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TITLE PAGE

CARIPRAZINE (RGH-188), A DOPAMINE D_3 RECEPTOR PREFERRING, D_3/D_2 DOPAMINE RECEPTOR ANTAGONIST-PARTIAL AGONIST ANTIPSYCHOTIC CANDIDATE: IN VITRO AND NEUROCHEMICAL PROFILE

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List of Abbreviations:

5-HT, serotonin; 5-HIAA, 5-hydroxyindolyl acetic acid, DA, dopamine; DOPAC, dihydroxy-phenyl acetic acid; HPLC-ED, high-pressure liquid chromatography with electrochemical detection; HVA, homovanillic acid

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ABSTRACT

Cariprazine (RGH-188, *trans*-N-[4-[2-[4-(2,3-dichlorophenyl)piperazin-1-yl]ethyl]cyclohexyl]-N',N'-dimethylurea hydrochloride), a novel candidate antipsychotic demonstrated approximately 10-fold higher affinity for human D_3 versus hD_{2L} and hD_{2S} receptors (pKi: 10.07, 9.16, 9.31, respectively). It displayed high affinity at h5-HT_{2B} receptors (pKi: 9.24) with pure antagonism. Cariprazine had lower affinity at human and rat hippocampal 5-HT_{1A} receptors (pKi: 8.59 and 8.34, respectively) and demonstrated low intrinsic efficacy. Cariprazine displayed low affinity at human 5-HT_{2A} receptors (pKi: 7.73). Moderate or low affinity for histamine H_1 and 5-HT_{2C} receptors (pKi: 7.63 and 6.87, respectively) suggest cariprazine's reduced propensity for adverse events related to these receptors. Cariprazine demonstrated different functional profiles at dopamine receptors depending on assay system. It displayed D_2 and D_3 antagonism in [^{35}S]GTPγS binding assays, but stimulated inositol phosphate (IP) production (pEC_{50}: 8.50, E_{max} 30%) and antagonized (±)-quinpirole-induced IP accumulation (pKb: 9.22) in murine cells expressing human D_{2L} receptors. It had partial agonist activity (pEC_{50}: 8.58, E_{max} 71%) by inhibiting cAMP accumulation in CHO cells expressing hD_3 receptors and potently antagonized 7-OH-DPAT–induced suppression of cAMP formation (pKb: 9.57). In these functional assays, cariprazine showed similar (D_2) or higher (D_3) antagonist-partial agonist affinity and greater (3- to 10-fold) D_3 versus D_2 selectivity compared with aripiprazole. In in vivo turnover and biosynthesis experiments, cariprazine demonstrated D_2-related partial agonist and antagonist properties, depending on actual dopaminergic tone. The antagonist-partial agonist properties of cariprazine at D_3 and D_2 receptors, with very high and preferential affinity to D_3 receptors, make it a candidate antipsychotic with a unique pharmacological profile among known antipsychotics.

INTRODUCTION

Dopamine D_3 receptors, cloned in the beginning of the 1990s (Sokoloff et al., 1990), are most abundant in the mesolimbic regions (i.e., nucleus accumbens, island of Calleja) where dysregulation of neurotransmission is thought to be associated with psychosis. The discovery that most antipsychotics, in addition to binding to D_2 receptors, also display reasonably high affinity for D_3 receptors, led to the assumption that these receptors may also be responsible for antipsychotic efficacy (Sokoloff et al., 1995). Unfortunately, the selective D_3 antagonists developed so far (e.g., SB-277011, S33084) have failed to demonstrate sufficient antipsychotic-like activity in various animal models (Reavill et al., 2000; Millan et al., 2000). However, studies with D_3 selective agents found that D_3 receptors are very likely associated with locomotor control, cognitive behavior, and drug abuse (Gyertyán and Sághy, 2008a; Joyce and Millan, 2005).

Recent human imaging studies found that approximately 60-75% occupancy of dopamine D_2 receptors is necessary for clinical antipsychotic efficacy (Kapur and Mamo, 2003). Blocking these receptors remains one of the primary targets in schizophrenia pharmacotherapy (Seeman, 2006). However, based on the known functions, properties, and localization of D_3 receptors, the hypothesis was developed that the presence of subnanomolar D_3 antagonism alongside nanomolar D_2 antagonism may yield an antipsychotic compound with a superior side effect profile (e.g., reduced extrapyramidal symptoms, improvement in cognition). Indeed, representative compounds (e.g., S33138 and RG-15) developed on the basis of this idea demonstrated antipsychotic-like activity and reduced side effect liability in animal models (Millan et al., 2008; Kiss et al., 2008; Gyertyán et al., 2008b).

Several attempts have been made to utilize partial D_2 agonists such as preclamol (Lahti et al., 1998) and terguride (Olbrich and Schanz, 1991) in the treatment of schizophrenia. However,

only partial and temporary efficacy has been demonstrated for these compounds, possibly due to their higher than optimal intrinsic activity. To date, however the optimal intrinsic activity required for a partial D_2 agonist to yield the desired antipsychotic efficacy to treat the major symptoms of schizophrenia remains unclear.

Aripiprazole, a D_2 partial agonist (Burris et al., 2002), received approval for the treatment of schizophrenia in the U.S. in 2002 (Keck and McElroy, 2003). Aripiprazole displayed subnanomolar affinity for the human dopamine D_2 receptors with relatively low intrinsic activity (Tadori et al., 2009). In addition, aripiprazole showed subnanomolar-nanomolar affinity for several other receptors in vitro with antagonist (e.g. for 5-HT_{2B}, 5-HT_{2A}, H1) or partial agonist profile (e.g. for D_3 and 5-HT_{1A}) (Shapiro et al., 2003). Understanding the exact in vitro mechanism of action of aripiprazole was complicated by the finding that, in different signaling pathway, it was a functionally selective compound at D_2 receptors (Urban et al., 2007).

Previously, we hypothesized that an effective antipsychotic agent with favorable properties (such as cognition improvement) and a beneficial side effect profile (e.g. greatly reduced liability to induce catalepsy) would combine subnanomolar affinity for dopamine D_3 receptors with nanomolar affinity for D_2 receptors (Kiss et al., 2008; Gyertyán et al, 2008b). From a series of compounds we selected cariprazine (former code name: RGH-188; Fig. 1.), a compound demonstrating subnanomolar affinity for D_3 receptors, nanomolar affinity for D_2 receptors with antagonist-partial agonist activity at both these dopamine receptor subtypes. In the present study, we have elucidated the in vitro receptor binding and functional profile (i.e. antagonist/agonist properties) of cariprazine at dopamine D_3/D_3-related signaling pathways in vitro. In addition, we investigated its action on the cerebral dopamine turnover and biosynthesis in mouse brain under various conditions. Throughout these studies, cariprazine was compared with known D_3/D_2 agonists, antagonists, first-generation and atypical antipsychotics with special

regards to aripiprazole, the only D_2/D_3 partial agonist/antagonist antipsychotic used in the clinic.

Methods

Animals

Male NMRI mice (20-25 g), 7- to 8-week-old male (180-220 g) Hanover Wistar rats, and Hartley guinea pigs (200-250 g) were obtained from Toxicoop Hungary Ltd. and acclimatized at the site for at least 3-4 days before any experiments started. They were kept under standard conditions (temperature: 21°C; relative humidity: 55-65%; 12:12 hours dark:light cycle) on commercial laboratory chow and tap water ad libitum. Animal maintenance and research were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. All procedures using animals were approved by the local ethics committee, and conformed to the rules and principles of the 86/609/EEC Directive.

Drugs

Cariprazine (RGH-188, *trans*-N-[4-[2-[4-(2,3-dichlorophenyl)piperazin-1-yl]ethyl]cyclohexyl]-N',N'-dimethylurea) HCl, aripiprazole HCl, S-(-)-pramipexole HCl, haloperidol, olanzapine, risperidone, L741626 (4-(4-Chlorophenyl)-1-(1H-indol-3-ylmethyl)piperidin-4-ol), SB-277011 (*trans*-N-[4-[2-(6-cyano-1,2,3,4-tetrahydro-2-isoquinolinyl)ethyl]cyclohexyl]quinoline-4-carboxamide), NSD-1015 (3-hydroxy-benzylhydrazine HCl), and reserpine were synthesized at Gedeon Richter Plc. or were the product of the company. Apomorphine HCl (Chemistry Department, University of Debrecen, Debrecen, Hungary), 7-OH-DPAT (R(+)-2-dipropylamino-7-hydroxy-1,2,3,4-tetrahydronaphtalene HBr), 8-OH-DPAT (R(+)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphtalen HBr), DOI ((-)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl), pyrilamine, dopamine (DA), (±)-quinpirole or (+)-quinpirole, γ-butyrolactone (GBL) and butaclamol were purchased from Sigma Chemicals (St. Louis, MO, USA). SB-204741 was obtained from Tocris (Avonmouth, UK). (+)-PHNO

(trans-1a,2,3,4a,5,6-hexahydro-9-hydroxy-4-propyl-4H-naphtho[1,2-b]-1,4-oxazine) was purchased from Toronto Research Chemical (Toronto, Canada). Radioligands, [^{3}H]spiperone (spec. act.: 15-16 Ci/mmol), [^{3}H]raclopride (spec. act.: 60-80 Ci/mmol), [^{3}H]8-Hydroxy-2-(di-n-propylamino)tetralin (spec. act.: 106 Ci/mmol), [^{3}H]ketanserine (spec. act.: 88 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences Inc. (Boston, MA, USA). [^{35}S]GTPγS (spec. act.: 1000-1150 Ci/mmol) [^{3}H]cAMP (spec. act.: 37.2 Ci/mmol) was purchased from Amersham Radiochemicals (Little Chalfont, Buckinghamshire, UK). [^{3}H]-Myo-inositol (spec. act.: 30 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). All other chemicals were of analytical grade and were obtained from commercial sources. Cariprazine was used in base-form in most experiments; use of its HCl salt is indicated separately. For in vivo experiments, drugs were dissolved in a minimum amount (~10-20 µL) of acetic acid and further diluted with saline. They were administered orally in a volume of 10 ml/kg.

Radioligand Binding Assays Using Rat Receptors. Rats were decapitated; brains were rapidly removed, chilled in ice-cold saline and the striatum, hippocampus, and frontal cortex of each rat were dissected on an ice-cooled surface.

Striata were homogenized by Ultra-Turrax (IKA-Werke GmbH & Co. KG. Germany) in 50 vol. (w/v) ice-cold buffer (50 mM Tris-HCl, 1 mM EGTA, 5 mM $MgSO_4x7H_2O$, pH: 7.4) and the homogenate was centrifuged at 40000g for 10 minutes at 4°C. The pellet was suspended in the same buffer and the homogenate was centrifuged at 40000g for 10 minutes at 4°C. This procedure was repeated once. The final pellet was resuspended in 30 vol. (w/v) of the same buffer and frozen in aliquots at -70°C until use.

Hippocampi were homogenized in 40 vol. (w/v) ice-cold 50 mM TRIS-HCl buffer (pH:

7.7) and the homogenate was centrifuged at 45000g for 10 minutes at 4°C. The pellet was suspended in the same buffer, and the homogenate was incubated at 37ºC for 10 minutes and centrifuged at 45000g for 10 minutes at 4°C. The final pellet was resuspended in 60 vol. (w/v) binding buffer consisting of 50 mM TRIS-HCl/4 mM $CaCl_2$/0.1 % ascorbic acid/10 µM pargyline (pH: 7.7) and frozen in aliquots at -70°C until use.

Frontal cortices were homogenized by a glass potter homogenizer with teflon pestle (B. Braun Melsungen, Germany) with six up-and-down strokes in 40 vol. (w/v) ice-cold 50 mM TRIS-HCl buffer (pH: 7.4) and the homogenate was centrifuged at 40000g for 15 minutes at 4°C. The supernatant was discarded and the pellet was washed twice by resuspension in 40 vol. (w/v) 50 mM TRIS-HCl buffer (pH: 7.4) followed by centrifugation. The final pellet was resuspended in 20 vol. (w/v) 50 mM TRIS-HCl (pH: 7.4) and frozen in 4 mL aliquots and stored at -70°C until use.

