SUPPLEMENTARY INFORMATION

Preclinical Pharmacodynamic and Pharmacokinetic Characterization of the Major Metabolites of Cariprazine

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Supplemental Methods

Data Analysis of In Vitro Receptor Binding Assays

The ligand displacement by the compounds was determined using a minimum of six concentrations in duplicates or triplicates, and experiments were repeated at least two times.

The specific radioligand binding is defined as the difference between total binding and the non-specific binding determined in the presence of an excess of unlabeled ligand. Results are expressed as a percent inhibition of specific binding obtained in the presence of cariprazine (RGH-188), didesmethyl-cariprazine (DDCAR), desmethyl-cariprazine (DCAR), or reference drugs.

IC_{50} values (i.e. concentration of compound giving 50% inhibition of specific binding) was calculated from concentration-displacement curves by sigmoidal fitting using GraphPad Prism Software 4.0.

K_i values (i.e. inhibition constants) were calculated using the Cheng-Prusoff equation $K_i = IC_{50}/[1+(L/K_D)]$, where [L] is the radioligand concentration and K_D the affinity of the labelled ligand for receptor.[1] K_D was determined from the Scatchard plot.

In Vitro Functional Assays: [^{35}S]GTPγS Binding, cAMP signaling, and Ca^{2+}-release

The conditions for determining functional responses at native rat striatal dopamine D_2, hippocampal 5-HT_{1A} and recombinant dopamine D_2 and D_3, and serotonin 5-HT_{1A} and 5-HT_{2B} receptors were adapted from the literature[2-5] and are summarized in Supplemental Table 3.

[^{35}S]GTPγS Binding Assay – Rat Striatum or Hippocampus

Male rats were decapitated, corpus striatum and hippocampus was dissected out and homogenized by a Dounce homogenizer immediately in ice-cold buffer (50 mM Tris, 5 mM $MgCl_2$, and 1 mM EDTA, pH 7.6). Both tissue homogenates were centrifuged at 40000 g, 4°C for 15 min. Membrane pellets were resuspended in the same buffer; hippocampal membranes were incubated for 10 min at 37°C in a shaking water bath to eliminate endogenous serotonin. Homogenates were centrifuged again under the same conditions. The final pellets were resuspended in ice-cold buffer containing 50 mM Tris, 100 mM NaCl, 7

mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT (pH 7.6) to yield a tissue concentration of 20 mg /mL and frozen at -70°C until use.

The assay was done in a buffer containing 50 mM Tris, 100 mM NaCl, 7 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT (pH 7.4). Each assay tubes contained 150 µL guanosine diphosphate (GDP; at a final concentration of 50 µM), 100 µL ligand and 125 µL of the membrane suspension (250 µg tissue/tube). The assay tubes were preincubated for 10 min at 30°C to ensure equilibrium. The non-specific binding was determined in the presence of GTPγS (10 µM); the basal binding was determined in the presence of buffer only. After addition of 50 pM [^{35}S]GTPγS in a volume of 25 µL, the membranes were incubated for an additional 60 min at 30°C. The reaction was terminated by rapid filtration through Packard GF/B UniFilter using a Packard harvester, and washed 4 times with 1 mL ice-cold buffer. After drying the filters at 40°C for 1 h, 40 µL of Microscint 20 (Packard) was added to the filters, and radioactivity of the filters was determined by a TopCount NXT (PerkinElmer).

[^{35}S]GTPγS Binding Assay - Recombinant Cells Expressing Human 5-HT_{1A}, D_2, and D_3 receptors

Cells were collected by a cell scraper in ice-cold PBS-EDTA and centrifuged at 2000 g, at 4°C for 15 min. The pellet was resuspended in a buffer containing 50 mM Tris, 5 mM $MgCl_2$, and 1 mM EDTA (pH 7.6) and homogenized with a Dounce homogenizer in a buffer containing. The membrane homogenate was washed 2 times with centrifugation at 40000 g, 4°C for 15 min. The final pellet was resuspended in a buffer containing 50 mM Tris, 100 mM NaCl, 7 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT (pH 7.6) to yield a concentration of 80 mg protein/mL. The membrane preparation was aliquoted and stored at -70°C until use. Protein concentration of the membrane preparations was determined by the bicinchoninic method.

The assay was done in 50 mM Tris, 100 mM NaCl, 7 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT (pH 7.4). Each assay tube contained 150 µL GDP (at a final concentration of 1 µM), 100 µL ligand and 125 µL of the membrane suspension (20 µg protein/tube). The assay tubes were preincubated at 30°C for 10 min to ensure equilibrium. Non-specific binding was determined in the presence of GTPγS (10 µM); basal binding was determined in the presence of buffer only. After the addition of 50 pM [^{35}S]GTPγS in a volume of 25 µL, the membranes were incubated at 30°C for an additional 60 min. The reaction was terminated by rapid filtration through Packard GF/B UniFilter using a Packard harvester and washed 4 times with 1 mL ice-cold buffer. After drying the filters at 40°C for 1 h, 40 µL of Microscint 20

(Packard) was added to the filters, and radioactivity of the filters was determined by a TopCount NXT (PerkinElmer).

Data Analysis for [^{35}S]GTPγS Binding Assays

Raw data were first converted to stimulation % above basal values. The stimulation % above basal values were further converted to % of maximal stimulation of [^{35}S]GTPγS binding by a single concentration of the full agonist.

For agonists, the EC_{50} values were calculated from concentration-response curves and were defined as the concentration of the agonist with half-maximal stimulation (expressed in % of maximal stimulation by the full agonist). Agonist efficacy (E_{max}) was also expressed relative to that of a full agonist. The pEC_{50} values were calculated as the mean ± standard error of the mean (SEM) of the pEC_{50} values of the individual experiments.

For antagonists, the IC_{50} values were calculated from concentration-response curves in the presence of a single concentration of the agonist. For each experiment, affinity (pK_b) values were calculated according to the Craig formula[6]; means and SEM values of these individual data are presented.

