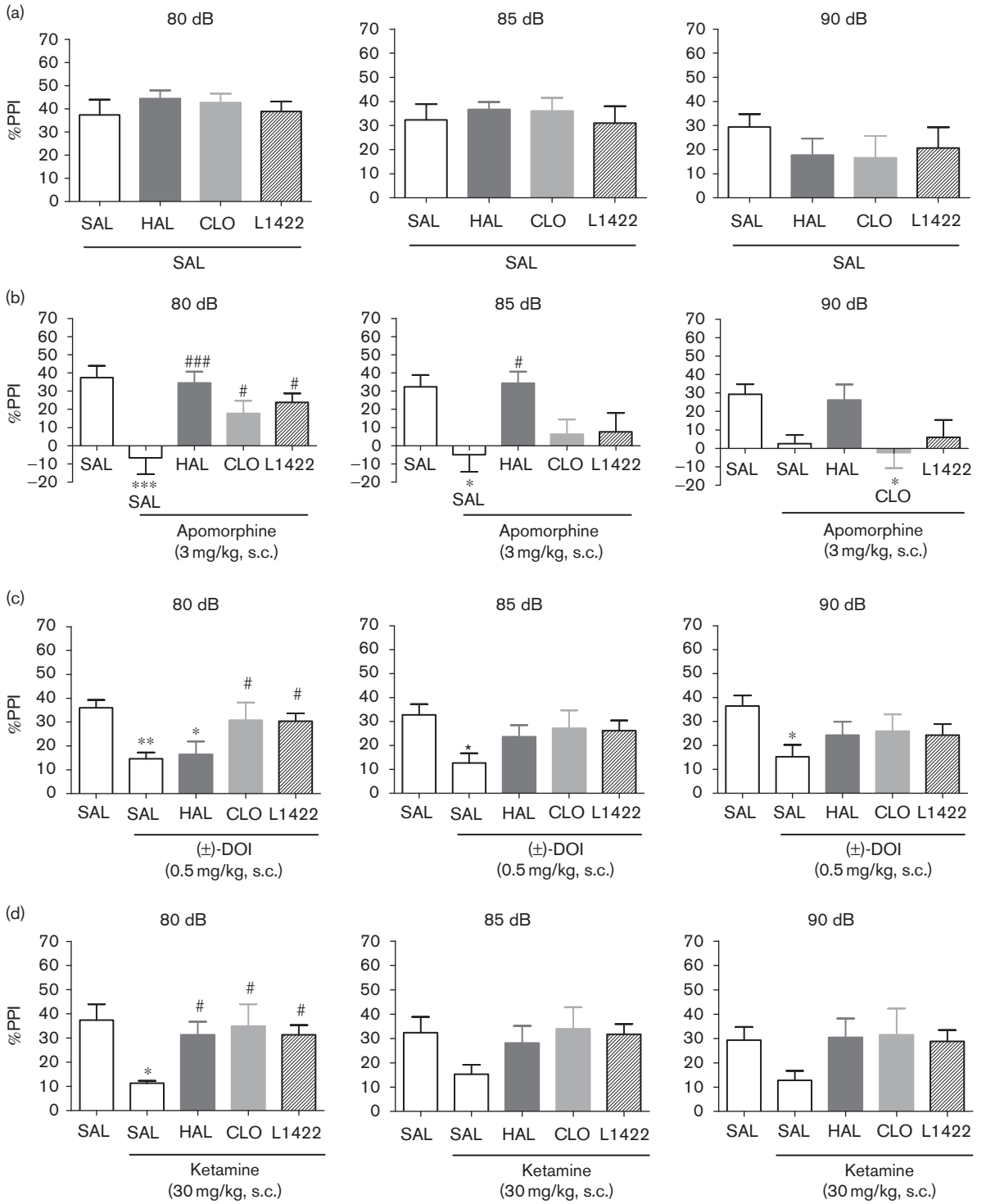
Ketamine-induced hyperlocomotion

Figure 4b shows a significant main effect of LASSBio-1422 [$F(8,80) = 18.25$, $P < 0.001$] on ketamine-induced hyperlocomotion. Post-hoc tests indicated a significant decrease in the ketamine-induced hyperlocomotion by the administration of LASSBio-1422 at the doses of 1 mg/kg ($P < 0.01$) and 5 mg/kg ($P < 0.001$) and of the positive control clozapine 1 mg/kg ($P < 0.001$) compared with vehicle–ketamine group. Haloperidol (0.01 mg/kg) did not block this behavior. Doses of clozapine (1 mg/kg, p.o.) and haloperidol (0.01 mg/kg) were chosen on the basis of absence of effects on spontaneous locomotion and the results obtained are in agreement with data in the literature (Satow *et al.*, 2009).