Membranes of Sf9 cells expressing the human recombinant D_3 receptors were purchased from PerkinElmer (Cat. No. 6110139).

Dopamine D_2 (striatum), 5-HT_{1A} (hippocampus), 5-HT_{2A} (frontal cortex), and rat recombinant D_3 binding assays were carried out using incubation conditions as summarized in Table 1. Incubations were stopped by filtration on a Whatman GF/B glass-fiber filter presoaked in 0.05 % polyethylene-imine. The filters were washed 3 times with 1 mL binding buffer (striatum, Sf9 membranes expressing rat D_3 receptor), 2 times with 5 mL binding buffer (hippocampus) and 3 times with 4 mL binding buffer (frontal cortex). Retained radioactivity was determined after addition of 4 mL Optiphase HiSafe (PerkinElmer) using an LKB-Wallac 1409 liquid scintillation counter.

Cariprazine binding was also tested at 62 neurotransmitter receptors, 5 ion-channel sites, 5

transporter sites and 5 enzymes at test concentration of 1 μM by MDS Pharma Service (Taiwan Ltd., Pharmacology Laboratories Peitou, Taipei, Taiwan, work order numbers: 1-1010647, 1-1010827-0, 1-1010891-0, 1-1011923-0, 1-1013302, 1-1014645; data on file at Gedeon Richter Plc.)

[^{35}S]GTPγS Binding. *Striatal and hippocampal membrane preparation*: Rats were decapitated; brains were rapidly removed and placed on ice. Striata and hippocampi were dissected out and immediately homogenized in ice-cold buffer containing 50 mM Tris, 5 mM $MgCl_2$, and 1 mM EDTA (pH=7.6) by a glass Dounce homogenizer. Tissue homogenates were centrifuged at 40000 g for 15 minutes at 4°C. Membrane pellets were resuspended in the same buffer. Hippocampal membranes were incubated for 10 minutes at 37°C in a shaking water bath to eliminate endogenous serotonin. Pellets were recentrifuged and the final pellets were resuspended in ice-cold buffer (pH=7.6) containing 50 mM Tris, 100 mM NaCl, 7 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiotreithol (DTT) to yield a concentration of 20 mg tissue weight/mL and frozen at -70°C until use.

HEK293-hD_2 and CHO-hD_3 cell membrane preparation: cells were grown at 37°C in a sterile, humidified incubator in 5% CO_2 atmosphere in DMEM-F12 medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), puromycin (Sigma), G418 (Calbiochem), and sodium pyruvate (Sigma). Cells were collected in PBS-EDTA, centrifuged at 2000g for 15 minutes at 4°C and the pellet resuspended and homogenized with a glass Dounce homogenizer in 50 mM Tris (pH=7,6), 5 mM $MgCl_2$, and 1 mM EDTA. The membrane homogenate was washed twice by centrifugation at 40000g for 15 minutes at 4°C. The final pellet (80 mg protein/mL) was resuspended in 50 mM Tris (pH=7.6), 100 mM NaCl, 7 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT, aliquoted and stored at -70°C until use.

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[^{35}S]GTPγS binding assays were done in 50 mM Tris (pH=7.4), 100 mM NaCl, 7 mM Mg_2Cl, 1 mM EDTA, and 1 mM DTT. Assay tubes (final volume 250 µL) contained 50 µM (striatum and hippocampus) or 1 µM (D_2 and D_3 cell membrane) GDP, the ligand to be examined, and membrane suspension (250 µg tissue/tube for the striatum and hippocampus and 20 µg protein/tube for hD_2 and hD_3 membranes). Samples were preincubated for 10 minutes at 30°C. After the addition of 50 pM [^{35}S]GTPγS, membranes were incubated for an additional 60 minutes at 30°C. Nonspecific binding was determined in the presence of 10 µM GTPγS; basal binding was determined in the presence of buffer only. The assay was terminated by rapid filtration through Packard UniFilter GF/B using a Packard harvester, and the membranes washed 4 times with 1 mL ice-cold buffer. After drying (40°C for 1 hour), 40 µL Microscint (Packard) was added to the filters, and the bound radioactivity was determined by a TopCount NXT (PerkinElmer).

Determination of IP Accumulation in A9 Cells Expressing Human D_{2L} Receptors. The murine cell line (A9 L hDF2 S.C. 18 cells, CRL-10225) expressing human D_{2L} dopamine receptor was purchased from ATCC (American Type Culture Collection 10801 University Boulevard, Manassas, VA, USA). The cells were co-transfected with an expression plasmid coding the Gqo5 protein (pCEP-Gqo5-Ha plasmid from Molecular Devices) that can activate phospholipase C-β (PLC-β), resulting in generation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), leading to Ca^{2+} release from intracellular stores. This double-transfected cell line (A9/1/49) was grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum, G418, and hygromicin B, and treated with 5 mM Na-butyrate 1 day before the experiment.

Cells were seeded on a 24-well tissue culture plate in 500 µL medium. Fifty µL medium containing 0.55 µCi [^{3}H]myo-inositol was added (final concentration: 1 µCi/mL) and incubated

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for 18-20 hours. Cells were then washed 3 times with buffer containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5 mM HEPES, 5 mM Na-HEPES, 20 mM glucose and 10 mM LiCl (pH= 7.4). Cells were then incubated for additional 60 minutes (37°C) in medium with test compounds alone (agonist test) or alongside 1000 nM (±)-quinpirole (antagonist test). Medium was then aspirated off, cells lysed by adding 400 µL of 0.1 M HCl/2 mM $CaCl_2$ and supernatants frozen at -72°C. After thawing and centrifugation at 1,000g for 10 minutes, 200 µL of each supernatant was loaded on 250 µL of AG1-X8 (formate form) anion exchange column. Effluent was discarded, and columns were washed twice with in 1.5 mL of distilled water. Inositol phosphates (IPs) were eluted with 2.5 mL of 1 M ammonium formate/0.1 M formic acid directly into scintillation vials, 10 mL Optiphase HiSafe 3 (PerkinElmer) added, and the radioactivity determined in a Tricarb 4900 scintillation counter (Perkin Elmer).

cAMP Accumulation in Cells Expressing Recombinant Human D_3 Receptors. CHO-K1 cells expressing recombinant human D_3 receptors were from Euroscreen (Brussels, Belgium). Cells were grown in DMEM -F12 medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), puromycin (Sigma), G418 (Calbiochem), and sodium pyruvate (Sigma) at 37°C in a a sterile, humidified incubator in 5% CO_2 atmosphere .Cells were harvested by trypsinization, seeded on Poly-D-Lysine-coated 96-well plates at a density of 25000 cells/well, and cultured for 2 days at 37°C. On the day of the experiment, the medium was removed and cells were preincubated in 60 µL Hank's Balanced Salt Solution (HBSS) for 10 minutes at 37°C and then subjected to test compounds (agonists, antagonists, and forskolin) in 60 µL HBSS complemented with 3-isobutyl-1-methylxanthine (100 µM) for 20 minutes at 37°C. After adding 20 µL 1 M perchloric acid to terminate the reaction, plates were frozen overnight at -20°C, thawed, and neutralized (pH= 7.4) with the addition of 50 µL ice-cold 0.5 M KOH. Samples were

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maintained at 4°C for 30 minutes and centrifuged at 700g at 4°C for 10 minutes. cAMP was determined by the method of Nordstedt and Fredholm (1990) using a cAMP binding protein from bovine adrenal cortex. Fifty µL of supernatant was incubated with 0.15 pmol/well [^{3}H]cAMP in 50 µL distilled water and 25 µg/well cAMP-binding protein (in 200 µL 50 mM Tris; pH=7.4) at 4°C for 130 minutes, and collected onto GF/B filters. Radioactivity of the samples was determined by a TopCount NXT (PerkinElmer).

Functional Activity at Histaminergic and Serotonergic Receptors. *H_1 antagonism*: the functional activity at histamine H_1 receptors was determined according to Yamauchi et al. (1994). Male Hartley guinea pigs (Toxicoop, Budapest, Hungary) weighing 200-250 g were sacrificed by cervical dislocation and the trachea was removed. Spirally cut trachea (5-6 cartilage rings) were set up in a 10 mL organ bath containing Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 11 mM glucose, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, pH:7.4) maintained at 37 °C and oxygenated with 95% O_2/5% CO_2. Resting tension was adjusted to 1.5 g and the change in isometric tension measured by isometric force transducer (HSE, Type D 7801). To prevent modulation of contractile responses by prostaglandins and acetylcholine released from epithelium and pre- or postsynaptic neurons, respectively, indomethacin (10 µM) and atropine (0.2 µM) were added for every experiment. During equilibration (90 minutes), the bath solution was changed every 15 minutes. After equilibration, histamine (3 µM) was repeatedly applied in 45-minute intervals so that after the contraction reached a plateau, the bathing solution was exchanged. After testing 3 contractions, different concentrations of the test substances were added and the application of histamine repeated after 30 minutes.

5-HT_{2A} antagonism: 20,000 CHO cells expressing human 5-HT_{2A} receptor (5-HT_{2A}-CHO) cells (Euroscreen) were seeded on a 24-well tissue culture plate in 500 µL medium. After 100 µL

medium containing 0.6 μCi [^{3}H]-myo-inositol (American Radiolabeled Chemicals) (final concentration: 1 μCi/ml) were added, cells were incubated for 18-20 hours. After loading with [^{3}H]-myo-inositol, cells were washed 3 times with IP buffer (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5 mM HEPES, 5 mM Na-HEPES, 20 mM glucose, 10 mM LiCl, pH 7.4).

Antagonist effect determination: Cells were treated with different concentrations of test compounds for 20 minutes at 37 °C and 5-HT_{2A} receptors were then stimulated by 30 nM DOI for 60 minutes at 37 °C. Determination of IP formation was as described above.

5-HT_{2B} antagonism: the functional activity at 5-HT_{2B} receptors was determined in rat isolated fundus strips (Baxter et al. (1994). Male Wistar rats weighing 250-350 g (Toxicoop Ltd., Budapest, Hungary) were killed by cervical dislocation and longitudinal strips (2-2.5 x 20 mm) were dissected from the greater curvature of the fundus and mounted in a 10 mL tissue bath containing oxygenated (95% O_2 /5% CO_2) Tyrode's solution (136.9 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1.0 $MgCl_2$, 5.6 mM glucose, 11.9 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 3 μM indomethacin) at 37 °C. After preincubation for 2 hours, with a starting preload of 2 grams, the baseline tension was recorded by K30 (Hugo Sachs) isometric force transducer coupled to a Multi-Pen recorder (Rikadenki). Strips were exposed to 5-HT (100 nM ≅ EC_{80}) 3 times. Serotonin was applied until a maximum contraction was achieved and then washed out after the tension started to decrease. After the third contraction and washout, the strips were incubated with the test substance (100 nM) for 35 minutes and the 5-HT application repeated. The determination of inhibitory effect of different concentrations of the test compounds was similar to the protocol of the pilot experiment except that 5-HT (100 nM) was repeatedly applied in 35 minute intervals, while increasing concentrations of test substances were applied after each 5-HT washout cycle.

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Determination of Dopamine, Serotonin, and Metabolites in Mouse Brain Regions. Dopamine (DA), dihydroxy-phenyl acetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindolyl acetic acid (5-HIAA) were determined by high-pressure liquid chromatography coupled with electrochemical detection (HPLC-ED) as described (Kiss et al., 2008), and expressed in pmol/g tissue. Ratios of (DOPAC+HVA)/DA and 5-HIAA/5-HT, respectively, were calculated as an index of DA and 5-HT turnover, respectively. Ratios from vehicle-treated animals were used as controls, and drug effects normalized to percent of control value.

Determination of DOPA Accumulation. Accumulation of DOPA after treatment with the aromatic amino acid decarboxylase inhibitor NSD-1015 has been used for the estimation of the in vivo dopamine biosynthesis rate. In these experiments, drug- or vehicle-treated animals received NSD-1015 intraperitoneally 30 minutes before decapitation. DOPA levels were determined from dissected brain regions by the HPLC-ED method (Kiss et al., 2008).