Results *w*ere calculated from concentration-response curves by sigmoidal fitting using the Origin 6.0 (MicroCal).

cAMP Signaling Measurements

In cells expressing recombinant human 5-HT_{1A} receptors, cAMP measurements were performed with a homogeneous time-resolved fluorescence (HTRF®) kit from Cisbio International (Codolet, France). The assay was done in Hank's Balanced Salt Solution (HBSS), supplemented with the phosphodiesterase inhibitor IBMX (Sigma-Aldrich) and FSK. All subsequent steps were carried out according to manufacturer's protocol. Briefly, cells were first washed with 100 µL of HBSS and then subjected to test compounds for 30 min at 37°C in a volume of 40 µL. Subsequently, a competitive immunoassay using cryptate-labeled anti-cAMP antibody and d2-labeled cAMP (dissolved in vendor-provided lysis buffer) was carried out. Following 60 min incubation at room temperature, the plate was read by using a PHERAstar FS microplate reader (BMG Labtech).

In cells expressing recombinant human D_2 receptors cAMP accumulation was performed with a homogeneous time-resolved fluorescence (HTRF®) kit from Cisbio International. The assay

was done in HBSS, supplemented with phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and forskolin (FSK). As above all steps were carried out according to the instructions provided with the HTRF® kit from Cisbio International.

In cells expressing recombinant human D_3 receptors cAMP accumulation was determined by using radiolabeled cAMP. On the day of the experiment, the cell culture medium was removed, and the cells were preincubated in 60 µL HBSS at 37°C for 10 min. Cells were then subjected to test compounds (agonists, antagonists, and 10 µM FSK) in 60 µL of HBSS supplemented with 100 µM IBMX at 37°C for 20 min. Following the addition of 20 µL of 1 M perchloric acid to terminate the reaction, plates were frozen for overnight at -20°C and thawed. 50 µL of ice-cold KOH (0.5 M) was added to neutralize the samples at 4°C for 30 min. Plates were then centrifuged at 700 g, 4°C for 10 min; 50 µL of the supernatant was incubated together with 0.15 pmol of [^{3}H]-cAMP per well in 50 µL distilled water and 25 µg/well cAMP-binding protein (cAMP-dependent protein kinase prepared from bovine adrenal cortices) in 200 µL of 50 mM Tris (pH 7.4) at 4°C for 130 min, then filtrated using GF/B filters. Radioactivity of the samples was determined by a TopCount NXT (PerkinElmer).

Data Analysis for cAMP Measurements

For HTRF experiments, raw data (measured at 665 and 620 nM) were first converted to $ratio_{665/620}$. This ratio between the acceptor fluorescence signal (A665 nm) and donor fluorescence signal (A620 nm) $X10^4$ represents the FRET between the conjugated cAMP and the anti-cAMP antibody and was calculated for each well of the assay plate. The ratios were further converted to % inhibition of the FSK-induced signal. The FSK-stimulated cAMP accumulation in the absence of agonist was defined as 100%. E_{max} values (% of maximal inhibition of FSK-stimulated cAMP) achieved for each drug were normalized to the response evoked by a maximally effective concentration of dopamine (DA; 1-10 µM) tested in the same experiment. The EC_{50} values (the concentration of agonist that produces 50% inhibition of FSK-stimulated cAMP accumulation) were calculated from concentration-response curves by sigmoidal fitting using the Origin 6.0 (MicroCal) software. The final EC_{50} values were calculated as mean ± SEM of at least 3 independent experiments performed in triplicates on different days. The pEC_{50} values were calculated as the mean ± SEM of the pEC_{50} values of the individual experiments.

For antagonists, the IC_{50} values were calculated from concentration-response curves in the presence of a single concentration of the agonist (100 nM dopamine).

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For the experiments using radiolabelled cAMP, the cellular cAMP content was expressed in pmol/well. Raw concentration-data were converted to % of maximal inhibition of the 10 µM FSK-induced stimulation of cAMP accumulation by a single dose (100 nM) of the full agonist 7-OH-DPAT (IC_{50} value was defined as the concentration of the agonist with half-maximal inhibition). Agonist efficacy (E_{max}, maximal agonist effect) was expressed as percent of maximal inhibition of FSK-induced cAMP accumulation by 100 nM 7-OH-DPAT.

In the antagonist tests, data were expressed in reversal % of the inhibitory effect of a single concentration of the agonist (100 nM 7-OH-DPAT) on the 10 µM FSK-induced cAMP accumulation. In this case, the IC_{50} value was determined as the concentration of the antagonist with half-maximal reversal of the 100 nM 7-OH-DPAT-induced inhibition of the FSK-stimulated cAMP accumulation.

Ca^{2+}-Release Assay

One day before the experiment the cells expressing human recombinant 5-HT_{2B} receptors were seeded into 96-well plates (30 000 cells/well). On the day of the experiment the growth medium was aspirated, and the plates were washed twice with 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, and 2 mM probenecid (pH 7.4) using a plate washer apparatus. Cells were loaded with Ca^{2+}-sensitive fluorescent dye, fluo-4/AM (2 µM) at 37°C for 45–60 min.

After dye loading, the plates were washed twice with assay buffer using the plate washer, then, depending on the experimental setup (agonist or antagonist test) assay buffer containing vehicle (0.3% DMSO) or test compounds were diluted in 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, and 2 mM probenecid (pH 7.4) and added to each well. The plates were incubated at 37°C for 10 min.

The plates then were transferred to a plate reader fluorimeter (FlexStation II) and the measurements began immediately. For the antagonists, compounds were tested for their inhibitory effects on the response evoked by 5-HT, administered at an EC_{80} concentration (5-HT_{EC80}), pre-determined on separate wells of the same plate. For the agonists, compounds administered *per se* were tested for their effects on baseline fluorescence, and

their effects were compared to those obtained with vehicle alone and a maximally effective 5-HT concentration (5-HT_{max}).

Data Analysis for Ca^{2+}-Release Assay

Primary data were expressed as ΔF/F (fluorescence change over baseline). ΔF/F values were converted to % response or % inhibition values by normalizing the data to the responses evoked by 5-HT_{max} and 5-HT_{EC80}, respectively.

Brain Dopamine Receptor Occupancy

Based on previous published literature,[7,8] brain occupancy of D_2 and D_3 receptors by cariprazine and DDCAR was et al. assessed by measuring inhibition of *in vivo* [^{3}H](+)-PHNO binding in rat striatum and cerebellum lobes 9 and 10 (L9, 10), respectively.