Prepulse inhibition

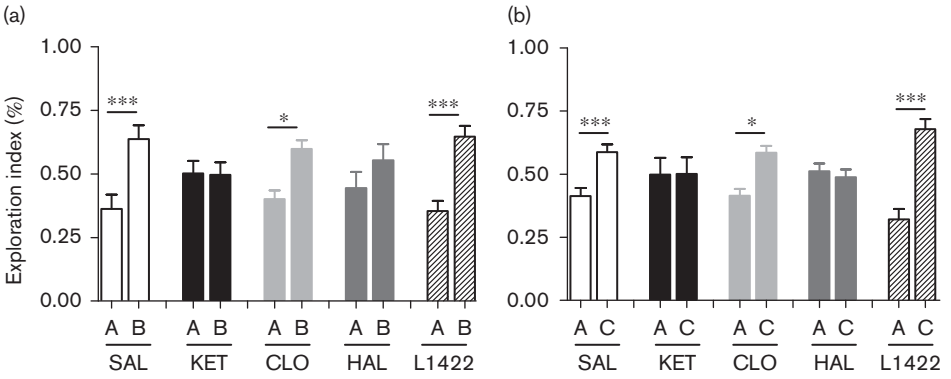
LASSBio-1422 (5 mg/kg, p.o.) and the antipsychotics tested did not alter the normal PPI response of animals [80 dB $F(3,27) = 0.41$, NS; 85 dB $F(3,27) = 0.19$, NS; 90 dB $F(3,27) = 0.60$, NS] (Fig. 5a). However, apomorphine (3 mg/kg, s.c.), (±)-DOI (0.5 mg/kg, s.c.), and ketamine (30 mg/kg, s.c.)

Fig. 5



Effect of LASSBio-1422 (L1422 5 mg/kg, p.o.) *per se* on prepulse inhibition (PPI) (a) and effect of LASSBio-1422 with apomorphine (b), (±)-DOI (c), and ketamine (d). Clozapine (CLO 15 mg/kg, p.o.) and haloperidol (HAL 0.5 mg/kg, p.o.) were used as reference drugs. Different from SAL + SAL: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; different from SAL + apomorphine, SAL + (±)-DOI or SAL + ketamine: $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$. Data are expressed as mean ± SEM. p.o, postoperatively; SAL, saline (vehicle); s.c., subcutaneously.

Fig. 6



Effect of LASSBio-1422 (L1422 5 mg/kg, p.o.) *per se* on short-term memory (a) and long-term memory (b) in the novel object-recognition test. Clozapine (CLO 1 mg/kg, p.o.) and haloperidol (HAL 0.01 mg/kg, p.o.) were used as reference drugs. On the horizontal axis, A is the familiar object, whereas B and C are the different (novel) objects. Different from object A in the same group: $^{*}P<0.05$, $^{***}P<0.001$. Data are expressed as mean ± SEM. p.o., postoperatively; SAL, saline (vehicle).

induced PPI impairment, as expected [apomorphine (3 mg/kg, s.c.): 80 dB $F(4,42)=6.37$, $P<0.01$; 85 dB $F(4,42)=4.50$, $P<0.001$; 90 dB $F(4,42)=3.64$, $P<0.05$ (Fig. 5b); (±)-DOI (0.5 mg/kg, s.c.: 80 dB $F(4,41)=5.23$, $P<0.01$; 85 dB $F(4,41)=2.67$; $P<0.05$, 90 dB $F(4,41)=2.28$, $P>0.05$ (Fig. 5c); ketamine (30 mg/kg, s.c.): 80 dB $F(4,39)=3.21$, $P<0.05$; 85 dB $F(4,39)=1.43$, $P<0.05$; 90 dB $F(4,39)=1.25$, $P>0.05$ (Fig. 5d)].