Data Analysis. IC_{50} values were calculated by nonlinear least-squares regression analysis (MDS Pharma, Data Analysis Toolbox; MDL Information Systems, San Leandro, CA). Some rat receptor binding assays, evaluation of functional activity was carried out by the Gedeon Richter Laboratory (Budapest, Hungary). Receptor binding isotherms and concentration or dose-response curves were analyzed by non-linear regression using Origin 6.0 (MicroCal Software Inc. Northampton, MA) or PRISM 4.0 software (GraphPad Software Inc. San Diego, CA); both programs gave identical results. From the IC-50 values, inhibition constants (Ki) were calculated by the Cheng and Prusoff (1973) equation; affinity (Kb) values were calculated according to the Craig (1993) formula. In turnover and biosynthesis experiments, comparison between control and treatment groups was made by Tukey-Kramer multiple comparison test. .

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RESULTS

In Vitro Receptor Binding Profile of Cariprazine. The affinity of cariprazine for receptors, channels, and transporters and its effect on certain enzymes are summarized in Table 2. Cariprazine showed picomolar affinity for human dopamine D_3 receptors, and subnanomolar affinity for both D_{2L} and D_{2S},subtypes of human D_2 receptors, D_{2L} and D_{2S}, and for human serotonin 5-HT_{2B} receptors. Cariprazine had nanomolar affinity for human 5-HT_{1A} receptors, moderate affinity for human 5-HT_{2A}, histamine H_1 and σ_1 receptors and low affinity for all tested adrenergic receptors. Cariprazine preference for rat D_3 versus D_2 receptors was similar to human receptors, though the overall affinity for rat receptors was about 10-fold lower than for human receptors. This difference was not observed for binding to human and rat 5-HT_{1A} receptors.

The receptor binding profile of cariprazine differed from aripiprazole, which was assayed alongside cariprazine (Table 2) Aripiprazole displayed highest affinity for both human dopamine D_2 subtypes and for human 5-HT_{2B}, followed by the human histamine H_1, dopamine D_3 and human and rat 5-HT_{1A} receptors. Furthermore, aripiprazole bound more potently than cariprazine to rat striatal dopamine D_2, human and rat 5-HT_{2A}, 5-HT_{2C} and adrenergic receptors.

Cariprazine Antagonizes Native Rat and Recombinant Human D_2 and D_3 Receptors: [^{35}S]GTPγS Binding Results. Dopamine, pramipexole, quinpirole, apomorphine, 7-OH-DPAT, and (+)PHNO all stimulated [^{35}S]GTPγS binding in rat striatal, HEK293-D_2 and CHO-D_3 cell membranes, consistent with their agonist properties, cariprazine and aripiprazole, did not show stimulation of [^{35}S]GTPγS binding (Fig. 2A, 2C, 2E, Table 3) and both compounds, similar to other antipsychotics (e.g., haloperidol, olanzapine and risperidone), inhibited dopamine-stimulated [^{35}S]GTPγS binding in these preparations (Fig 2B, 2D, and 2F, Table 3). Among the compounds tested, cariprazine displayed the highest antagonist potency for CHO-D_3 receptors.

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Aripiprazole, followed by cariprazine, was the most potent antagonist at HEK-D_2 cells. Comparison of the antagonist potency (i.e., pKb) values obtained in these experiments indicated that the reported D_3 receptor selective compound SB-277011 had the highest (100-fold) selectivity toward D_3 versus D_2 receptors, followed by cariprazine (2.4-fold). Aripiprazole and L741626, a selective D_2 antagonist, were the most D_2 versus D_3 selective compounds in these tests. In the rat striatal membrane preparation, cariprazine showed the highest antagonist potency, followed by aripiprazole, haloperidol, L741626, and risperidone.

Antagonist-Partial Agonist Activity of Cariprazine at hD_2 and hD_3 Receptors: IP and cAMP Formation. In murine A9 cells expressing human D_2 receptors co-transfected with Gqo5 protein, dopamine, pramipexole, 7-OH-DPAT fully stimulated IP formation. Cariprazine proved to be partial agonist in this assay; it stimulated IP formation with a high potency (pEC_{50}: 8.50) with relatively low efficacy (E_{max}: 30%). Aripiprazole showed similar efficacy (max: 34%) but its potency (pEC_{50}: 7.66) at these human D_2 receptors was about 7-fold less than cariprazine (Fig 3A, Table 4). IP formation stimulated by (±)-quinpirole was concentration-dependently and most effectively inhibited by haloperidol (pKb: 10.47) followed by cariprazine and aripiprazole. Although aripiprazole displayed higher binding affinity for human D_2 receptors (Table 2), it had less antagonist activity (pKb: 8.52) than cariprazine (pKb: 9.22) in this cell-based functional assay system (Fig 3B, Table 4).

In vitro functional activity of cariprazine at dopamine D_3 receptors was tested by measuring cAMP production in CHO cells transfected with human D_3 receptors. Dopamine and the D_3/D_2 agonists 7-OH-DPAT, quinpirole, and pramipexole all demonstrated full agonist activity (i.e., they inhibited forskolin-stimulated cAMP production, Fig 3C, Table 4). Cariprazine proved to be a potent partial agonist with relatively high intrinsic activity (E_{max}: 70.9%).

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Aripiprazole demonstrated partial agonist properties with similar intrinsic efficacy to cariprazine but with an 11-fold lower EC_{50} value (Fig. 3C, Table 4). As expected from partial agonists, cariprazine and aripiprazole also displayed antagonist properties and partially reversed 7-OH-DPAT–induced inhibition of forskolin-stimulated cAMP accumulation in D_3-CHO cells by up to 27 % and 24 % for cariprazine and aripiprazole, respectively. Pure antagonists, such as haloperidol, fully antagonized the effect of 7-OH-DPAT (Fig. 3D, Table 4). Among the tested compounds, cariprazine displayed the highest antagonist potency in reversing 7-OH-DPAT response, approximately 60-fold higher than aripiprazole.

Functional Activity at Serotonin and Histamine H_1 Receptors. In the [^{35}S]GTPγS binding assay using rat hippocampal membrane preparation both cariprazine and aripiprazole showed partial agonism at 5-HT_{1A} receptors with somewhat higher potency than the 5-HT_{1A} full agonist 8-OH-DPAT (Fig 4A).

In the in vitro functional assays using CHO cells expressing human 5-HT_{2A} receptors, neither cariprazine nor aripiprazole stimulated IP formation, indicating negligible intrinsic activity of both compounds at these receptors. Cariprazine showed weak 5-HT_{2A} receptor antagonist activity, inhibiting the DOI-induced IP formation. Consistent with the in vitro binding results, aripiprazole had approximately 7-fold higher potency than cariprazine in this assay (Fig. 4B).

Both cariprazine and aripiprazole showed high affinity for human 5-HT_{2B} receptors (Table 2). Their functional activity was assayed using isolated rat stomach fundus, a tissue known to express high levels of 5-HT_{2B} receptors (Fig. 4C). Neither drug caused contraction in this preparation when applied at 100 nM final concentration (not shown). However, both cariprazine and aripiprazole, similar to the selective 5-HT_{2B} antagonist SB-204741, concentration-dependently antagonized contractions induced by 100 nM serotonin.

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The functional activity of cariprazine at histamine H_1 receptors was compared with aripiprazole using isolated guinea pig trachea preparation (Fig. 4D). Histamine produced half-maximal contraction (EC_{50}) at 1.45 μM, whereas neither cariprazine nor aripiprazole caused contraction in this preparation (not shown). Pyrilamine, a prototypical H_1 antagonist, potently antagonized the histamine-induced contractions, whereas both cariprazine and aripiprazole were less potent antagonists.

Effects on Cerebral Dopamine and Serotonin Turnover. Cariprazine dose-dependently increased the DA turnover (i.e., [DOPAC+HVA]/DA ratio) in both mouse striatum and olfactory tubercles. The elevated levels of fronto-cortical DOPAC are also consistent with the D_2 receptor antagonist property of cariprazine. Conversely, cariprazine at the higher dose range (i.e., at 3 and 10 mg/kg) appeared to reduce 5-HT turnover (i.e., 5-HIAA/5-HT ratio) (Fig 5).

The increase in DA turnover evoked by cariprazine treatment (1 mg/kg, p.o.) persisted for at least 8 hours; at this time, increases of ~75 % and 125% were noted in striatum and olfactory tubercles, respectively. Similar increases in DOPAC levels were found in the frontal cortex (~130 % above control), but DOPAC levels returned to baseline in 2 hours. Serotonin turnover was moderately but statistically significantly reduced (from 15% to 32%) in all 3 regions at time points of 2, 4 and 8 hours (data not shown).

Haloperidol and the atypical antipsychotics risperidone and olanzapine caused a maximal 3- to 4-fold increase in dopamine turnover in the mouse striatum and olfactory tubercles. In contrast, cariprazine and aripiprazole produced lower maximal enhancement of dopamine turnover in these 2 regions. Furthermore, the latter 2 drugs produced consistently higher rates of dopamine turnover in the olfactory tubercles than in the striatum (Fig 6).

Cariprazine Has Dopamine Partial Agonist and Antagonist Properties in the GBL

Model In Vivo. Treatment with GBL induces cessation of impulse flow in the nigrostriatal tract accompanied by increased dopamine biosynthesis in the terminal region (as determined by DOPA accumulation after inhibition of aromatic amino acid decarboxylase by NSD-1015). This method allows for the assessment of activity of presynaptic biosynthesis- and release-modulating dopamine D_2 receptors in the striatum (Walters and Roth 1976). The dopamine D_2 full agonist apomorphine dose-dependently reduced the GBL-induced enhancement of DOPA accumulation with almost complete blockade at 3 mg/kg subcutaneously (s.c.) (data not shown). Cariprazine and aripiprazole given before GBL dose-dependently but only partially reduced the GBL-induced DOPA accumulation in mouse striatum (Fig. 7A). Significant reduction in GBL-induced increase of DOPA accumulation was achieved by 3 and 10 mg/kg p.o. cariprazine and 10 mg/kg p.o aripiprazole. Conversely, oral cariprazine given before apomorphine and GBL, dose-dependently and fully antagonized the effect of apomorphine-evoked reduction of GBL-induced enhancement of DOPA accumulation, while partial antagonism was achieved by aripiprazole even at relatively high doses (i.e., at 10 and 30 mg/kg) (Fig. 7B).

Cariprazine Is a Partial Dopamine Receptor Agonist in the Reserpine Model. Reserpine treatment, via disruption of vesicular storing mechanisms, leads to depletion of striatal dopamine and causes an increase in dopamine biosynthesis (Hjorth et al., 1988). Reserpine (1 mg/kg, sc.) given to mice 18 hours before decapitation enhanced dopamine biosynthesis in the mouse striatum by approximately 90-130% above control as measured by DOPA accumulation rate following NSD-1015 treatment. Lower increases (approximately 50-80% above control) of DOPA formation was found in the olfactory tubercles (not shown).

The potency and efficacy of cariprazine for reducing striatal DOPA accumulation in reserpinized mice was compared to drugs that have different functional interactions with dopamine D_2 receptors (Fig. 8.).

Apomorphine, a full dopamine D_2 receptor agonist, dose-dependently reduced DOPA accumulation and, at maximal doses, fully blocked DOPA accumulation to 100% below the non-reserpinized control levels. In contrast, dopamine D_2 pure antagonists such as the typical antipsychotic haloperidol and the atypical antipsychotics risperidone and olanzapine did not change the reserpine-induced enhancement of striatal DA biosynthesis.

Cariprazine potently and dose-dependently, but only partially, reduced the DA biosynthesis in the striatum of reserpine-treated mice. It is noteworthy that cariprazine administration adjusted DOPA accumulation to the control levels with no further decrease, reaching a plateau beginning at 0.3 mg/kg and extending to 10 mg/kg, the highest dose tested.

Aripiprazole, similar to cariprazine, dose-dependently and partially inhibited the reserpine-induced increase of DA biosynthesis in the striatum. However, unlike cariprazine, aripiprazole at doses above 1 mg/kg reduced DA biosynthesis below that of non-reserpine controls. At the highest dose tested (30 mg/kg), response to aripiprazole produced a response that was 80% of the maximum effect elicited by the full agonist apomorphine.