[^{3}H](+)-PHNO was obtained from two sources. [^{3}H](+)-PHNO (specific activity: 61.8 Ci/mmol) was custom synthesized by catalytic titration (Ubichem Research) from (+)-(4aR,10bR)-4-allyl-3,4,4a,5,6,10b-hexahydro-2H-naphth[1,2-b]-1,4-oxazin-9-ol hydrochloride, which was a gift from Dr. A. Wilson (PET Centre, Centre for Addiction and Mental Health, 250 College St., Toronto, Ontario, Canada, M5T 1R8). Later, [^{3}H](+)-PHNO (specific activity: 63–64 Ci/mmol) was purchased from Moravek Biochemical Inc. The ligand from the two sources gave identical in vivo results. The radioligand was diluted in saline immediately before use and kept on ice throughout the experiments.

Stock solutions of test compounds were dissolved in minimal amount (~10–20 µL) glacial acetic acid, made up to final volume with distilled water and further dilutions were made with distilled water. Dose-response curves consisting of 5–6 different doses were generated. Rats (5–6/group) were given vehicle or different doses of cariprazine or DDCAR orally (p.o.) in a volume of 0.5 mL/100 g body weight and then returned to their home cage.

Rats were given vehicle or different doses of cariprazine or DDCAR orally in a volume of 0.5 mL/100 g body weight and then returned to their home cage. Thirty minutes later, animals were gently placed in a metal restraining box with easy access to the tail. Through caudal vein, they received [^{3}H](+)-PHNO (nominally 5 µCi/rat in 0.2 mL saline). Thirty minutes after i.v. administration of [^{3}H](+)-PHNO, animals were decapitated and brains were quickly removed, chilled in ice cold saline, and dissected by free-hand on ice-cold surface. Both striata, and cerebellum (CB) lobes L9,10 were dissected, the remaining part of cerebellum was saved for determination of non-specific binding. Tissue specimens were frozen on dry-

ice, weighed and dissolved in 1 mL 0.6 N NaOH in scintillation vials under continuous horizontal shaking. After complete dissolution, 5 mL of Optiphase Hisafe3 (PerkinElmer) was added to the samples, followed by thorough mixing; samples were left to equilibrate overnight. TriCarb 2900TR liquid scintillation counter (PerkinElmer) was used to determine radioactivity.

In case of inhibition experiments, expressed in percent radioactivity accumulated in vehicle-treated animals according to the following formula[7]:

$$Accumulation\ (\%) = \frac{Drug\ treated_{(DPM/mg)} - CB_{(DPM/mg)}}{Vehicle\ treated_{(DPM/mg)} - CB_{(DPM/mg)}} \times 100\%\,,$$

where ***Drug treated***$_{(DPM/mg)}$ is the radioactivity of striatum or CB L9, 10 samples from drug treated animals; ***Vehicle treated***$_{(DPM/mg)}$ is the radioactivity from animals treated with vehicle; and $CB_{(DPM/mg)}$ is the mean DPM/mg of the cerebellum without CB L9, 10 from vehicle-treated animals.

Group means were analyzed by one-way ANOVA followed by Tukey-Kramer post-hoc multiple comparison test. ED_{50} values were determined by sigmoidal fitting

Dopamine and Serotonin Turnover and DOPA Accumulation

Cariprazine- and DDCAR-induced changes in levels of dopamine, serotonin, and their respective metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and 5-hydroxyindole-acetic acid (5-HIAA) in rat brain regions such as striatum and prefrontal cerebral cortex and their effects on NSD-105-induced DOPA accumulation in striatum of reserpine-treated rats were determined using high performance liquid chromatography with electrochemical detection (HPLC-ED) as described previously with minor modifications.[9,10]

Stock solutions were prepared in minimal amount (~10–20 µL) of glacial acetic acid and diluted in saline to its final volume. Dose-response curves consisting of 5–6 different ascending doses were generated. Rats (5–6/group) were given vehicle or cariprazine or DDCAR p.o. in a volume of 0.5 mL/100 g body weight and then returned to their home cage.

In dopamine biosynthesis experiments, rats (5–6/group) were treated with reserpine (1 mg/kg, subcutaneously [s.c.], 0.2 mL/100 g body weight). The animals received cariprazine or DDCAR p.o. 18 h later; then, after another 30 min, they were treated with an aromatic amino acid decarboxylase inhibitor, NSD-1015 (100 mg/kg, intraperitoneally [i.p.]).

Rats were treated with ascending doses cariprazine or DDCAR and sacrificed 1 h later after dosing. In biosynthesis (i.e., DOPA accumulation) experiments, animals were decapitated 30 min after the NSD-1015 injection. Brains were quickly removed and chilled in ice-cold saline, striata were dissected by free hand on an ice-cold surface and immediately frozen on dry ice. Tissue samples homogenized by Ultra-Turrax homogenizer in 10 vol. HPLC mobile phase containing 1 nmol/mL isoproterenol as the internal standard. Samples were frozen at -20°C, and then thawed at room temperature. Homogenates were centrifuged at 16000 g, 4°C for 10 min (Eppendorf centrifuge 5415), supernatants (0.8–1 mL) were then filtered through a piece of Whatman G/C filter plugged into 1-mL pipette tip by vacuum directly into HPLC minivials.

For monoamines (DA, NE, 5-HT) and their metabolites (DOPAC, HVA and 5-HIAA) high pressure liquid chromatography coupled with electrochemical detection (HPLC-ED) was used.

Tissue contents (given in pmol/g tissue) were calculated by the pre-programmed integrator using internal standards. Standard calibration (1.25 pmol for each substance of interest) was done at the start and at the end of each run. Data (tissue contents in pmol/g tissue from the integrator report) were further processed by Microsoft Excel. Turnover index for dopamine ([DOPAC+HVA]/dopamine] and serotonin (5-HIAA/serotonin) were calculated for each individual sample with their means and SEM. Graphical presentations were made by Origin 6.0 (MicroCal). Student t-test was used for statistical comparison between various treatment groups.