Post-hoc tests showed that LASSBio-1422 prevented the deficit induced by apomorphine only when the prepulse intensity was 80 dB ($P<0.05$). The same profile of action was observed for clozapine (80 dB $P<0.05$; 85 dB, NS; 90 dB, NS). Haloperidol fully prevented the disruption of PPI induced by apomorphine at 80 dB ($P<0.001$) and 85 dB ($P<0.05$) (Fig. 5b). In the serotonergic model, LASSBio-1422 was effective in preventing the impairment of PPI at 80 dB ($P<0.05$), as was clozapine ($P<0.05$). Haloperidol did not significantly restore the PPI deficit at any of the prepulse intensities. LASSBio-1422 also prevented the ketamine-induced impairment of PPI at 80 dB ($P<0.05$), as did clozapine ($P<0.05$) and haloperidol ($P<0.05$).

Novel object recognition

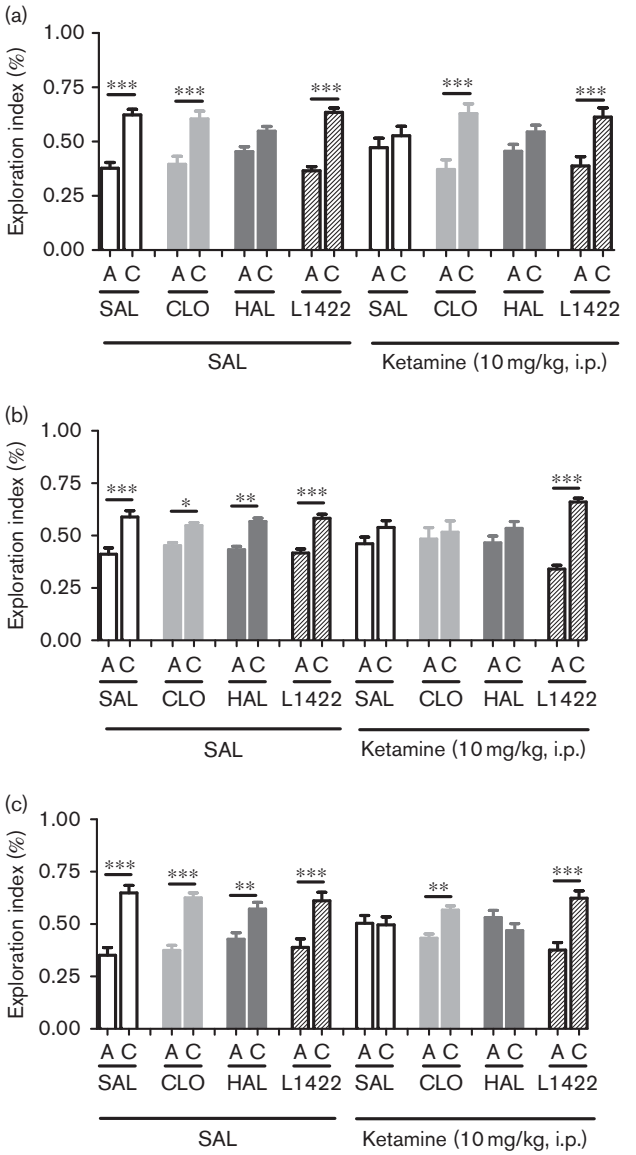
Effect of LASSBio-1422 on short and long-term memory: In the training session of the short-term and long-term memory tests, all groups explored both objects equally, as expected (short-term: $F(9,79)=0.26$, NS; long-term: $F(9,85)=1.03$, NS; data not shown). In the short-term memory test session (Fig. 6a), the statistics indicated a significant main effect of object [$F(1,97)=14.98$, $P<0.001$] and a significant treatment × object interaction [$F(4,97)=5.35$, $P<0.001$]. Haloperidol and ketamine impaired recognition memory as the animals explored both objects equally: familiar and novel; the animals treated with clozapine and LASSBio-1422 spent more time exploring the novel object, like the control group. The same results were observed for the long-term memory [treatment, $F(4,86)=0.07$, NS; object, $F(1,86)=33.21$, $P<0.001$; interaction, $F(4,86)=3.01$, $P<0.01$] (Fig. 6b).

Effect of LASSBio-1422 on acquisition: In the training session, all groups explored both objects equally, as expected (data not shown). In the test session (Fig. 7a), the ANOVA showed a significant main effects of object [$F(1,127)=94.57$, $P<0.001$] and a significant treatment × object interaction [$F(7,127)=3.01$, $P<0.001$]. The animals that received ketamine + saline and haloperidol + saline presented a memory impairment as these animals could not discriminate the novel object. Mice that received LASSBio-1422 and clozapine showed a preference for the novel object. LASSBio-1422 and clozapine administered simultaneously with ketamine protected against the memory impairment induced by this drug as these animals showed a preference for the novel object. Haloperidol did not protect the animals against the deleterious effect of ketamine.