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DISCUSSION

In Vitro Receptor Profile and Functional Activity of Cariprazine

Cariprazine demonstrated picomolar affinity for human dopamine D_3 and subnanomolar affinity for rat D_3 receptors with 6-, 8-, 7-, and 31-fold selectivity against human D_{2S}, D_{2L}, 5-HT_{2B}, 5-HT_{1A} receptors, respectively, and over 100-fold selectivity against other tested receptors (Table 2). Cariprazine has a markedly different receptor profile and D_3 selectivity than aripiprazole, which demonstrated highest affinity for D_2 receptors with much less selectivity against other receptors (Shapiro et al., 2003). Very high affinity for and selectivity toward the dopamine D_3 receptor distinguishes cariprazine from compounds like bifeprunox (D_2 partial agonist) (Marquis et al., 2005), which was reported to possess antipsychotic-like properties.

Dopamine D_2 and D_3 receptor agonists (e.g., dopamine, pramipexole, apomorphine, 7-OH-DPAT, quinpirole, (+)PHNO) fully activated [^{35}S]GTPγS binding in membrane preparation from rat striatum, HEK293-D_2, and CHO-D_3 cells. In these tests, cariprazine and aripiprazole did not show G-protein activation at concentrations up to 10 μM. Lack of agonist efficacy of aripiprazole at D_2 receptors using [^{35}S]GTPγS binding is consistent with findings reported by Lin et al. (2006). However, like compounds with D_2 and D_3 antagonist properties (e.g., haloperidol, L741626, SB-277011, olanzapine, risperidone), cariprazine and aripiprazole antagonized dopamine-stimulated [^{35}S]GTPγS binding. Similar antagonist potency and D_2 or D_3 selectivity have been reported for aripiprazole (Shapiro et al, 2003), SB-277011 (Reavill et al, 2000), and L741626 (Millan et al., 2000). Among the compounds tested, SB-277011 displayed the highest D_3 antagonist selectivity (about 100-fold), followed by cariprazine (2.4-fold); however, cariprazine was 27-times more potent at D_3 receptors. Consistent with published data, aripiprazole demonstrated the highest D_2 potency and selectivity (Shapiro et al. 2003; Burris et al.

2002; Tadori et al., 2005, 2008). Antagonist potencies from the [^{35}S]GTPγS binding assays are consistent with receptor binding affinities for cariprazine and aripiprazole.

In mouse A9 cells expressing human D_{2L} receptors (co-transfected with Gqo5 protein), dopamine, pramipexole, 7-OH-DPAT, and quinpirole concentration-dependently stimulated IP formation. In these cells, pramipexole, 7-OH-DPAT, and dopamine fully activated IP formation whereas quinpirole was less efficacious. Shapiro et al. (2003) also found lower intrinsic efficacy for quinpirole than for dopamine in MES-235 cells expressing D_{2L} receptors by measuring outward K^+ currents. Both cariprazine and aripiprazole demonstrated partial agonist activity with relatively low intrinsic efficacy (E_{max}: 30% and 34 %, respectively) and high or medium potency (pEC_{50}: 8.50 and 7.66, respectively). Potency and efficacy values obtained for aripiprazole are slightly different than those reported earlier (Burris et al., 2002; Tadori et al., 2005); differences are likely due to dissimilarities in cellular systems and assay methodologies. Our system contains a transfected "promiscuous" Gqo5 protein, which brings about artificial stochiometry and may explain different results obtained with cariprazine and aripiprazole in native tissues. Both cariprazine and aripiprazole concentration dependently antagonized (±)-quinpirole-induced IP accumulation with high affinity consistent with results from [^{35}S]GTPγS binding assays.

In CHO cells expressing human D_3 receptors, cariprazine displayed partial agonist activity, inhibiting forskolin-induced cAMP accumulation with high potency and intrinsic efficacy (E_{max}) of 71%. Aripiprazole showed similar intrinsic efficacy, but 10-fold lower potency. Tadori et al. (2008) also found aripiprazole to be a partial agonist at human D_3 receptors but with somewhat lower efficacy and potency. Consistent with the hD_3 binding data, cariprazine demonstrated high antagonist potency for D_3 receptors in this functional assay. Moreover, cariprazine showed approximately 3-fold higher antagonist selectivity for D_3 versus D_2 receptors, whereas the reverse was true for aripiprazole.

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Results from in vitro receptor binding and functional experiments clearly demonstrate high and preferential affinity of cariprazine for dopamine D_3 versus D_2 receptors and underline that functional activity of compounds like cariprazine (and aripiprazole) largely depends on the assay systems (Urban et al., 2007).

Cariprazine, like aripiprazole, displayed nanomolar affinity for human and rat 5-HT_{1A} receptors; both compounds demonstrated relatively low intrinsic efficacy (E_{max}: 38.6 %) in the [^{35}S]GTPγS binding assay using rat hippocampal membrane preparation. At high doses, both aripiprazole and cariprazine reduced serotonin turnover rate in the striatum, olfactory tubercle, and prefrontal-frontal cortex of mouse brain (data not shown), assumedly by partial agonism at 5-HT_{1A} autoreceptors (Fig. 5). These changes, however, were apparent only at doses 10- and 30-fold higher than the ED_{50} for antipsychotic-efficacy (e.g., inhibition of apomorphine-induced climbing) in mice (Gyertyán et al., unpublished data).

Reduced cataleptogenic properties of some newly developed antipsychotics are partly attributed to partial agonist activity at 5-HT_{1A} receptors (Bardin et al. 2006). Neurochemical data reported here strongly suggest that, at lower doses, the in vivo partial agonist activity of cariprazine at 5-HT_{1A} receptors may contribute minimally to the antipsychotic-like and side effect profile; at higher doses, 5-HT_{1A} receptor partial agonism and its contribution to the favorable side effect profile (i.e., lack of extrapyramidal symptoms) of cariprazine cannot be excluded.

Affinity for 5-HT_{2A} receptors is considered an important component of atypicality of second generation antipsychotics (Meltzer et al., 2003). Cariprazine displayed approximately 10- to 60-fold lower affinity for h5-HT_{2A} receptors in vitro compared with marketed atypicals including risperidone, olanzapine, clozapine, and aripiprazole (Shahid et al., 2009), and it demonstrated weak antagonist activity (pK_b: 6.85). Human 5-HT_{2A} affinity and antagonist potency of aripiprazole was 10- and 6-fold higher (pKi: 8.75 and pKb: 7.72, respectively) than

cariprazine. Considering that aripiprazole at clinically effective doses produced 54-60% occupancy of 5-HT_{2A} receptors in human PET studies (Mamo et al., 2007), it is anticipated that the clinical effects of cariprazine due to 5-HT_{2A} receptor activity would be considerably less.

Cariprazine displayed high affinity for serotonin 5-HT_{2B} receptors and it behaved as a pure antagonist. Consistent with data from Shapiro et al. (2003), aripiprazole also demonstrated high affinity for 5-HT_{2B} receptors. The atypical antipsychotics olanzapine, clozapine, and risperidone have also been reported to display relatively high affinity for 5-HT_{2B} receptors (Wainscott et al., 1996). Whether the 5-HT_{2B} receptor antagonism of cariprazine (and aripiprazole), in addition to its D_3, D_2, and 5-HT_{1A} affinities, contributes to its antipsychotic-like activity and side effect profile is presently unclear.

Cariprazine demonstrated approximately 10-fold lower affinity for histamine H_1 and 5-HT_{2C} receptors than aripiprazole; in the guinea pig trachea preparation, cariprazine showed H_1 antagonist activity. Therefore, it is anticipated that cariprazine may have low propensity for causing sedation and body weight gain, side effects commonly associated with high H_1 and 5-HT_{2C} antagonist activity (Kroeze et al., 2003).

In Vivo Dopamine Antagonist-Partial Agonist Properties of Cariprazine

Cariprazine and aripiprazole, at doses within the in vivo antipsychotic-like efficacy range (Gyertyán et al., unpublished data), moderately enhanced dopamine turnover (and biosynthesis) in mouse striatum, olfactory tubercles, and the cortical area, showing antagonist activity at "normosensitive" D_2 receptors. Maximal dopamine turnover increase produced by cariprazine in both regions was approximately 2- to 3-fold lower than that achieved by risperidone, olanzapine, and haloperidol. Both cariprazine and aripiprazole, unlike risperidone, olanzapine, or haloperidol, produced greater enhancement of dopamine turnover (and biosynthesis) in mouse olfactory

tubercle (i.e., limbic region) compared to striatum. The greater olfactorial versus striatal dopamine turnover enhancing action of aripiprazole in this experiment is consistent with results reported by Nakai et al. (2003). Altogether these data indicate that cariprazine, similar to aripiprazole, has lower potential for inhibiting dopamine neurotransmission in the striatum relative to the limbic region as compared with other antipsychotics (e.g., risperidone, olanzapine, haloperidol), suggesting that cariprazine may have low propensity for causing extrapyramidal symptoms.

The moderate turnover (and biosynthesis) enhancing actions of cariprazine and aripiprazole resembled those produced by the D_2 partial agonists SDZ-208-211, SDZ 208-212, and terguride (Svensson et al, 1991). Both cariprazine and aripiprazole proved to be low efficacy partial agonists at presynaptic autoreceptors in the GBL-model. Conversely, cariprazine fully, while aripiprazole only partially, antagonized the biosynthesis-reducing effect of the D_2 full agonist apomorphine in GBL-treated animals. Additionally, in reserpine-treated mice, cariprazine partially inhibited increased striatal dopamine synthesis, with reversal to the control level (at 0.3 mg/kg and higher), while aripiprazole at the highest dose tested caused nearly full reversal. These differences suggest that cariprazine has greater in vivo D_2 antagonist activity than aripiprazole.

Cariprazine demonstrated high affinity for dopamine D_3 and D_2 receptors in binding experiments, with about 10-fold selectivity for the D_3 versus D_2 subtype. In this regard, cariprazine characteristically differs from aripiprazole, the prototype D_2 partial agonist antipsychotic, and other clinically used typical and atypical antipsychotics with D_2 or D_2/5-HT_{2A} antagonism. Cariprazine displayed lower 5-HT_{2A}, 5-HT_{2C}, and H_1 receptor affinity relative to marketed antipsychotics. In functional assays, it possessed both antagonist and partial agonist properties at dopamine D_3 and D_2 receptors depending on the assay/signaling system and showed high D_3 antagonist potency. In in vivo neurochemical experiments, cariprazine, similar to

aripiprazole, displayed D_2-related partial agonist properties in conditions with low dopaminergic tone and behaved as an antagonist in conditions with high dopaminergic tone, demonstrating higher D_2 antagonist efficacy than aripiprazole. Potent antagonist-partial agonist activity at D_2 and D_3 dopamine receptors, with high affinity for D_3 subtypes, are properties that may render cariprazine as a potential antipsychotic candidate with a distinct profile. Positive results have been reported in Phase II bipolar mania and schizophrenia trials; clinical development of cariprazine is on-going.

DISCLOSURE/CONFLICT OF INTEREST:

The authors are employees of Gedeon Richter Plc. and Forest Laboratories Inc. and declare no conflict of interest.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Chemical structure of cariprazine (RGH-188) HCl

trans-N-[4-[2-[4-(2,3-dichlorophenyl)piperazin-1-yl]ethyl]cyclohexyl]-N',N'-dimethylurea hydrochloride

Fig. 2. Cariprazine does not stimulate [^{35}S]GTPγS binding in membrane preparation from rat striatal tissue, HEK-293 cell expressing human D_{2L} receptors, and CHO cells expressing human D_3 receptors but antagonizes dopamine-(10 μM)-stimulated [^{35}S]GTPγS binding in these preparations.

Fig. 3. Partial stimulation by cariprazine of the IP formation (A) and inhibition of the (±)quinpirole-(1 μM)-induced IP formation (B) in murine A9 cells expressing human D_{2L} receptors co-transfected with Gqo5 protein. Cariprazine partially inhibits the forskolin induced cAMP accumulation (C) and partially reverses the 7-OH-DPAT-(100 nM)-induced inhibition of forskolin (10 μM)-induced cAMP accumulation (D) in CHO cells expressing human D_3 receptors.