Electrophysiology

Rats were anaesthetized by an initial dose of chloral-hydrate (Aldrich) applied in saline (400 mg/kg, i.p.), followed by infusion of supplementary doses of anesthetic i.p. (60 mg/kg every 30 min) via a cannula. Single 50 µg/kg s.c. dose of atropine sulfate (Egis) was administered in the unconscious state of animals to prevent mucus formation in airways. The trachea was cannulated and animals were allowed to breathe spontaneously with oxygen-enriched room-air throughout the experiments. Respiratory functions were monitored continuously by a rodent capnograph (Type 340, Harvard Apparatus) and end tidal CO_2 was kept between 3–5%. The left femoral vein and the contralateral femoral artery were exposed and cannulated for intravenous (i.v.) infusion of saline (5 mL/kg/h) or recording of the arterial blood pressure by a DC bridge amplifier (model no. EXP-HG-1, Experimetria Ltd.). Maintenance of sufficient depth of anesthesia was regularly checked and judged from the

absence of withdrawal reflexes and the level of respiratory rate, which was kept lower than 120 beats per minute. Rectal body temperature was monitored and maintained at 37±0.2°C using a heating pad (temperature controller 3B, Supertech Ltd.). Animals exhibiting lower than 60 mmHg mean arterial blood pressure for a period longer than 5 min were excluded from further experimentation. At the end of the experiment animals were euthanized with an i.v. dose of potassium chloride (2 mL of 1 M).

The head of the rat was mounted in a stereotaxic frame (model 1730, David Kopf Instruments) and A 3×5 mm rectangular craniotomy over the left parietal bone was performed with a saline-cooled drill. The dura was carefully opened, and the brain surface was covered by bacteriological agar gel (1.5%) to prevent dehydration

To perform unitary extracellular recordings from putative dopamine neurons, glass-coated tungsten electrodes (Tunglass-1; Kation Scientific LLC) were used. Impedance of these electrodes was typically between 0.8–2.8 MΩ measured *in vitro* at 1 kHz in saline. To search for dopamine neurons, the electrode was repeatedly lowered to the *pars compacta* of the substantia nigra under stereotaxic control (coordinates according to the atlas of Paxinos & Watson: AP, -4.8 to -6.1; L, 1.8 to 2.6; V to the level of the exposed tissue, 7.0 to 8.5 mm) using a motoric microdrive (Model 2662; David Kopf Instruments). A neuron encountered in the SNc region was considered dopaminergic if it fulfilled the following characteristics: (i) slow (2.0–9.0 Hz) firing rate, with or without burst firing; (ii) action potentials having biphasic or triphasic waveform with a duration of at least 1.1 ms (measured from spike initiation to the maximal negative phase of the action potential) and (iii) a characteristic low-pitch sound when monitored through an audioamplifier as determined by Ungless and Grace.[11] The signal from the recording electrode was fed through a preamplifier (gain: 100X, Supertech Ltd.), to an end stage filter-amplifier (bandwidth, 100 to 10 kHz; gain, 100; Linearamp, Supertech Ltd.). The filtered and amplified electrical signal was routed to a loudspeaker and through an interface to a personal computer. Recording from one neuronal unit per animal was evaluated. Ongoing activity was followed for a minimum period of 5 min before any pharmacological intervention. Verification of electrode placement was carried out in some cases.

Cariprazine and DDCAR were freshly dissolved in physiological saline prior to use. Drugs were injected i.v. in a maximal volume of 2 mL/kg.

Establishment of dose-response relationship of the effects of cariprazine and that of DDCAR on firing activity of single dopamine cells was carried out upon cumulative dosing. Vehicle of

the drug was first injected after establishing a stable baseline period of at least 5 min and then successive doses of drugs were administrated (cariprazine: 2.5, 5, 10, 20, 40, and 80 µg/kg; DDCAR: 2.5, 5, 10, 20, 40, 80, and 120 µg/kg) with separation of 2-min intervals. Additionally, time-course of neuronal effects induced by a single dose of cariprazine (10 µg/kg, i.v.) and DDCAR (120 µg/kg, i.v.) was also investigated in separate experiments.

Accurate inclusion and exclusion criteria were pre-set based on physiological parameters and neuronal characteristics prior to testing. Electrophysiological recordings were made by using Spike2 Version 8.04 (Cambridge Electronic Design Limited). Firing activity was quantified relative to baseline and drug-induced changes in neuronal activity were defined as percent changes from this baseline using values obtained in the last 30-second of the 2-min post-treatment period. Dose-response curves were constructed by plotting the firing rates normalized to the mean firing rate of the 'vehicle' period against doses. ED_{50} values were calculated for each animal using a sigmoid curve fitting method. In dose-response curve experiments, statistical differences were assessed by using repeated-measures ANOVA in a general linear model and Dunnett's test was used for *post-hoc* analysis. One-way ANOVA with Helmert contrast was used to determine the time when the steady-state of firing activity following single-dosing with cariprazine or DDCAR.

Summary data are presented as mean ± SEM. Level of significance was set to $\alpha=0.05$. All statistical analyses are conducted using Statistica for Windows Version 12 (TIBCO Software Inc).

Animal Behavioral Assessment

Cariprazine and DDCAR (as bases) were suspended in a minimal volume of 2–4% Tween solution; lower doses were obtained by appropriate dilution. The drugs were injected in a volume of 5 mL/kg (in rats) and 10 mL/kg (in mice).

Amphetamine (0.5 mg/kg) and phencyclidine (2.0 mg/kg or 2.5 mg/kg) dissolved in distilled water was administered s.c. in a volume of 1 mL/kg.

MK-801 dissolved in distilled water was administered i.p. at a dose of 0.1 or 0.2 mg/kg in a volume of 10 mL/kg.

Locomotor activity was measured in a six-channel activity monitor manufactured by Experimetria. The apparatus consisted of acrylic cages (48.5 cm x 48.5 cm x 40 cm) equipped with 2 x 30 pairs of photocells along all the bottom axis of the cage. Additional

arrays of photocells (30 pairs) were placed along two opposite sides of the cage at different heights (6.5, 12, 18, and 23 cm) in order to detect rearing responses. The photocell beam, when broken, signaled a count, which was then recorded by a computer. The signals were processed by a motion analyzing software, which determined the spatial position of the animal at 1-Hz sampling frequency and computed the distance travelled and time spent by the rats with ambulation, local movement (e.g., grooming), immobility, rearing, and other activities.

Mean ± SEM of horizontal activity (e.g., ambulation time) and vertical activity data (e.g., number of rearings) in each group was calculated.

Percent inhibition for the whole 60-min period were used for determining dose-response relationships, and ED_{50} values were determined by linear regression (Origin 6.0).