Effect of LASSBio-1422 on consolidation: In the training session, all groups explored both objects equally, as expected (data not shown). In the test session (Fig. 7b), the ANOVA showed a significant effect of object [$F(1,129)=76.40$, $P<0.001$] and a significant treatment × object interaction [$F(7,129)=4.35$, $P<0.001$)]. When administered immediately after training, LASSBio-1422 ($P<0.001$) reversed the memory impairment induced by ketamine, different from clozapine (NS) and haloperidol (NS).

Effect of LASSBio-1422 on retrieval: In the training session, all groups explored both objects equally, as expected (data not shown). In the test session (Fig. 7c), the statistics showed a significant effect of object [$F(1,125)=85.49$, $P<0.001$] and a significant treatment × object interaction

Fig. 7



Effect of LASSBio-1422 (L1422 5 mg/kg, p.o.) on long-term memory impairment induced by ketamine (10 mg/kg, i.p.). (a) LASSBio-1422 was administered 1 h before the training session (affecting memory encoding and consolidation). (b) LASSBio-1422 was administered immediately after the training trial (affecting memory consolidation). (c) LASSBio-1422 was administered 1 h before the memory retrieval trial. Clozapine (CLO 1 mg/kg, p.o.) and haloperidol (HAL 0.01 mg/kg, p.o.) were used as reference drugs. On the horizontal axis, A and C are different objects. Different from object A in the same group: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Data are expressed as mean ± SEM. i.p., intraperitoneally; p.o., postoperatively; SAL, saline (vehicle).

[$F(7,125)=7.17$, $P<0.001$)]. When treated 1 h before the long-term memory test, LASSBio-1422 reversed the ketamine-induced memory deficit ($P<0.001$) as did clozapine ($P<0.05$), but different from haloperidol (NS).

Exploration time: As a control, the total exploration time of both objects was recorded. There were no significant drug effects [acquisition: training $F(7,63)=0.78$, NS; LTM $F(7,63)=1.69$; consolidation: training $F(7,64)=0.80$, NS; LTM $F(7,64)=1.50$, NS; retrieval: training $F(7,58)=2.18$, NS; LTM $F(7,58)=1.81$, NS; data not shown].

Table 2 Effect of LASSBio-1422 (15 mg/kg, postoperatively) on locomotor activity of mice

Treatments	Dose	Crossings	Rearings	Groomings
Saline	1 ml/100 g	330.8 ± 175.0	104.6 ± 41.4	19.5 ± 7.9
Haloperidol	4 mg/kg	71.0 ± 110.7***	6.8 ± 14.0***	1.4 ± 2.7***
Haloperidol	0.5 mg/kg	151.1 ± 117.9**	32.9 ± 30.4***	6.5 ± 2.8**
Clozapine	15 mg/kg	145.0 ± 71.5*	25.6 ± 29.3***	2.8 ± 2.4***
Clozapine	5 mg/kg	183.5 ± 45.0*	87.6 ± 24.2	11.8 ± 3.7*
LASSBio-1422	15 mg/kg	249.5 ± 93.8	71.4 ± 35.4	23.1 ± 11.7

Data are expressed as mean ± SD.
Clozapine and haloperidol were used as reference drugs.
Analysis of variance post-hoc Student–Newman–Keuls test.
Different from saline: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Table 3 Effect of LASSBio-1422 (15 mg/kg postoperatively) in catalepsy test at 30, 60, and 90 min after treatment

Treatments	Dose	30 min (T_{30})	60 min (T_{60})	90 min (T_{90})
Saline	1 ml/100 g	1.1 ± 0.4	1.1 ± 0.5	2.3 ± 1.7
Haloperidol	0.5 mg/kg	11.5 ± 11.5**	19.2 ± 13.9***	24.5 ± 9.9***
Clozapine	15 mg/kg	13.3 ± 12.3**	12.0 ± 8.1**	3.5 ± 3.7
Clozapine	5 mg/kg	3.1 ± 3.9	1.5 ± 0.9	1.6 ± 1.5
LASSBio-1422	15 mg/kg	1.6 ± 1.6	4.2 ± 4.2	4.1 ± 3.9

Data are expressed as mean ± SD.
Clozapine and haloperidol were used as reference drugs.
Two-way repeated measures analysis of variance post-hoc Student–Newman–Keuls test.
Different from saline at the same time of measure: $^{**}P<0.01$; $^{***}P<0.001$.