Fig. 4. Cariprazine and aripiprazole display low efficacy partial agonist activity at hippocampal 5-HT_{1A} receptors as determined by [^{35}S]GTPγS binding[1] (A). Antagonism by cariprazine of DOI-induced IP formation in CHO cells expressing human serotonin 5-HT_{2A} receptors[2] (B) and serotonin-induced contraction in rat isolated stomach fundus strips (C). Cariprazine and

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aripiprazole are antagonists with moderate potency at histamine H_1 receptors[4] (histamine-induced smooth muscle contraction in guinea pig trachea) (D).

[1] Maximal stimulation (Emax) is given relative to maximal stimulation by 8-OH-DPAT.**: 8-OH-DPAT produced maximal stimulation of 80.4±21.2 % above basal. Data are from 3-4 independent experiments.

[2] Inhibition is given in percent of DOI-(30 nM)-induced IP formation (n=2-3).

[3] Inhibition is given in percent of maximal contraction induced by 100 nM serotonin. (n=3-6).

[4] Histamine produced half maximal inhibition (EC_{50}) at 1.45 μM (n=2). Contraction induced by 3 μM histamine was taken as 100 % in each tissue preparation. (n=3-6).

Fig 5. Cariprazine enhances the turnover of dopamine and slightly reduces serotonin turnover in mouse striatum, limbic brain and frontal cortex.

Cariprazine was given orally 1 hour prior to decapitation. The absolute values of dopamine turnover indices (i.e. [(DOPAC+HVA)/DA] were 0.203±0.008 for striatum and 0.152±0.006 for olfactory tubercles, respectively. The dopamine content of frontal cortex showed high scattering (probably due to small but variable "contamination" with striatal tissue) therefore the alterations of cortical DOPAC are shown (control levels: 364±45 pmol/g). The serotonin turnover indices were 0.581±0.020 for striatum, 0.342±0.011 for olfactory tubercles and 0.433±0.035 for frontal cortex.

Differs from vehicle-treated control *: $p<0.05$; (n=5).

Fig. 6. In contrast with olanzapine, risperidone and haloperidol, cariprazine and aripiprazole elicit less enhancement of dopamine turnover ([DOPAC+HVA)/DA] in the mouse striatum and in the "limbic" part. However, both cariprazine and aripiprazole caused higher rate of dopamine turnover increase in the olfactory tubercle than in the striatum..

All drugs were given orally. Cariprazine was given 2 hours prior to decapitation, while other antipsychotics were administered 1 hour prior to killing. Data were taken from seven experiments where the absolute values of turnover indices varied between 0.177±0.007 and 0.221±0.012 for striatum and 0.157±0.006 and 0.190±0.013 for olfactory

tubercles

Differs from vehicle-treated control (which was taken 100%) *: $p<0.05$; (n=4-5).

Fig. 7. In mouse striatum cariprazine and aripiprazole partially reduce GBL-induced DOPA accumulation (Panel A) but they antagonize apomorphine (APM)-evoked synthesis reduction in this model (Panel B).

All animals received NSD-1015 (100 mg/kg, i.p.) 30 min. before sacrifice. GBL (750 mg/kg, i.p. 40 min. before killing) was administered to all animals except controls (C) which received NSD only. Cariprazine and aripiprazole HCl were given orally 1 h before decapitation. Apomorphine HCl (APM) was given in dose of 0.3 mg/kg, s.c. 45 min. prior to sacrifice. (n=5)

Panel A: differs from Control (C=NSD-only,Δ) $^{\#}$: $p<0.001$; differs from NSD+GBL-treatment (◆) *: $p<0.05$; N=5.

Panel B: differs from Control (C=NSD-only, Δ) $^{\#}$: $p<0.001$; differs from NSD+GBL-treatment (◆) ^{x}: $p<0.001$; *: differs from apomorphine+GBL+NSD-treatment (⊡) *: $p<0.05$. (n=5.)

Fig. 8. Cariprazine "restores" the enhanced DOPA accumulation to the control level in the striatum of reserpine-treated mice.

Data were taken from separate experiments for individual drugs. In these experiments reserpine treatment induced 90-130 % enhancement of DOPA accumulation relative to the appropriate controls (i.e., "NSD-only" groups). Effects of drugs were expressed in the percent of reserpine-induced accumulation above "NSD-only" group (i.e., in percent of incremental accumulation caused by reserpine). All drugs, except apomorphine, were given orally 1 h before decapitation. Apomorphine HCl was given subcutaneously 1 hour before killing. Reserpine (1 mg/kg, s.c. 18h before killing) was administered to animals except "NSD-controls". NSD-1015 (100 mg/kg, i.p.) was given to all animals 30 min. before killing. Differs from NSD+reserpine-treated group *: $p<0.05$; (n=5.)

TABLES

TABLE 1.

Summary of binding assay conditions for rat receptors

Receptor	Tissue (µg Protein/Assay)	Incubation Buffer	Incubation Time; Temperature; pH	Radioligand	Nonspecific	Reference
		mM		nM	µM	
rD_3	Sf9 (8)	50 Tris-HCl; 10 $MgCl_2$, 1 EDTA ; 120 NaCl	60 min; 27° C; pH:7.4	[^{3}H]Spiperone (0.85)	Haloperidol (10)	Sokoloff et al. (1990)
rD_2	Striatum (250)	50 Tris-HCl; 1 $MgCl_2$, 5 KCl; 2 $CaCl_2$, 120 NaCl; 0.1 % ascorbic acid	15 min; 37° C; pH 7.4	[^{3}H]Spiperone (0.5)	(+)Butaclamol (10)	Creese et al. (1984)
r5-HT_{1A}	Hippocampus (450)	50 Tris-HCl; 4 $CaCl_2$; 0.01 pargyline, 0.1 % ascorbic acid,;	15 min; 37° C; pH 7.7	[^{3}H]8-OH-DPAT (2.0)	Serotonin (10)	Hall et al. (1985)
r5-HT_{2A}	Cerebral cortex (170)	50 Tris-HCl	15 min; 37° C; pH 7.4	[^{3}H]Ketanserin (1.1)	Methysergide (1)	Gozlan et al. (1986)

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TABLE 2

Affinities for cariprazine and aripiprazole at various receptors, channels, transporters and enzymes.

Cariprazine and aripiprazole demonstrated negligible (percent displacement less than 20 % at 1 μM test concentration) affinities for adenosine A_1, A_{2A}, and A_3; adrenergic α_{2B}, α_{2C}; cannabinoid CB_1 and CB_2; cholecystokinin CCK_1 and CCK_2; corticotropin (CRF1); dopamine D_1, $D_{4.2}$, D_5; estrogen ERα and ERß; GABA (A and B), galanin (GAL1 and GAL2) glucocorticoid; glutamate (AMPA, kainate, NMDA); histamine (H_2, H_3 and H_4) muscarinic (M_1, M_2, M_3, M_4 and M_5); nicotinic; orphanin ORL_1; opiate δ, κ and μ; potassium channel HERG; progesterone; serotonin 5-HT_3, 5-HT_4, 5-HT_{5A}, 5-HT_6; sigma σ_2 receptors; Ca^{2+}-L-type (benzothiazepine and phenylalkylamine); Ca^{2+}-N-type, sodium (site 1 and site-2) channels; norepinephrine, adenosine, choline transporters. Cariprazine did not inhibit (inhibition < 10 % at 10 μM test concentration) Ca^{2+}-ATPase; Na^+/K^+-ATPase; acetylcholinesterase; HMG-CoA reductase; acyl-CoA-cholesterol acyltransferase.

Receptor	Species	Source	Radioligand (nM)	pKi	
				Cariprazine	Aripiprazole
D_3	Human	CHO cells	[^{3}H]Spiperone (0.7)	10.07	9.03
	Rat	Sf9	[^{3}H]Spiperone (0.85)	9.15	8.47
D_{2S}	Human	CHO cells	[^{3}H]Spiperone (0.16)	9.16	9.72
D_{2L}	Human	CHO cells	[^{3}H]Spiperone (0.16)	9.31	9.68
D_2	Rat	Striatum	[^{3}H]Spiperone (0.7)	8.03	8.20
5-HT_{1A}	Human	CHO cells	[^{3}H]8-OH-DPAT (1.5)	8.59	8.97
5-HT_{1A}	Rat	Hippocampus	[^{3}H]8-OH-DPAT (2.0)	8.34	8.20
5-HT_{2A}	Human	CHO-K1 cells	[^{3}H]Ketanserine (0.5)	7.73	8.75
5-HT_{2A}	Rat	Frontal cortex	[^{3}H]Ketanserine (1.1)	7.26	7.64
5-HT_{2B}	Human	CHO-K1 cells	[^{3}H]LSD (1.2)	9.24	9.60
5-HT_{2C}	Human	CHO-K1 cells	[^{3}H]Mesulergine (1.0)	6.87	7.81
5-HT_6	Human	HeLa cells	[^{3}H]LSD (1.5)	<6.0	7.23
5-HT_7	Human	CHO cells	[^{3}H]LSD (1.5)	6.95	n.d.
H_1	Human	CHO-1 cells	[^{3}H]Pyrilamine (1.2)	7.63	9.07
α_{1A}-AR	Rat	Submax. gland	[^{3}H]Prazosin (0.25)	6.88	7.31
α_{1B}-AR	Rat	Liver	[^{3}H]Prazosin (0.25)	<6.0*	n.d.
α_{1D}-AR	Human	HEK-293	[^{3}H]Prazosin (0.6)	6.68	n.d.
α_{2A}-AR	Human	Sf9	[^{3}H]MK-912 (1.0)	<6.0*	7.55
ß-AR	Human	Brain	[^{3}H]DHA (0.25)	<6.0*	7.44
σ_1	Human	Jurkat cells	[^{3}H]Haloperidol (8.0)	7.74	7.07
DAT	Human	CHO cells	[^{125}I]RTI-55 (0.15)	<6.0	6.75
SERT	Human	HEK-293 cells	[^{125}I]RTI-55 (0.15)	<6.0	7.38

*: Cariprazine resulted in 65%, 50% and 64 % displacement at α_{1B}-AR α_{2A}-AR and ß-AR receptors, respectively in concentration of 1 μM.

MK-912, ((2S,12bS)-1',3'-dimethylspiro[1,3,4,5',6,6',7,12b-octahydro-2H-benzo[b]furo[2,3-a]quinolizine-2,4'-pyrimidin]-2'-one); DHA (dihydroalprenolol); RTI-55 ((-)-3beta-(4-Iodophenyl)tropane-2beta-carboxylic acid methyl ester); LSD (lysergic acid diethylamide); DAT (dopamine transporter); SERT (serotonin transporter).

TABLE 3

Agonist and antagonist efficacies and potencies of selected agonist and antagonists in [^{35}S]GTPγS binding assay using membranes from rat striatum, HEK293 cell expressing human D_2 and CHO cell expressing human D_3 receptors.