Spontaneous Locomotor Activity

Male Wistar rats (n=10 animals/dose group) were used. Sixty minutes after the p.o. administration of drugs (0.075-0.15-0.3-0.6-1.2-2.4 mg/kg po. for CAR; 0.2-0.4-0.8-1.6-3.2-6.4 mg/kg p.o. for DDCAR) or vehicle, the animals were individually placed in one of the photocell cages for 1 h. Horizontal and vertical movements were determined for 1 h at 15-min intervals.

Amphetamine-Induced Hypermotility

Male rats (n=10 animals/dose group) were treated with amphetamine (0.5 mg/kg, s.c.) 1 h after the p.o. administration of drug (0.05-0.1-0.15-0.2-0.3 mg/kg p.o. for CAR; 0.2-0.4-0.6-0.8-1.6 mg/kg p.o.) for DDCAR) or vehicle. Then, the animals were individually placed in photocell cages for 1 h.

Phencyclidine-Induced Hypermotility

Male Wistar rats (n=10 animals/dose group) were used. Half an hour after the p.o. administration drug (0.05-0.1-0.15-0.2-0.4-0.8 mg/kg p.o. for CAR, or (0.1-0.2-0.3-0.4-0.8 mg/kg p.o. for DDCAR) or vehicle, animals were individually habituated in the photocell cages for 30 min. After habituation, animals were treated with PCP (2 mg/kg, s.c.) or MK-801 (0.1 or 0.2 mg/kg, i.p.) and replaced into the into the experimental apparatus for the 1-h measurement period.

MK-801-Induced Hypermotility

Male NMRI mice (n=10 animals/dose group) were used. Half an hour after the p.o. administration drug (0.025-0.05-0.1-0.2 mg/kg p.o. for CAR or: 0.1-0.2-0.3-0.4 mg/kg p.o. for DDCAR) or vehicle, animals were individually habituated in the photocell cages for 30 min. After habituation, animals were treated with MK-801 (0.2 mg/kg, i.p.) and replaced into the experimental apparatus for the 1-h measurement period.

Apomorphine-Induced Climbing

Cariprazine was dissolved in distilled water, DDCAR was suspended 2% Tween 80 solution. Apomorphine was dissolved in 0.1% (w/v) ascorbic acid solution. All the drugs were injected in a

Mice (n=12 animals/group) were placed into cylindrical cages, 12 cm diameter, 15 cm high, with walls of vertical metal bars of 2 mm diameter and 1 cm apart. After 10 min of habituation, the mice were treated with apomorphine s.c. and moved into the cages. The measurement of climbing behavior started 10 min after apomorphine treatment and lasted for 16 min. The climbing behavior was scored at 1-min intervals as follow: 0 = four paws on the floor, 1 = forefeet grasping the wall, and 2= four paws grasping the wall. Animals were also rated for repetitive sniffing as a measure of stereotypy according to the following scale: 0 = no sniffing; 1 = moderate sniffing, little snout contact with cage walls or floor; and 2 = constant sniffing, persistent snout contact. Scores for both behaviors were summed for each individual and group means were calculated.

Mice were administered p.o. with drug (0.1-0.2-0.3-0.4-0.8 mg/kg p.o. for CAR; 0.4-0.8-1.6-3.2 mg/kg p.o. for DDCAR) or saline 60 min before the apomorphine injection (1.5 mg/kg, s.c.); then, the climbing test was done as described above. A dose-response curve was plotted for each compound and ED_{50} values were determined by linear regression.

Conditioned Avoidance Response in Rat

Cariprazine and DDCAR were suspended in a minimal amount of 2% Tween 80 solution; lower doses were obtained by appropriate dilution. The compounds were administered p.o. in a volume of 5 mL/kg.

The experiments were performed in the 4G shuttle box equipment. The apparatus consisted of two similar compartments separated by a wall that was fitted with a central opening and had a grid floor, which was used to shock the animals. Both compartments included a light source. In the cariprazine model, animals learned to avoid an aversive stimulus (e.g., electric

footshock) preceded by a warning signal (e.g., white-constant light) in a two-compartment shuttle box. To avoid the footshock, the rat must move from one compartment to the other. The footshock occurred 10 s after the warning signal (conditioned stimulus, CS) during which the animal could cross to the other compartment (avoidance response, AR) without receiving any punishment (e.g., footshock). If it fails to cross during this 10-s period, a 0.6 mA electric shock (unconditioned stimulus, US) would be delivered to the animal for a maximum of 5 s with the CS still on. If, during these 5 s, the rat moves to the other compartment, then this would be recorded as a so-called escape response (ER). If the animal remains for 15 s in the same compartment, the trial is terminated and an escape failure is recorded. Before the next trial, there is a 10-s long inter-trial interval (ITI) when the animal can freely change compartment without being punished. The boxes are connected to the control unit, which is controlled by an IBM PC (Win XP). 4G Shuttle Box software was used to collect, save, and process the measured data into data files. These raw data files were converted into Excel files with the following parameters: number and latency of AR, number and latency of ER, number of escape failures, number of crossings during ITI.

Rats were conditioned to complete 48 trials daily. The animals were conditioned for several days before receiving treatment. If the animal had stable avoidance performance (≥75% successful AR) for ≥3 consecutive days, it would be subjected to drug treatment.

Animals were administered p.o. with CAR (n=5 animals/dose group; doses: 0.5-1.0-2.0 mg/kg p.o.) or DDCAR (n=9 animals/dose group; doses: 0.75-1.5-3.0-6.0) 1 h before the beginning of the daily session. Animals received drug treatment once a week.

Scopolamine-Induced Learning Deficit in the Water-Labyrinth Test

Cariprazine was suspended in 6% Tween solution at a concentration corresponding to 1 mg/kg dose; this suspension was diluted further as necessary and was administered p.o. in a volume of 5 mL/kg, 1 h before each daily training session. DDCAR was suspended in 5% Tween solution and was administered p.o. in a volume of 5 mL/kg, 1 h before each daily training session.

Scopolamine, dissolved in distilled water, was injected i.p. (2 mL/kg) at a dose of 3 mg/kg, 30 min prior to the first daily trial.

Experiments were performed in a 3-choice-point water-labyrinth. The water tank was made of rust-proof metal, with the following dimensions: 1 m long, 50 cm wide, and 50 cm deep. It was filled with water, with a temperature of 25±1°C, to a depth of 27 cm. The labyrinth

system was constructed from removable, vertical metal plates. The animals had to maneuver through 3-choice points in order to reach a platform, which allowed them to escape from water.