Effects of LASSBio-1422 on models of potential antipsychotic adverse effect

Spontaneous locomotor activity: As shown in Table 2, the statistical analysis indicated significant drug effects on locomotor activity [crossings $F(5,47)=5.85$, $P<0.001$] and exploratory behavior [rearing: $F(5,47)=12.97$, $P<0.001$; grooming: $F(5,47)=14.92$, $P<0.001$]. However, post-hoc tests showed that LASSBio-1422, even when used at a high dose (15 mg/kg), did not significantly alter the number of crossings ($P=0.11$), rearing ($P=0.056$), or grooming ($P=0.28$). However, clozapine 5 mg/kg ($P<0.02$) and 15 mg/kg ($P<0.05$), and haloperidol 0.5 mg/kg ($P<0.01$) and 4 mg/kg ($P<0.001$) caused motor impairment. Haloperidol and clozapine also affected rearing (clozapine 15 mg/kg, $P<0.001$; haloperidol 0.5 mg/kg, $P<0.001$, 4 mg/kg, $P<0.001$) and grooming (clozapine 15 mg/kg, $P=0.001$; haloperidol 0.5 mg/kg $P=0.003$, 4 mg/kg, $P<0.001$).

Catalepsy test: Statistics showed a significant effect of treatment [$F(5,161)=15.13$, $P<0.001$], no significant effect of time [$F(2,161)=1.31$, NS], and a significant treatment × time interaction [$F(10,161)=4.69$, $P<0.001$]. Post-hoc analyses indicated that, after 30 min, haloperidol 0.5 mg/kg ($P<0.01$) and clozapine 15 mg/kg ($P<0.01$) increased the time spent by mice at the imposed uncomfortable

Table 4 Effect of LASSBio-1422 (15 mg/kg, postoperatively) in the rota-rod test

Treatments	Dose	Permanence T_0	Time (s) T_{60}	Number of T_0	Falls T_{60}
Saline	1 ml/100 g	227.3 ± 55.5	235.1 ± 67.7	1.0 ± 1.0	1.0 ± 1.6
Haloperidol	0.5 mg/kg	255.3 ± 59.3	91.4 ± 78.6***	1.1 ± 1.4	13.4 ± 12.5***
Clozapine	15 mg/kg	238.3 ± 74.5	86.9 ± 89.8***	1.3 ± 1.4	21.3 ± 17.5***
Clozapine	5 mg/kg	249.6 ± 65.5	216.3 ± 74.5	0.9 ± 1.1	1.0 ± 0.9
LASSBio-1422	15 mg/kg	288.6 ± 22.8	273.7 ± 45.5	0.2 ± 0.4	0.7 ± 1.1

Data are expressed as mean ± SD.
Clozapine and haloperidol were used as reference drugs.
Two-way repeated measures analysis of variance post-hoc Student–Newman–Keuls test.
Different from the first measure (baseline, T_0) at the same treatment group: ***$P < 0.001$.

Table 5 Effect of LASSBio-1422 (15 mg/kg, postoperatively) on barbiturate sleeping time and latency to sleep

Treatments	Dose	Latency to sleep (min)	Sleeping time (min)
Saline	1 ml/100 g	4.4 ± 0.7	34.2 ± 14.4
Haloperidol	4 mg/kg	3.6 ± 1.077	86.2 ± 48.0***
Haloperidol	0.5 mg/kg	5.0 ± 2.8	29.5 ± 11.3
Clozapine	15 mg/kg	3.5 ± 1.3	71.8 ± 23.7*
Clozapine	5 mg/kg	4.8 ± 3.0	45.6 ± 29.5
LASSBio-1422	15 mg/kg	6.2 ± 2.7	42.4 ± 22.0

Data are expressed as mean ± SD.
Clozapine and haloperidol were used as reference drugs.
Analysis of variance post-hoc Student–Newman–Keuls test.
Different from saline: *$P < 0.05$; ***$P < 0.001$.

position. After 60 min, haloperidol 0.5 mg/kg ($P < 0.001$) and clozapine 15 mg/kg ($P < 0.01$) maintained the cataleptic effect. After 90 min, only haloperidol 0.5 mg/kg maintained its effect ($P < 0.001$). In contrast, LASSBio-1422 (15 mg/kg) did not cause catalepsy at any time evaluated (Table 3).