	Rat Striatum				HEK-D_2 Cells				CHO-D_3 Cells			
	Agonism		Antagonism		Agonism		Antagonism		Agonism		Antagonism	
	E_{max}	pEC_{50}	AA_{max}	pKb	E_{max}	pEC_{50}	AA_{max}	pKb	E_{max}	pEC_{50}	AA_{max}	pKb
Dopamine	154±16	5.49±0.17		n.t.	117±9	6.01±0.07		n.t.	101±1	7.64±0.09		n.t.
Pramipexole	56±5	6.05±0.13		n.t.	119±13	6.60±0.17		n.t.	121±17	8.32±0.09		n.t.
Apomorphine	177±5	6.13±0.07		n.t.	107±13	8.10±0.10		n.t.	125±8	8.05±0.07		n.t.
7-OH-DPAT	48±14	6.87±0.16		n.t.	79±7	7.16±0.09		n.t.	66±2	8.73±0.12		n.t.
Quinpirole	62±9	6.09±0.14		n.t.	96±14	6.19±0.08		n.t.	118±6	8.02±0.13		n.t.
PHNO	138±9	6.96±0.08		n.t.	122±12	7.94±0.34		n.t.	75±12	8.91±0.05		n.t.
Cariprazine	*N.S.*		107±3	8.95±0.17	*N.S.*		100±1	9.12±0.15	*N.S.*		99±2	9.50±0.07
Aripiprazole	*N.S.*		105±3	8.79±0.27	*N.S.*		101±2	9.33±0.10	*N.S.*		113±4	8.71±0.05
Haloperidol	n.t.		88±1	8.44±0.11	n.t.		105±3	8.81±0.07	n.t.		124±2	8.51±0.08
L741626	n.t.		83±1	8.16±0.11	n.t.		119±3	8.13±0.08	n.t.		122±11	7.39±0.14
SB-277011	n.t.		116±7	6.13±0.14	n.t.		54±14	6.07±0.04	n.t.		82±5	8.07±0.21
Olanzapine	n.t.		121±8	7.72±0.15	n.t.		105±5	7.78±0.16	n.t.		130±28	7.30±0.06
Risperidone	n.t.		121±2	8.22±0.15	n.t.		112±2	8.05±0.08	n.t.		136±23	7.57±0.02

E_{max}: maximal stimulation relative to 10 μM dopamine; AA_{max}: percent maximal antagonism achieved. Given are the means ± SEM from 3-6 independent experiments in duplicates or triplicates. N.S.: no stimulation; n.t.: not tested

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TABLE 4

Agonist and antagonist efficacies and potencies of selected agonists and antagonists in the IP accumulation using mouse A9 cells expressing human D_2 receptors and CHO cells expressing human D_3 receptors.

	Mouse A9 Cells*				CHO-D_3 Cells**			
	Agonism		Antagonism		Agonism		Antagonism	
	E_{max}	pEC_{50}	AA_{max}	pKb	E_{max}	pEC_{50}	AA_{max}	pKb
Dopamine	159±16	6.95±0.08		n.t.	102±1	7.73±0.01		n.t.
Pramipexole	130±17	7.30±0.11		n.t.	106±3	8.65±0.07		n.t.
7-OH-DPAT	145±9	7.69±0.17		n.t.	105±4	8.50±0.09		n.t.
Quinpirole	100±3	7.01±0.07		n.t.	101±2	8.80±0.02		n.t.
Cariprazine	30±3	8.50±0.32	81±3	9.22±0.18	71±4	8.58±0.30	27±2	9.57±0.28
Aripiprazole	34±7	7.66±0.35	84±4	8.52±0.12	69±2	7.53±0.06	24±3	7.80±0.01
Haloperidol		n.t.	108±4	10.47±0.28		n.t.	110±4	8.15±0.03
SB-277011		n.t.		n.t.		n.t.	52±6	7.56±0.08

*: In these experiments (±)-quinpirole was used and E_{max} represents maximal stimulation relative to 1 µM (±)-quinpirole.
**: In these experiments (+)quinpirole was used. E_{max} represents the maximal inhibition of forskolin-10 µM)-induced cAMP accumulation.
AA_{max}: percent maximal antagonism achieved against (±)quinpirole in mouse A9 cells and maximal reversal of 7-OH-DPAT-(100 nM)-induced inhibition of forskolin-10 µM- induced cAMP accumulation in CHO-D_3 cells, respectively.
n.t.: not tested.
Shown are the means ±SEM from 3-6 independent experiments in duplicates or triplicates.

Fig 1

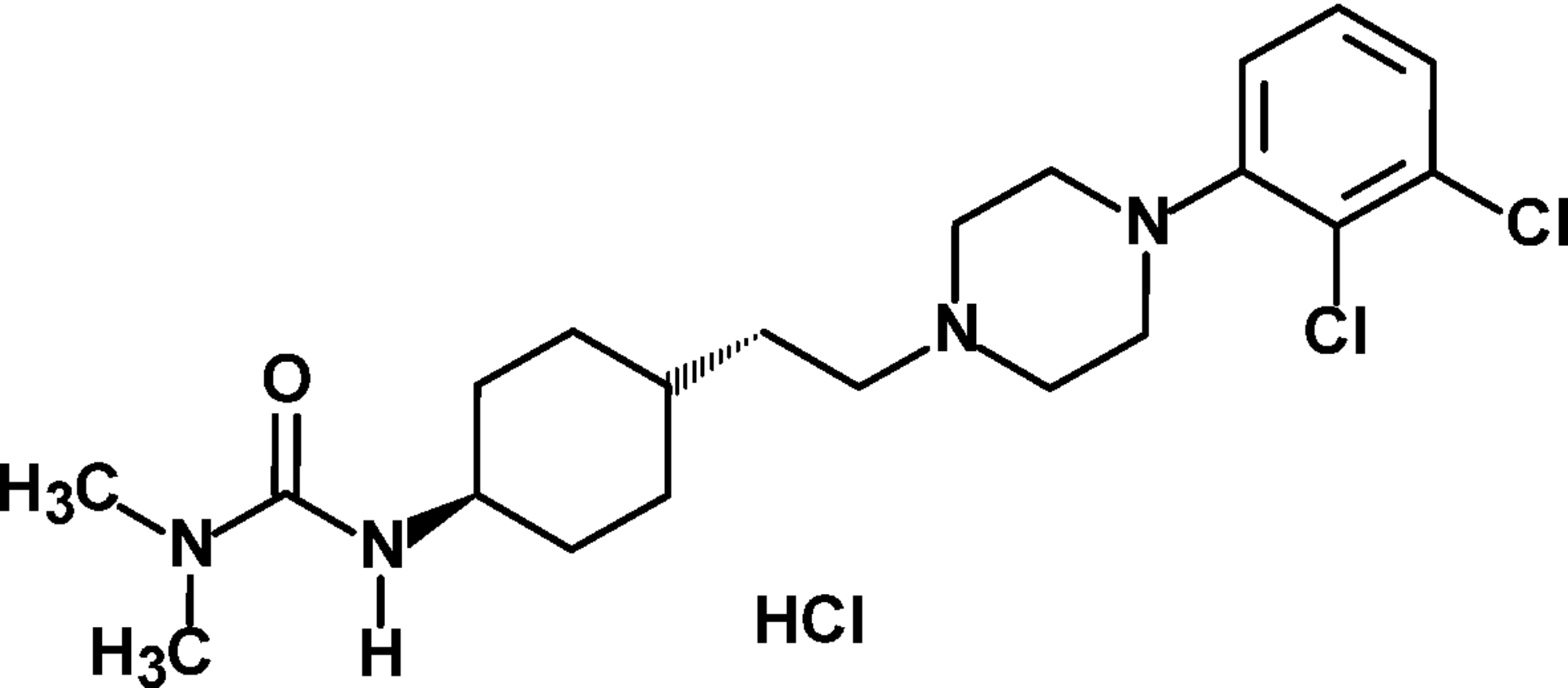


Fig 2.

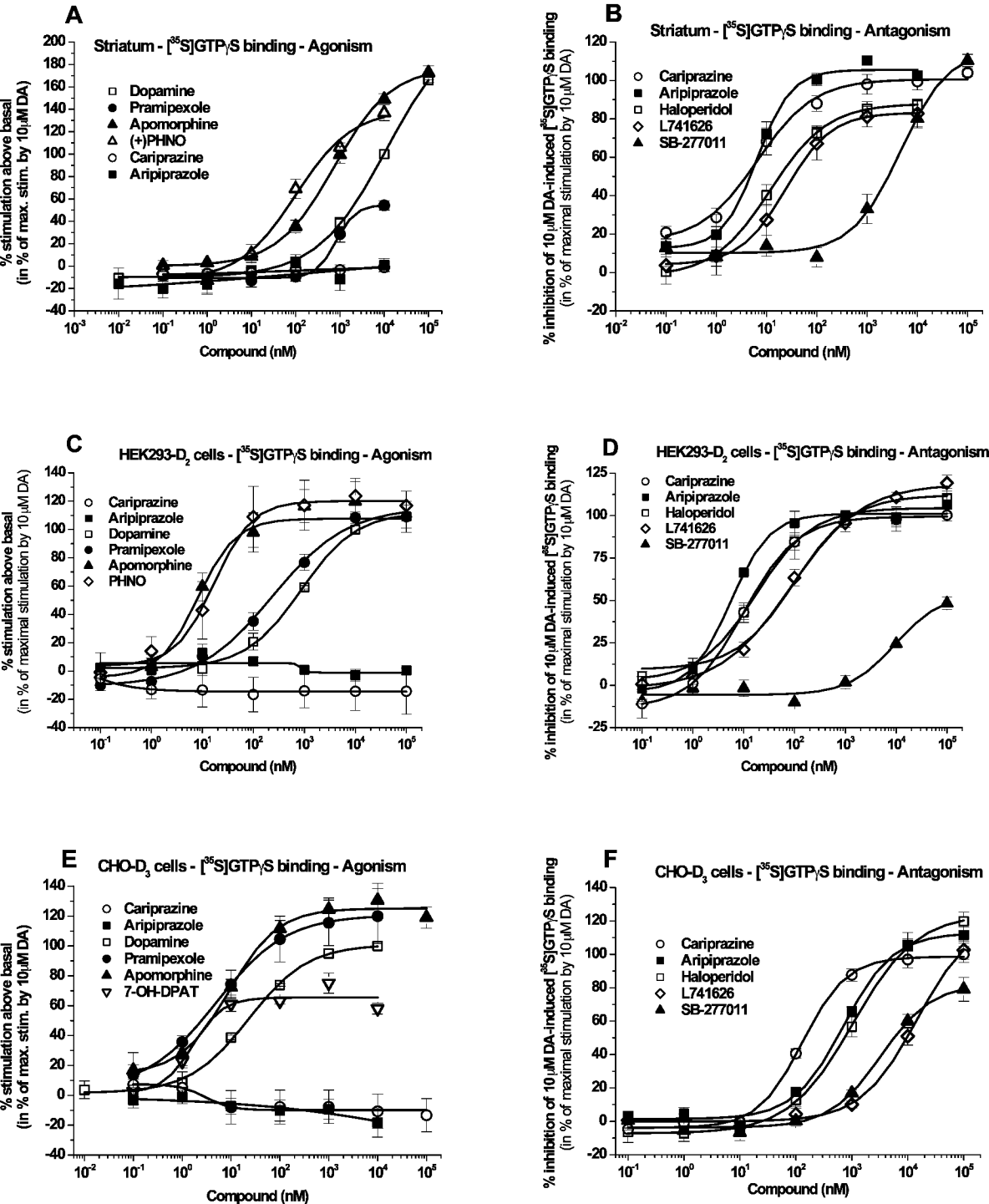

A
Striatum - [35S]GTPγS binding - Agonism
% stimulation above basal (in % of max. stim. by 10μM DA)
Compound (nM)
Dopamine
Pramipexole
Apomorphine
(+)PHNO
Cariprazine
Aripiprazole
B
Striatum - [35S]GTPγS binding - Antagonism
% inhibition of 10μM DA-induced [35S]GTPγS binding (in % of maximal stimulation by 10μM DA)
Compound (nM)
Cariprazine
Aripiprazole
Haloperidol
L741626
SB-277011
C
HEK293-D2 cells - [35S]GTPγS binding - Agonism
% stimulation above basal (in % of maximal stimulation by 10μM DA)
Compound (nM)
Cariprazine
Aripiprazole
Dopamine
Pramipexole
Apomorphine
PHNO
D
HEK293-D2 cells - [35S]GTPγS binding - Antagonism
% inhibition of 10μM DA-induced [35S]GTPγS binding (in % of maximal stimulation by 10μM DA)
Compound (nM)
Cariprazine
Aripiprazole
Haloperidol
L741626
SB-277011
E
CHO-D3 cells - [35S]GTPγS binding - Agonism
% stimulation above basal (in % of max. stim. by 10μM DA)
Compound (nM)
Cariprazine
Aripiprazole
Dopamine
Pramipexole
Apomorphine
7-OH-DPAT
F
CHO-D3 cells - [35S]GTPγS binding - Antagonism
% inhibition of 10μM DA-induced [35S]GTPγS binding (in % of maximal stimulation by 10μM DA)
Compound (nM)
Cariprazine
Aripiprazole
Haloperidol
L741626
SB-277011

Fig 3.