The procedure was carried out during 4 consecutive days. Adaptation (Day 1): The metal plates constituting the labyrinth system were removed from the water and rats were conditioned to swim from the start point to the platform a total of 3 times. Animals were left on the platform for 20 s and then, they were allowed to rest in their cage for 30 min between swimming sessions.

Training procedure (Day 2–4): On the training days the labyrinth system was in place. One daily session, consisting of 3 trials, was performed and 30 ± 5 min rest periods were allowed between trials. For each trial, rats were placed into the water at the start point of the labyrinth system and had to swim to the escape platform. Variables reflecting the learning performance, such as the number of directional turning errors and swimming time were measured. An error was defined as swimming through a choice-point in the wrong direction (e.g., away from the platform or towards a blind alley). If the rat made an error, it could swim back to the choice-point and try again; however, once a rat swam over a choice-point into the correct direction (leading out of the labyrinth), the way back was manually closed by a metal plate.

Swimming time was the interval from the entry into the labyrinth until the exit from the water and could not exceed 5 min for any trial. If the rat did not find the platform during this period, the experimenter would intervene, and the number of errors was recorded either as its real value or 12, whichever was higher and swimming time recorded 5 min for this trial. Occasionally, the rat would hang onto the metal plates and remained in this position during the trial period. At the end of the trial, these rats were also guided to the escape platform by the experimenter. However, when a rat consistently adopted this strategy in 7–9 trials out of the 9 trials, the result was excluded from the experiment.

All the experiments included a solvent control, a memory-impaired group, and an impaired group.

Catalepsy Test

Cariprazine and DDCAR (as base) were suspended 2–5% Tween solution and injected in a volume of 5 mL/kg.

Catalepsy with the test compounds was measured with the help of a 10-cm high podium. Thirty min after the treatment with drug or vehicle, the animals were placed in extra-ordinary position with both forepaws on the podium. Rats were considered to be cataleptic if they did not correct their body posture within 30 s; yes or no was registered. This procedure was repeated at 1, 2, 3, 4, and 5 h after treatment.

Pharmacokinetics

Plasma samples were obtained from rats at the following time points: 5 min, 20 min, 1 h, 3 h, 5 h, 7 h, 10 h, and 24 h after i.v. administration; 0.5 h, 1 h, 2 h, 3 h, 5 h, 7 h, 10 h, and 24 h after p.o. administration in bioavailability study; 10 min, 1 h, 2 h, 5 h, and 8 h after i.v. administration of DDCAR; and 1 h, 2 h, 4 h, 8 h, 24 h, and 32 h after p.o. administration of cariprazine or DDCAR in brain penetrability study. In mice the sampling times were 0.5 h, 1 h, 2 h, 4 h, 8 h and 24 h after p.o. administration. Blood samples were taken from the retroorbital plexus (rats) or sinus (mice) into lithium-heparin containing tubes and were centrifuged at ~800 g, 4°C for 20 minutes to obtain plasma.

Whole brain was also removed from the animals in brain penetration studies. Brain homogenate samples were prepared by homogenizing the whole brain with deionized water (brain:water 1:2.5). Plasma and brain homogenate samples were stored at -18°C until analysis.

Plasma and brain homogenate samples were prepared for analysis by protein precipitation with acetonitrile or liquid-liquid extraction with tert-butyl-methyl ether or 1-chlorobutane using deuterated internal standard.

Samples were analyzed for cariprazine and DDCAR, or for DDCAR alone, respectively, by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). In all studies, the mass spectrometer was operated in positive-ion, multiple reaction monitoring (MRM) mode and the mass transitions were monitored at *m/z* 427 → 382.

In rat bioavailability study, Agilent 1100 LC system with PE Sciex API 365 mass spectrometer was used. The analytes were separated on an XTerra RP18 (4.6x150 mm, 5 µm) column under isocratic conditions. The mobile phase was 1 mM ammonium-acetate in methanol- water 9:1. The lower limit of quantification (LLOQ) was 0.5 ng/mL.

In rat brain penetration and DDCAR (i.v.) studies, Agilent 1200 LC coupled with Sciex 5500 QTRAP MS system was used. The separation was performed on a Zorbax Eclipse Plus C18

(50x4.6 mm, 1.8 µm) column with eluent of acetonitrile:water 1:1 and 5% acetic acid methanol 9:1 with gradient elution. The LLOQ was 0.1 (cariprazine) and 0.2 (DDCAR) ng/mL in plasma, 0.07 (cariprazine) and 0.7 (DDCAR) ng/g in brain.

In the mouse study, Agilent 1100 LC system with Sciex API 4000 MS was used. The analytes were separated with gradient elution on XBridge C18 (75x4.6 mm, 3.5 µm) column with mobile phase of water:acetonitrile:1 M ammonium-acetate 100:2:0.5 and acetonitrile. The LLOQ was 0.25 (cariprazine) and 0.05 (DDCAR) ng/mL in plasma and 0.9 (cariprazine) and 0.2 (DDCAR) ng/g in brain.

Microdialysis Experiment

A dual-probe microdialysis (MD) study was conducted to estimate the free extracellular levels of cariprazine and DDCAR in awake, freely moving rats (n=5).

Animal surgery was performed in two phases. In the first phase, implantation of intracerebral microdialysis probe guide cannula was carried out, followed by the gastric cannulation. Between the two surgeries and before the dialysis experiment, 2–6 days of recovery periods were allowed. Probes were stereotaxically implanted in the right prefrontal cortex (2 mm membrane length) and left striatum (4 mm membrane length) according to the coordinates of a rat brain atlas[12] with dual-armed stereotaxic instrument under pentobarbital anesthesia. Gastric cannulation was performed under isoflurane anesthesia by implanting indwelling pyrogen-free catheter in the forestomach. After cannulation, the catheter was externalized subcutaneously and connected to extended catheter with a syringe.

Prior to the day of MD experiment, probes were inserted in the brain regions through the guide cannulas while the animals were under light isoflurane anesthesia. The probes were connected to the syringe of the infusion pump and the needle of the collector with Teflon tubes and were perfused overnight with artificial cerebrospinal fluid (aCSF) at a flow rate of 0.33 µL/min. Next day the perfusion rate was set to 1.0 µL/min and 1-h stabilization period was allowed. Cariprazine was administered intragastrically at 1 mg/kg dose in d.w. via the surgically implanted catheter and the MD samples were collected at 20-min intervals from 60 min predose to at least 300 min postdose. The MD experiments were performed with the Culex automated sampling system (Bioanalytical Systems Inc.).