Rota-rod test: The results showed significant effects on the stay time [treatment, $F(4,93) = 9.83$, $P < 0.001$; time, $F(1,93) = 27.89$, $P < 0.001$; treatment × time interaction, $F(4,93) = 7.45$, $P < 0.001$] and the number of falls [treatment, $F(4,93) = 8.97$, $P < 0.001$; time, $F(1,93) = 19.74$, $P < 0.001$; treatment × time interaction, $F(4,93) = 7.90$, $P < 0.001$]. As expected, post-hoc analysis indicated that haloperidol 0.5 mg/kg ($P < 0.001$) and clozapine 15 mg/kg ($P < 0.001$) decreased the maximum stay time on the apparatus and increased the number of falls (haloperidol $P < 0.001$, clozapine $P < 0.001$). LASSBio-1422 did not affect the maximum stay time or the number of falls (Table 4).

Barbiturate sleeping time: Statistical analysis did not show any effect on the latency to sleep [$F(5,55) = 2.18$ $P > 0.05$], but there was a significant drug effect on sleeping time [$F(5,55) = 6.52$ $P < 0.001$]. Post-hoc tests showed that haloperidol 4 mg/kg ($P < 0.001$) and clozapine 15 mg/kg ($P < 0.05$) increased the sleeping time, different from LASSBio-1422 (15 mg/kg), which did not modify the pentobarbital sodium (40 mg/kg, intraperitoneally) sleeping time (Table 5).

Discussion

In this work, we evaluated a new *N*-phenylpiperazine derivative, LASSBio-1422, obtained by functionalization of the lead compound LASSBio-579, aiming at developing new drugs to treat positive and cognitive symptoms of schizophrenia. We showed that LASSBio-1422 has affinity for D_4 and 5-HT_{1A} receptors, and is effective in different animal models predictive of efficacy in the treatment of schizophrenia positive and cognitive symptoms: apomorphine-induced climbing (Moore and Axton, 1988), ketamine-induced hyperlocomotion (Geyer and Ellenbroek, 2003), PPI (Porsolt *et al.*, 2010), and novel object-recognition task (Jones *et al.*, 2011).

Apomorphine-induced hyperactivity in mice presents a certain translational validity as direct or indirect activation of dopamine receptors can exacerbate psychotic symptoms in patients with schizophrenia (Porsolt *et al.*, 2010). The administration of NMDA receptor antagonists (PCP, ketamine, MK-801) can induce a behavioral syndrome in rodents, characterized by an increase in locomotor activity, head weaving, ataxia, body rolling, and stereotyped movements. This syndrome has been used widely as an animal model of the schizophrenia-like symptoms (Harris and Batki, 2000; Lahti *et al.*, 2001) since hyperactivity seems to mimic psychotic agitation. The NMDA antagonist-induced hyperactivity can be prevented by both typical and atypical antipsychotics; however, the typical antipsychotics are effective only at doses that also impair locomotion (Leite *et al.*, 2008; Satow *et al.*, 2009). In contrast, in behavioral tests using dopamine agonists, there is no differentiation between typical and atypical antipsychotics (Costall *et al.*, 1978).

Typical and atypical antipsychotics can also be differentiated in rodents using a single model such as PPI, which models the preattentive deficits observed in schizophrenic patients, with clear translational and face validity (Porsolt *et al.*, 2010; Jones *et al.*, 2011). Apomorphine and ketamine disrupt PPI in rodents, which can be prevented by typical and atypical antipsychotics, whereas the disruption of PPI be 5-$HT_{2A/2C}$ agonists is prevented only by atypical antipsychotics (Geyer *et al.*, 2001; Martin *et al.*, 2003; Auclair *et al.*, 2006). As expected, haloperidol prevented only the apomorphine and ketamine-induced PPI deficits, whereas clozapine also prevented the PPI impairment induced by (±)-DOI, a 5-$HT_{2A/2C}$ agonist. LASSBio-1422 (5 mg/kg) presented the same profile as clozapine, which reinforces

its putative atypicality. Most importantly, this finding points to the potential of LASSBio-1422 to alleviate the disruption of preattentional processes presented by schizophrenic patients, which is of interest, given that improving attention in neuropsychiatric patients remains crucial for their treatment (Young *et al.*, 2011).