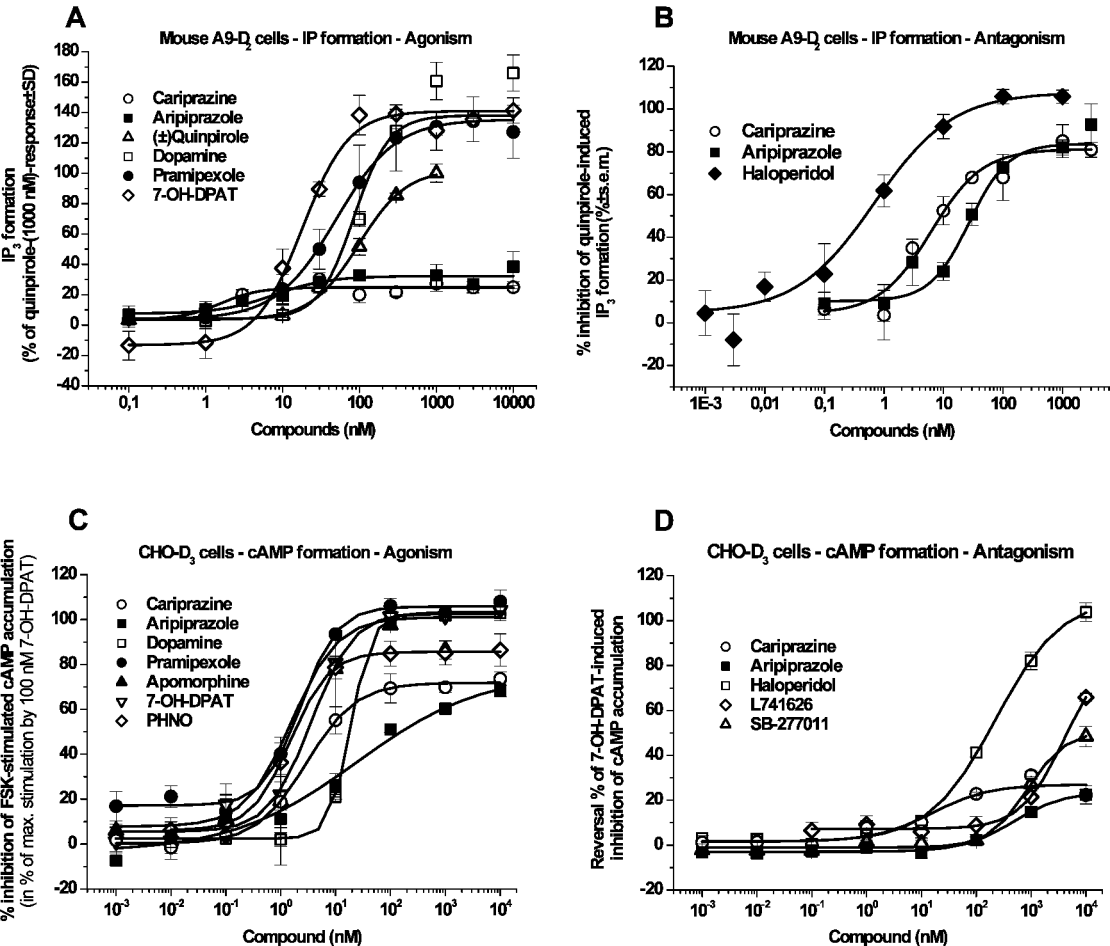

A
Mouse A9-D2 cells - IP formation - Agonism
IP3 formation (% of quinpirole-(1000 nM)-response±SD)
Cariprazine
Aripiprazole
(±)Quinpirole
Dopamine
Pramipexole
7-OH-DPAT
Compounds (nM)
B
Mouse A9-D2 cells - IP formation - Antagonism
% inhibition of quinpirole-induced IP3 formation(%±s.e.m.)
Cariprazine
Aripiprazole
Haloperidol
Compounds (nM)
C
CHO-D3 cells - cAMP formation - Agonism
% inhibition of FSK-stimulated cAMP accumulation (in % of max. stimulation by 100 nM 7-OH-DPAT)
Cariprazine
Aripiprazole
Dopamine
Pramipexole
Apomorphine
7-OH-DPAT
PHNO
Compound (nM)
D
CHO-D3 cells - cAMP formation - Antagonism
Reversal % of 7-OH-DPAT-induced inhibition of cAMP accumulation
Cariprazine
Aripiprazole
Haloperidol
L741626
SB-277011
Compound (nM)

Fig 4.

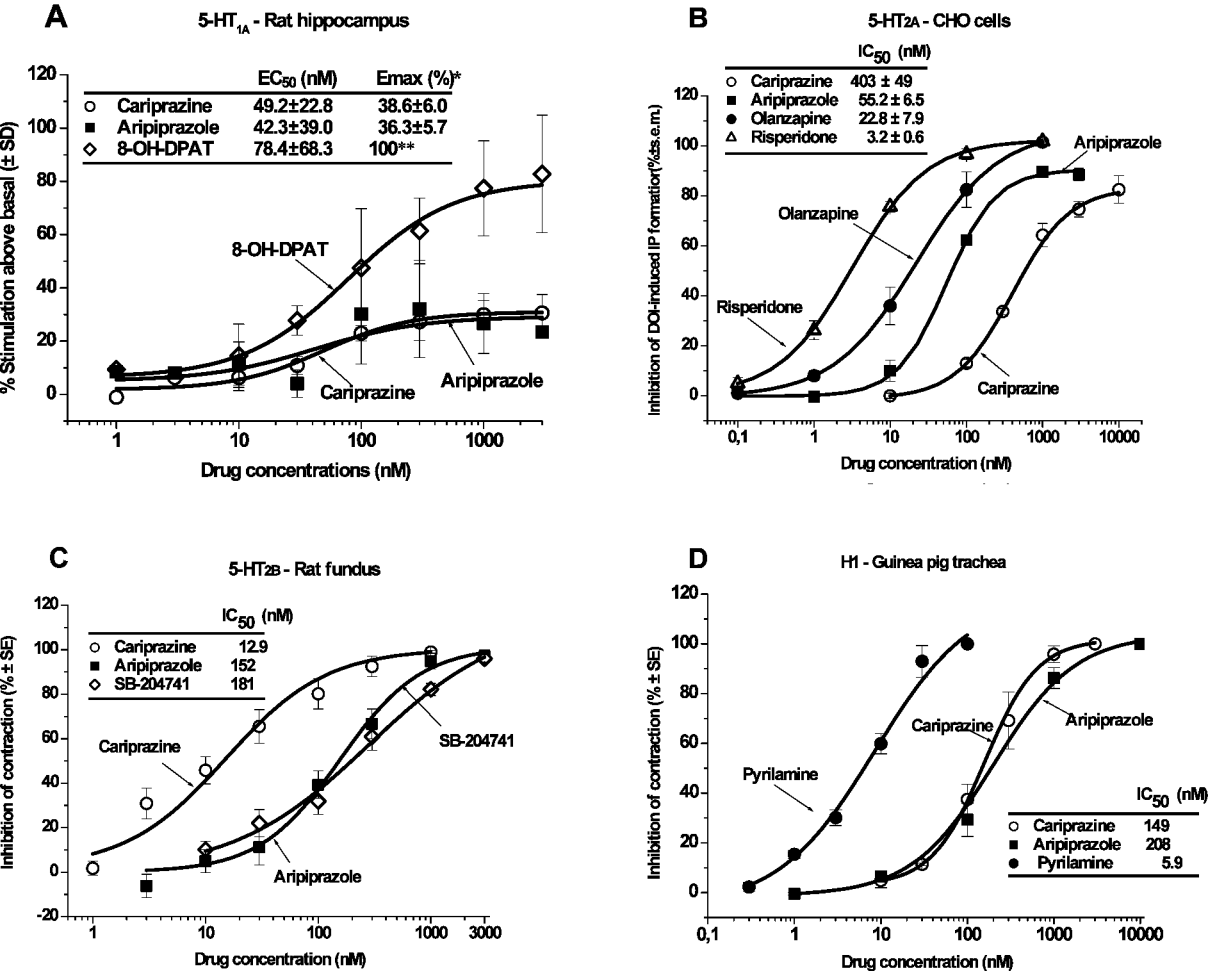

A
5-HT1A - Rat hippocampus
EC50 (nM)
Emax (%)*
Cariprazine 49.2±22.8 38.6±6.0
Aripiprazole 42.3±39.0 36.3±5.7
8-OH-DPAT 78.4±68.3 100**
% Stimulation above basal (± SD)
8-OH-DPAT
Cariprazine
Aripiprazole
Drug concentrations (nM)
B
5-HT2A - CHO cells
IC50 (nM)
Cariprazine 403 ± 49
Aripiprazole 55.2 ± 6.5
Olanzapine 22.8 ± 7.9
Risperidone 3.2 ± 0.6
Inhibition of DOI-induced IP formation(%ss.e.m)
Aripiprazole
Olanzapine
Risperidone
Cariprazine
Drug concentration (nM)
C
5-HT2B - Rat fundus
IC50 (nM)
Cariprazine 12.9
Aripiprazole 152
SB-204741 181
Inhibition of contraction (% ± SE)
Cariprazine
SB-204741
Aripiprazole
Drug concentration (nM)
D
H1 - Guinea pig trachea
Inhibition of contraction (% ± SE)
Pyrilamine
Cariprazine
Aripiprazole
IC50 (nM)
Cariprazine 149
Aripiprazole 208
Pyrilamine 5.9
Drug concentration (nM)

Fig 5.

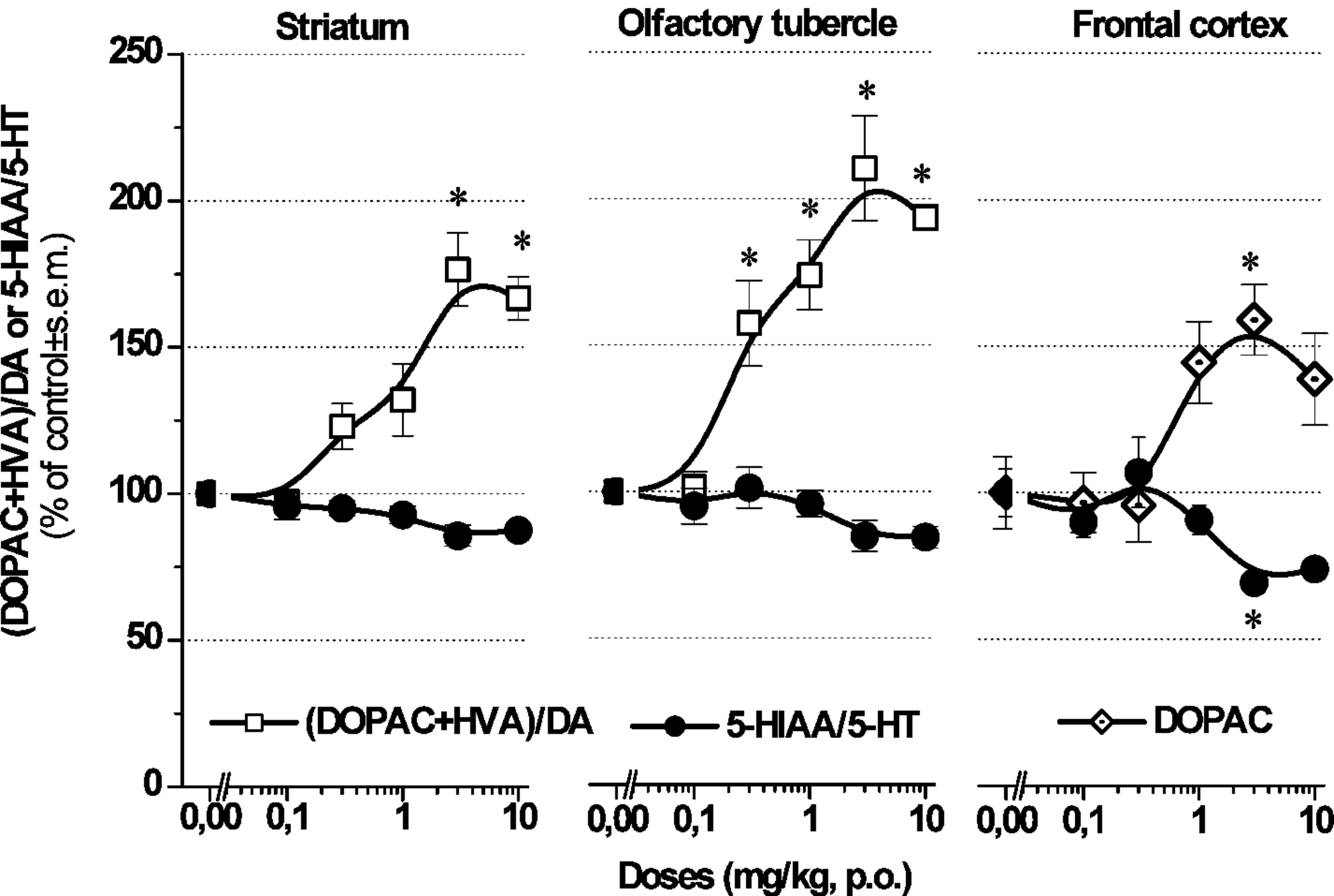

Striatum
Olfactory tubercle
Frontal cortex
(DOPAC+HVA)/DA or 5-HAA/5-HT
(% of control±s.e.m.)
250
200
150
100
50
0
0,00
0,1
1
10
(DOPAC+HVA)/DA
5-HIAA/5-HT
DOPAC
Doses (mg/kg, p.o.)