In vitro recovery of the MD probes was determined after the experiment. Type and size of the tubing set was the same as used in the *in vivo* study. Probes (2 and 4 mm membrane length)

were fixed in a calibration solution (100 nM in aCSF) of cariprazine and thermostatted at 37°C. Drug-free aCSF was pumped through the probes at 1 µL/min. Following 1-h equilibration period, 5 20-µL fractions were collected. Recovery was calculated as percentage ratio of the dialysate to the calibration solution. The individual recovery of the probes was used for the calculation of the extracellular concentrations. Concentration values measured in dialysate samples were divided by the calculated recovery and multiplied by 100.

Dialysate concentrations of cariprazine and DDCAR were determined by LC-MS/MS. Agilent 1100 LC coupled with Sciex API 4000 MS was used for the analysis. The analytes were separated on Zorbax Eclipse XDB C18 (75x4.6 mm, 3.5 µm) column with 0.025% acetic acid in 5 mM ammonium-acetate and methanol as the mobile phase and using gradient elution. The mass spectrometer was operated in positive-ion, MRM mode. Detection was performed at mass transitions at *m/z* 427 → 382 (cariprazine) and 399 → 382 (DDCAR). The LLOQ in microdialysate samples was 0.4 ng/mL for cariprazine and 0.8 ng/mL for DDCAR.

Abbreviations

Cariprazine (RGH-188), *trans*-*N*-[4-[2-[4-(2,3-dichlorophenyl)piperazin-1-yl]ethyl]cyclohexyl]-*N*',*N*'-dimethylurea hydrochloride; 5-HT, serotonin; hD_2, human recombinant dopamine D_2 receptor; hD_3, human recombinant dopamine D_3 receptor; rD_3 rat recombinant dopamine D_3 receptor; h5-HT_{1A}, human recombinant serotonin 5-HT_{1A} receptor; h5-HT_{2B}, human recombinant serotonin 5-HT_{2B} receptor; DCAR, desmethyl-cariprazine; DDCAR, didesmethyl-cariprazine; DOPA, 3,4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxyphenyl acetic acid; HVA, homovanillic acid; 5-HIAA, 5-hydroxy-indolyl acetic acid; CHO, Chinese hamster ovary; HEK, human embryonic kidney; [^{3}H](+)-PHNO, [^{3}H](+)-*trans*-1a,2,3,4a,5,6-hexahydro-9-hydroxy-4-propyl-4*H*-naphtho[1,2-b]-1,4-oxazine; DDT, dithithreitol; GDP, guanosine-diphasphate; HBSS, Hank's Balanced Salt Solution; IBMX, 3-isobutyl-1-methylxanthine; FSK, forskolin, HTRF, homogeneous time-resolved fluorescence; DMSO, dimethylslfoxide; HEPES: 2-[4-(hydroxyethyl)piperazin-1-yl]ethanesulfonic acid.

Supplemental Tables

Supplemental Table 1. Summary of Experimental Conditions for the In Vitro Receptor Binding Assays (Recombinant Receptors)[a]

Receptor[b]	Cell or tissue	[^{3}H]Radioligand (nM)	Non-specific (μM)	K_d (nM)	Incubation Buffer[c]	Incubation Conditions (Time, Temperature)	Reference
rα_{1A}	submax. gland	Prazosin (0.25)	Phentolamine (10)	0.17	A	60 min, 25°C	Michel, et al. (1989)[13]
hD_{2L}	CHO	Spiperone (0.16)	Haloperidol (10)	0.08	B	120 min, 25°C	Grandy, et al. (1989)[14]
hD_{2S}	CHO	Spiperone (0.16)	Haloperidol (10)	0.09	B	120 min, 25°C	Grandy, et al. (1989)[14]
hD_3	CHO	Spiperone (0.7)	(S)-(–)-Sulpiride (25)	0.36	B	120 min, 37°C	Sokoloff, et al. (1990)[15]
hH_1	CHO-K1	Pyrilamine (1.2)	Pyrilamine (1)	1.1	C	180 min, 25°C	De Backer, et al. (1993)[16]
h5-HT_{1A}	CHO	8-OH-DPAT (1.5)	Metergoline (10)	2.0	D	60 min, 25°C	Martin, et al. (1994)[17]
h5-HT_{2A}	CHO	Ketanserin (0.5)	Mianserin (1)	0.2	E	60 min, 25°C	Bonhaus, et al. (1995)[18]
h5-HT_{2B}	CHO-K1	LSD (1.2)	Serotonin (10)	2.1	F	60 min, 37°C	Bonhaus, et al. (1995)[18]
h5-HT_{2C}	CHO-K1	Mesulergine (1)	Mianserin (1)	1.1	G	60 min, 25°C	Wolf, et al. (1997)[19]
h5-HT_6	HeLa	LSD (1.5)	Serotonin (5)	1.3	H	120 min, 37°C	Monsma, et al. (1993)[20]
h5-HT_7	CHO	LSD (5.5)	Serotonin (10)	7.4	I	120 min, 25°C	Roth, et al. (1994)[21]
σ1	Jurkat	Haloperidol (8)	Haloperidol (10)	5.8	J	240 min, 25°C	Hashimoto, et al. (1993)[22]

Notes: [a]Assays were performed by MDS Pharma (or MDS Pharma Services – Discovery at the time of the experiments were performed); [b]Lowercase r and h indicate if the receptor was the rat or human version; [c]Buffers: A, 50 mM Tris (pH 7.4); B, 50 mM Tris-HCl, 1.4 mM ascorbic acid, 150 mM NaCl, 0.001% BSA (pH 7.4); C, 2 mM $MgCl_2$, 100 mM NaCl, 250 mM sucrose, 50 mM Tris-HCl (pH 7.4); D, 50 mM Tris-HCl, 10 mM $MgSO_4$, 0.5 mM EDTA, 0.1 % ascorbic acid (pH 7.4); E, 50 mM Tris-HCl (pH 7.0); F, 50 mM Tris-HCl, 4.4 mM $CaCl_2$, 0.1 % ascorbic acid (pH 7.4); G, 50 mM Tris HCl, 0.1 % ascorbic acid, 10 μM pargyline; H, 50 mM Tris-HCl, 150 mM NaCl, 2 mM ascorbic acid, 0.001 % BSA (pH 7.4); I, 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM EDTA (pH 7.4); J, 5 mM K2$HPO_4 \cdot 3H_2O/KH_2PO_4$ (pH 7.5).