Furthermore, LASSBio-1422 was also effective in preventing memory deficits induced by ketamine in the novel object-recognition task, a rodent model of visual learning and memory (Porsolt *et al.*, 2010; Jones *et al.*, 2011; Sood *et al.*, 2011). Glutamate antagonists, such as ketamine and PCP, induce clear learning/memory deficits in humans and animals (Morgan and Curran, 2006). Schizophrenic patients generally show considerable deficits in remembering stories, verbal paired associates, and visual designs, compared with untreated patients, which may be related to deficiencies in episodic memory (Sood *et al.*, 2011). Typical antipsychotics have generally been found to be ineffective against cognitive symptoms in schizophrenic patients, but there is evidence from rodent and primate studies that the atypical antipsychotics are more effective in reversing and even preventing cognitive impairment (Seeman, 2010; Meltzer, 2013). In addition, a clinical study showed the efficacy of atypical antipsychotics, such as clozapine and olanzapine, but not typical antipsychotics, in attenuating cognitive deficits associated with chronic schizophrenia.

The antipsychotic clozapine, but not haloperidol, has been reported to reverse the deficits induced by PCP (Grayson *et al.*, 2007) and ketamine (Antonio *et al.*, 2016) in the object-recognition task. Grayson *et al.* (2007) have tested the effect of these drugs, administered before training only, after a subchronic treatment with PCP, and in our protocol, ketamine was administered just once (before training) to prevent hippocampal degeneration and neurotoxicity induced by repeated treatment with this drug (Olney *et al.*, 1989). LASSBio-1422 was administered at different stages of memory formation: encoding, consolidation, and retrieval. As expected, a single administration of ketamine impaired both short-term and long-term memories. LASSBio-1422 did not affect short-term or long-term memory *per se*, but improved the deficit induced by ketamine on long-term memory, when administered in the different stages of memory formation (acquisition, consolidation, and retrieval). This profile was not fully shared by clozapine (1 mg/kg), which did not protect against the ketamine-induced memory impairment when administered immediately after the training session, indicating that it is not able to counter attack the deleterious effect of ketamine on the memory consolidation process.

The highest tested dose of LASSBio-1422 did not alter animals' exploratory behavior; neither did it impair motor function, or cause sedative/hypnotic effects, suggesting that it has a better safety profile than typical antipsychotics such as haloperidol. LASSBio-1422 could fit with second-generation antipsychotics that induce extrapyramidal effects only at high doses, such as risperidone and olanzapine (Owens, 1996; Farah, 2005). In a certain way, it could be advantageous also relative to the atypical antipsychotics as a meta-analysis comparing several antipsychotic drugs showed that clozapine, quetiapine, and zotepine are even more sedating than haloperidol (Leucht *et al.*, 2009).

It is noteworthy that LASSBio-1422 has a binding profile different from the known atypical antipsychotic drugs. It does not bind to AMPA, kainate, NMDA, glicine, and $mGluR_2$ receptors, which have been investigated as possible targets for antipsychotic action (Moghaddam and Javitt, 2012). Indeed, LASSBio-1422 has affinity for D_4 and, to a lesser extent, for 5-HT_{1A} receptors, considered important for the activity of some atypical antipsychotics such as aripiprazole (Lerond *et al.*, 2013), in both cases with a K_i similar to that of clozapine. However, LASSBio-1422 differs from clozapine as it has a much lower affinity for D_2 and 5-HT_{2A} receptors. It is important to note that the binding affinity profile of LASSBio-1422 also differs considerably from its parent compound LASSBio-579 (Neves *et al.*, 2010; 2013) as it showed a relative selectivity for D_4 receptors compared with D_2 and D_3 receptors.