Fig 6.

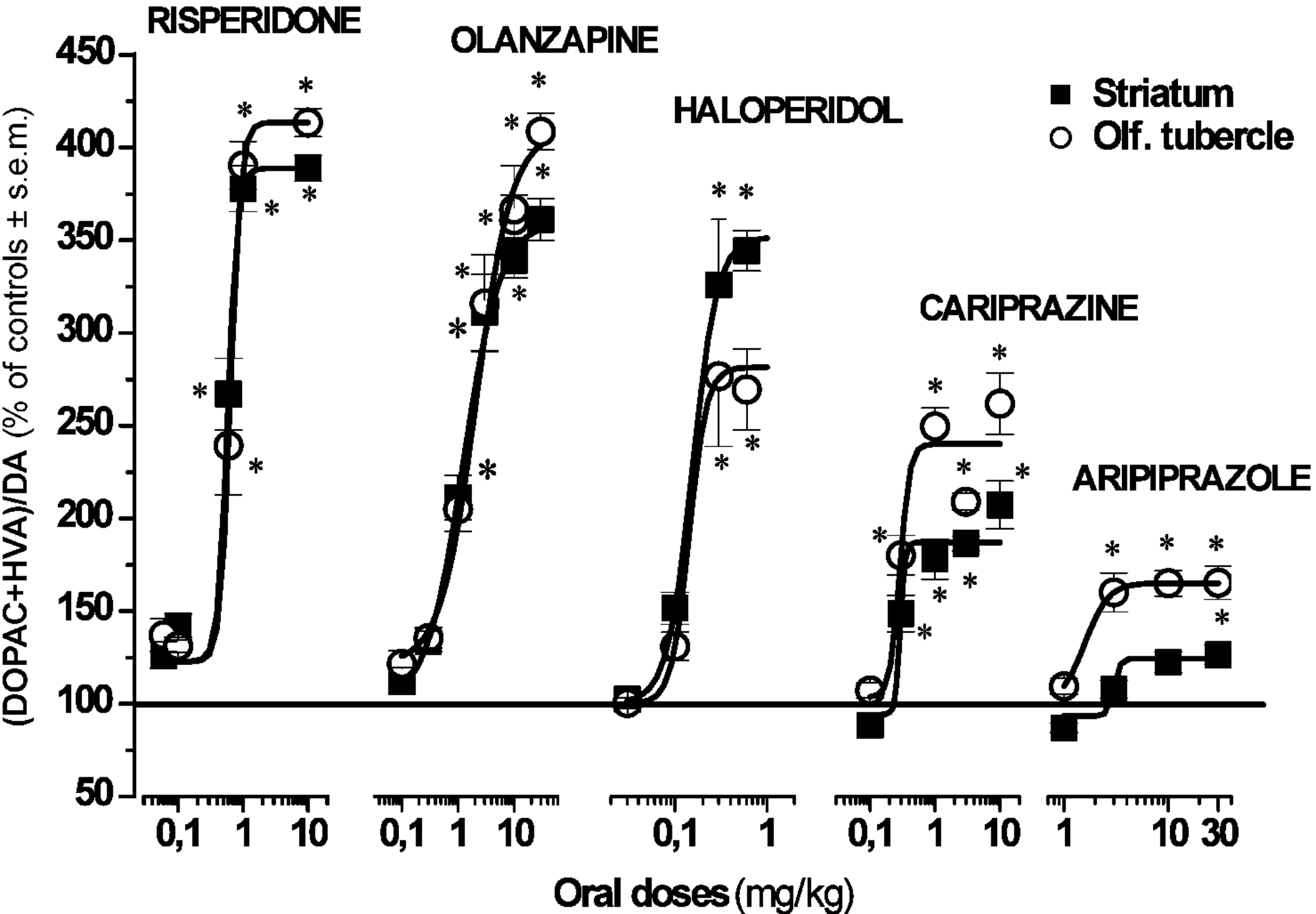

RISPERIDONE
OLANZAPINE
HALOPERIDOL
CARIPRAZINE
ARIPIPRAZOLE
Striatum
Olf. tubercle
(DOPAC+HVA)/DA (% of controls ± s.e.m.)
450
400
350
300
250
200
150
100
50
0,1
1
10
0,1
1
10
0,1
1
0,1
1
10
1
10
30
Oral doses (mg/kg)

Fig 7.

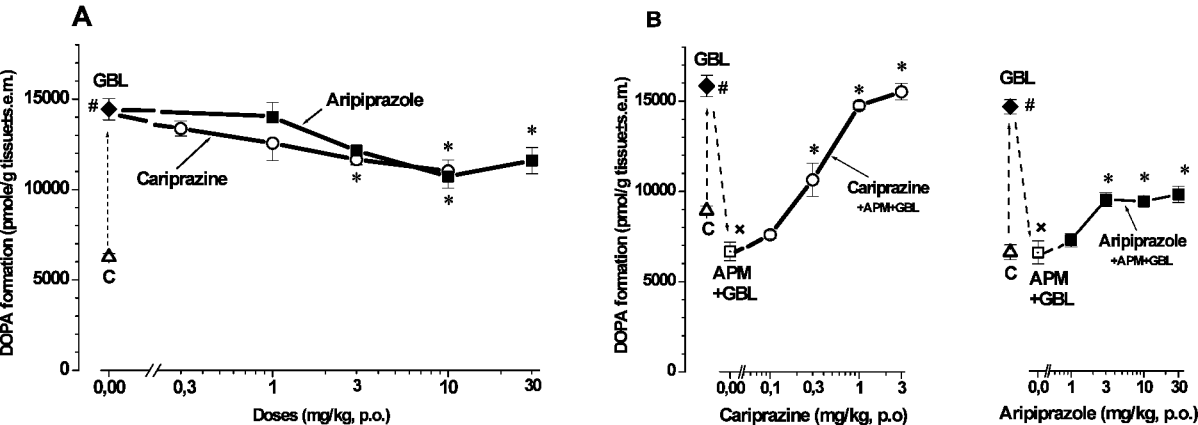
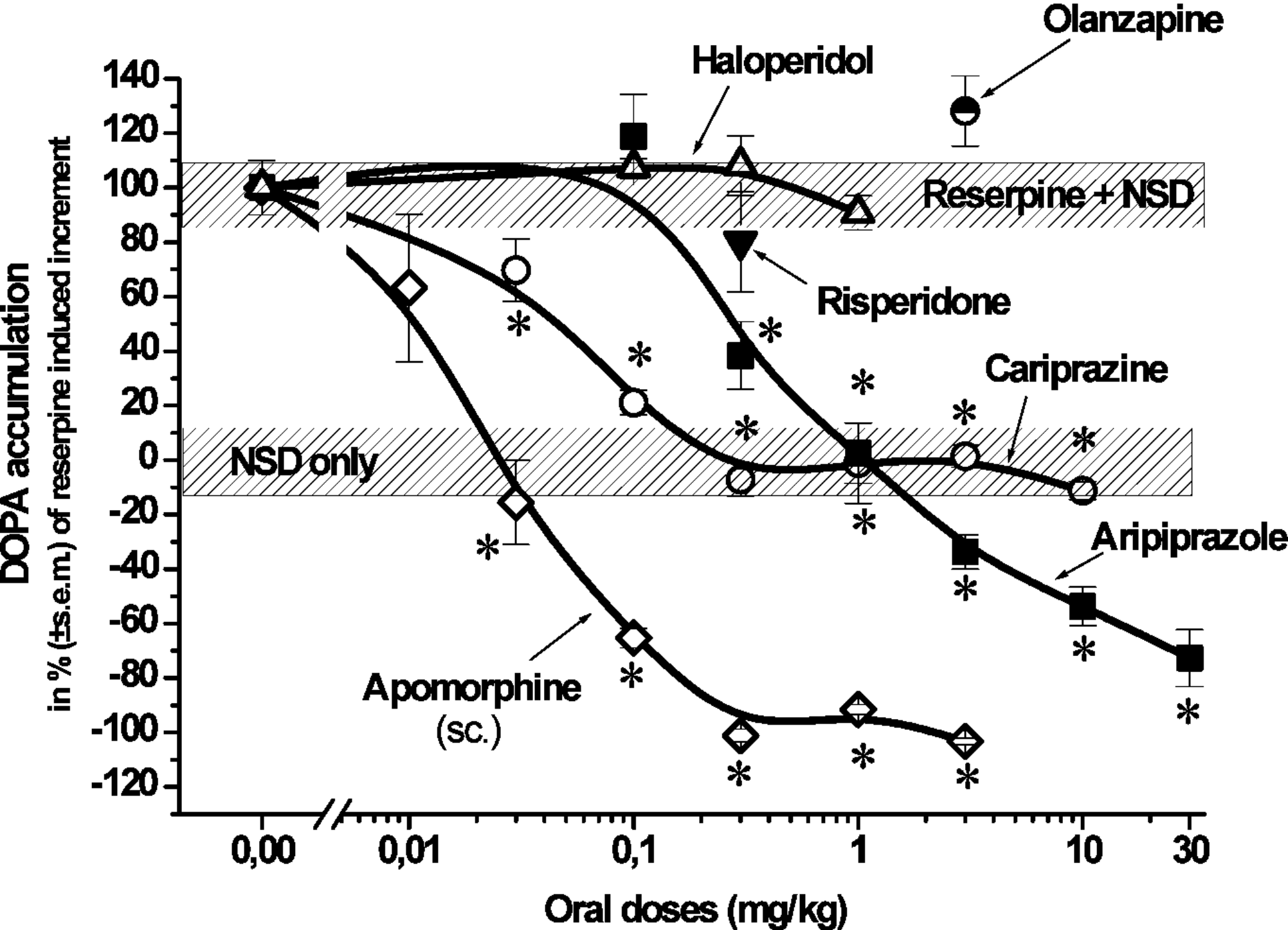

A
GBL
#
Aripiprazole
Cariprazine
C
DOPA formation (pmole/g tissuets.e.m)
15000
10000
5000
0
0,00
0,3
1
3
10
30
Doses (mg/kg, p.o.)
B
GBL
#
C
APM
+GBL
Cariprazine
+APM+GBL
DOPA formation (pmol/g tissuets.e.m)
0,00
0,1
0,3
1
3
Cariprazine (mg/kg, p.o)
GBL
#
C
APM
+GBL
Aripiprazole
+APM+GBL
0,0
1
3
10
30
Aripiprazole (mg/kg, p.o.)

Fig 8.


Olanzapine
Haloperidol
Reserpine + NSD
Risperidone
Cariprazine
NSD only
Aripiprazole
Apomorphine
(sc.)
DOPA accumulation
in % (±s.e.m.) of reserpine induced increment
140
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
0,00
0,01
0,1
1
10
30
Oral doses (mg/kg)