Abbreviations: 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin; LSD, Lysergic acid diethylamide; submax, submaxilliary.

Supplemental Table 2. Summary of Experimental Conditions for the In Vitro Receptor Binding Assays (Native Rat Brain Receptors)[a]

Rat Receptor	Brain Tissue	[^{3}H]Radioligand (nM)	Non-Specific (µM)	K_d (nM)	Incubation Buffer[b]	Incubation Conditions (Time, Temperature)	Reference
α1	cerebral cortex	Prazosin (0.3–0.5)	phentolamine (10)	0.22–0.37	A	30 min, 25°C	Greengrass, et al. (1979)[23]
D_2	striatum	Spiperone (0.7)	(+)-butaclamol (1)	0.48–0.7	B	20 min, 37°C	Creese, et al. (1979)[24]
D_3	Sf9	Spiperone (0.85)	haloperidol (10)	0.85–1.5	C	60 min, 27°C	Per vendor instruction (Perkin-Elmer, Cat. No. 3110139)
5-HT_{1A}	hippocampus	8-OH-DPAT (2.0)	serotonin (10)	1.9–3.0	D	15 min, 37°C	Hall, et al. (1985)[25]; Gozlan, et al. (1983)[26]
5-HT_{2A}	frontal cortex	Ketanserin (0.5–1.1)	methysergide (1)	0.49–1.1	E	15 min, 37°C	Leysen, et al. (1986)[27]; Gozlan, et al. (1986)[28]
5-HT_{2C}	choroid plexus	Mesulergine	mianserin (1)	1.1–1.9	F	30 min, 37°C	Pandey, et al. (1993)[29]

Notes: [a]Assays were performed at Gedeon Richter Plc; [b]Buffers: A, 50 mM Tris-HCl (pH: 7.4); B, 50 mM Tris-HCl, 1 mM EGTA, 5 mM $MgSO_4 \cdot 7H_2O$ (pH 7.4); C, 50 mM Tris-HCl, 1 mM EDTA, 10 mM $MgCl_2$, 120 mM NaCl (pH 7.4); D, 50 mM Tris-HCl, 4 mM $CaCl_2$, 0.1 % ascorbic acid, 10 µM pargyline (pH 7.7); E, 50 mM Tris-HCl (pH 7.4); F, 50 mM Tris-HCl, 4 mM $CaCl_2$, 0.1 % ascorbic acid (pH 7.4).

Abbreviations: 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin; Sf9, Spodoptera frugiperda.

Supplemental Table 3. Summary of Experimental Conditions for the In Vitro Functional Assays

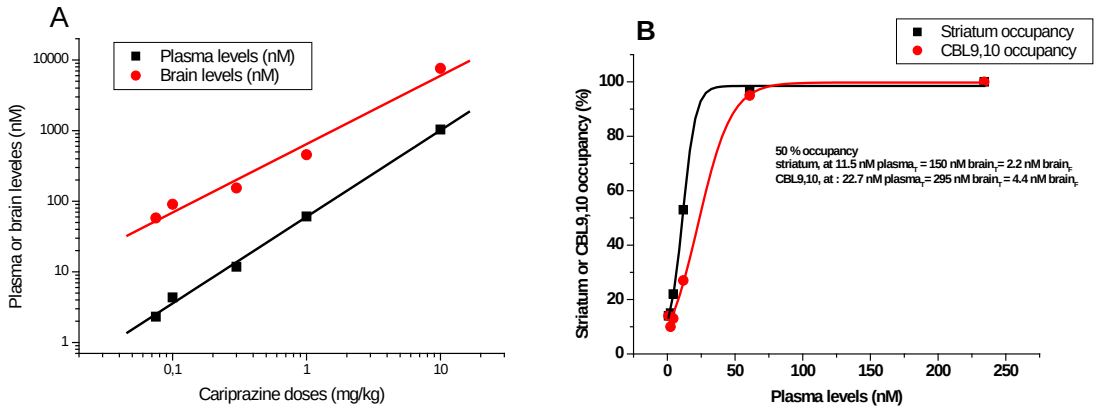
Receptor	Tissue or cell line	Functional assay	Homogenization Buffer[a]	Incubation Buffer[a]	Incubation Conditions (Time, Temperature)	References
rD_2 $r5\text{-}HT_{1A}$ $h5\text{-}HT_{1A}$ hD_2 hD_3	rat striatum hippocampus CHO HEK293 CHO-K1	$[^{35}S]GTP\gamma S$ binding	A-H	A-I	10 min; 30°C	Alper, et al. (1998)[2]; Malmberg, et al. (1998)[3]; Rinken, et al. (1999)[4]
hD_2 hD_3 $h5\text{-}HT_{1A}$	HEK293 CHO CHO	cAMP production	NA	B C C	30 min; 37°C 10 min; 37°C 30 min; 37°C	Instruction provided by the HTRF kit (Cisbio, Cat. No. 62AM6PEC)
$h5\text{-}HT_{2B}$	CHO	Ca^{2+}-release	NA	D	10 min; 37°C	Kurko, et al. (2009)[5]

Notes: [a]Buffers: A-H: 50 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA (pH 7.6); A-I: 50 mM Tris, 100 mM NaCl, 7 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT (pH 7.4); B, 140 mM NaCl, 5 mM KCl, 5 mM HEPES-Na, 5 mM HEPES, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose (pH 7.4), supplemented with various concentrations of IBMX and 1 µM FSK; C, Hank's Balanced Salt Solution (HBSS), supplemented with various concentrations of IBMX and FSK; D, 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, 2 mM probenecid (pH 7.4).

Abbreviations: FSK, forskolin; HTRF, homogeneous time resolved fluorescence; NA, not applicable.

Supplemental Figure

Supplemental Figure 1. Relationship Between Cariprazine Dose, Plasma and Brain Levels at 1 hour Post-Dose, and Occupancy



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