Therefore, our results reinforce other studies showing that dopamine D_4 receptor antagonists are effective in animal models of cognitive symptoms of schizophrenia, reversing apomorphine-induced blockade of PPI (Mansbach *et al.*, 1998), inhibiting locomotor hyperactivity (Zhang *et al.*, 2002), and improving memory in a delayed alternation task in rats (Zhang *et al.*, 2004). Also, the current study is in line with genetic, behavioral, electrophysiological, and molecular evidence pointing to an important role for the D4 receptor subtype in the processing of emotionally salient information, learning, and memory (Lauzon and Laviolette, 2010; Sood *et al.*, 2011). D_4 receptors are enriched in the prefrontal cortex (PFC) and hippocampus (van Tol *et al.*, 1991; Defagot *et al.*, 2000), brain regions involved in cognitive processes (Goldman-Rakic, 1996). Within the PFC, dopamine can modulate the firing of GABAergic interneurons through a D_4 receptor, thus indirectly modulating cortical output (Mrzljak *et al.*, 1996). D_4 receptors may be a key element in regulating the balance between glutamatergic excitation and GABAergic inhibition (Thomas *et al.* 2009). Yuen and Yan (2009) suggested that impairments in GABAergic inhibition to PFC pyramidal neurons are implicated in the abnormal neural synchrony and working memory disturbances in schizophrenia. The lack of extrapyramidal side-effects could also be attributed, at least in part, to D_4 antagonism (Wong and van Tol, 2003) as D_4 receptors are more prominently expressed in limbic regions (frontal cortex and hippocampus) than in extrapyramidal motor control regions (caudate nucleus and putamen) (Wong and van Tol, 2003).

Serotonin pathways also appear to be relevant for the action of LASSBio-1422 as it binds with moderate affinity to 5-HT_{1A} receptors and prevented the impairment of PPI by (±)-DOI, a 5-$HT_{2A/2C}$ agonist. The increase in PFC dopamine release produced by atypical antipsychotics, such as clozapine, olanzapine, and ziprasidone, but not by haloperidol, seems to involve 5-HT_{1A} receptor activation (Ichikawa *et al.*, 2001; Diaz-Mataix *et al.*, 2005). This finding has been considered a potential basis for the action of some of the atypical antipsychotics (Sakaue *et al.*, 2000; Almeida *et al.*, 2008). Bantick *et al.* (2001) suggested that serotonin 5-HT_{1A} receptors are an important target for cognitive dysfunction in schizophrenia. Uehara *et al.* (2014) showed that tandospirone, a 5-HT_{1A} receptor partial agonist, improved cognitive deficits in rats. In addition, brexpiprazole, a serotonin-dopamine activity modulator with 5-HT_{1A} receptor partial agonism, attenuated PCP-induced cognitive deficits in mice, suggesting that it could ameliorate cognitive deficits observed in schizophrenia and other neuropsychiatric diseases (Yoshimi *et al.*, 2014). Therefore, an action of LASSBio-1422 on 5-HT_{1A} receptors might underlie, at least in part, the cognitive properties of LASSBio-1422 shown in the current study. This could also explain the lack of extrapyramidal effects. The ability of 5-HT_{1A} agonists to decrease extrapyramidal symptoms induced by atypical antipsychotics is one of the best-documented benefits of this receptor modulation in the treatment of schizophrenia (Haleem *et al.*, 2004; Newman-Tancredi, 2010; Shimizu *et al.*, 2010). 5-HT_{1A} receptor agonism plays a meaningful role in reducing the cataleptogenic effects of antipsychotics such as aripiprazole and bifeprunox in rodents and nonhuman primates (Prinssen *et al.*, 1998; Shapiro *et al.*, 2003; Bardin *et al.*, 2005; Newman-Tancredi *et al.*, 2005; Auclair *et al.*, 2009). Furthermore, attenuation of haloperidol-induced catalepsy by clozapine has been proposed to be partially 5-HT_{1A} receptor mediated (Millan *et al.*, 1998), despite its modest affinity for this receptor ($K_i = 0.26$ mol/l) (Gomes *et al.*, 2013). Neves *et al.* (2013) also proposed this mechanism for LASSBio-579, which reversed the catalepsy induced by the 5-HT_{1A} antagonist WAY 100,635.

Conclusion

LASSBio-1422 is effective in rodent models of positive and cognitive symptoms of schizophrenia at doses devoid of extrapyramidal and sedative effects. Its ability to bind to D_4 and 5-HT_{1A} receptors may at least in part explain its effects on these animal models. The beneficial effects of LASSBio-1422 on cognition identified in this study encourage further studies using this molecular scaffold for the treatment of attention and cognition disorders. The investigation of other neurochemical targets, as well as the effects of this compound in animal models of negative symptoms of schizophrenia, should further substantiate the usefulness of this molecular scaffold in the search for new antipsychotic drugs.

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Conflicts of interest

There are no conflicts of interest.

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