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1. Title Page

Brexpiprazole I: *In vitro* and *in vivo* characterization of a novel serotonin-dopamine activity modulator

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2. Running title page

a) Running title: Brexpiprazole, a novel serotonin-dopamine activity modulator

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d) A list of nonstandard abbreviations

5-HT, serotonin

7-OH-DPAT, 7-hydroxy-2-(di-n-propylamino)tetralin

8-OH-DPAT, 2-dipropylamino-8-hydroxy-1,2,3,4- tetrahydronaphthalene

[³⁵S]GTPγS, guanosine 5'-O-(3-[³⁵S]thio)-triphosphate

aCSF, artificial cerebrospinal fluid

ANOVA, analysis of variance

CHO, Chinese hamster ovary

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cIC₅₀, corrected IC₅₀ value

CNS, central nervous system

DHFR, dehydrofolate reductase

D, dopamine

D_{2L}, long form of human D₂ receptor

DMSO, dimethyl sulfoxide

DOI, 2,5-dimethoxy-4-iodoamphetamine

DOPAC, 3,4-Dihydroxy-phenyl-acetic acid

EPS, extrapyramidal symptoms

HeLa, human cervical epithelial adenocarcinoma

HPLC, high-performance liquid chromatography

HTRF, homogeneous time resolved fluorescence

HVA, homovanillic acid

IMDM, Iscove's modified Dulbecco's medium

IP₁, inositol monophosphate

LSD, lysergic acid diethylamide

MEM, minimum essential medium

mPFC, medial prefrontal cortex

MRM, multiple-reaction-monitoring

MS/MS, tandem mass spectrometry

NSD-1015, 3-hydroxybenzylhydrazine dihydrochloride

PCR, polymerase chain reaction

PET, positron emission tomography

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SD, Sprague-Dawley

e) A recommended section assignment: Neuropharmacology

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3. Abstract

Brexpiprazole (OPC-34712, 7-{4-[4-(1-benzothiophen-4-yl)piperazin-1-yl]butoxy}quinolin-2(1H)-one) is a novel drug candidate in clinical development for psychiatric disorders with high affinity for serotonin, dopamine and noradrenaline receptors. In particular, it bound with high affinity ($K_i < 1$ nM) to h5-HT_{1A}, h5-HT_{2A}, hD_{2L}, h α_{1B} and h α_{2C} -adrenergic receptors. It displayed partial agonism at h5-HT_{1A} and hD₂ receptors in cloned receptor systems, and potent antagonism of h5-HT_{2A} receptors and h $\alpha_{1B/2C}$ -adrenoceptors. Brexpiprazole also had affinity ($K_i < 5$ nM) for hD₃, h5-HT_{2B}, h5-HT₇, h α_{1A} and h α_{1D} adrenergic receptors, moderate affinity for hH₁ ($K_i = 19$ nM), and low affinity for hM₁ receptors ($K_i > 1000$ nM). Brexpiprazole potently bound to rat 5-HT_{2A} and D₂ receptors *in vivo*, and *ex vivo* binding studies further confirmed high 5-HT_{1A} receptor binding potency. Brexpiprazole inhibited DOI-induced head-twitches in rats, suggestive of 5-HT_{2A} antagonism. Furthermore, *in vivo* D₂ partial agonist activity of brexpiprazole was confirmed by its inhibitory effect on reserpine-induced DOPA accumulation in rats. In rat microdialysis studies, brexpiprazole slightly reduced extracellular dopamine in nucleus accumbens, but not in prefrontal cortex, while moderate increases of the dopamine metabolites, homovanillic acid and 3,4-dihydroxy-phenyl-acetic acid, in these areas, also suggested *in vivo* D₂ partial agonist activity. In particular, based on a lower intrinsic activity at D₂ receptors and higher binding affinities for 5-HT_{1A/2A} receptors than aripiprazole, brexpiprazole would have a favourable antipsychotic potential without D₂ receptor agonist- and antagonist-related adverse effects. In conclusion, brexpiprazole is a serotonin-dopamine activity modulator with a unique pharmacology, which may offer novel treatment options across a broad spectrum of central nervous system disorders.

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4. Introduction

The main strategy for treatment of schizophrenia is based on antagonizing dopamine D₂ receptors. In addition, most second generation antipsychotics are antagonists of serotonin 5-HT_{2A} receptors and α_1 -adrenoceptors, and individual compounds have a variety of effects on other monoamine receptors, such as 5-HT_{1A} receptors. These broad target effects have the objective of either improving antipsychotic efficacy (with additional effects on affective symptoms or cognitive deficits) or mitigating adverse effects (e.g. extrapyramidal symptoms [EPS]) (Arnt and Skarsfeldt, 1998; Meltzer, 1999; Roth et al., 2004; Arnt et al., 2008; Wong et al., 2008; Newman-Tancredi, 2010; Newman-Tancredi and Kleven, 2011).

However, due to tolerability issues, treatment with D₂ receptor antagonists is not considered to be the optimal strategy to modulate dopaminergic activity, and the discovery and development of D₂ receptor partial agonists has provided a well-tolerated treatment with stabilizing effects on dopamine function (Stahl, 2001; Citrome, 2013). So far, only one D₂ partial agonist, aripiprazole, with moderate D₂ intrinsic activity, has reached the market (Burriss et al., 2002; Potkin et al., 2003), while other compounds with higher D₂ intrinsic activity are in development (Citrome, 2013) or have been discontinued during development, often because of lack of sufficient clinical efficacy, e.g. bifeprunox (Newman-Tancredi et al., 2007; Casey et al., 2008).

A key issue for the D₂ partial agonists is to ascertain how much intrinsic activity (or relative efficacy) is ideal in leading to optimal stabilization of dopaminergic transmission. If the D₂ intrinsic activity is too high, this can lead to lack of robust antipsychotic activity as well as pronounced adverse effects related to increased D₂ receptor tonus, e.g. nausea, vomiting, and motor side effects such as hyperkinesias and restlessness (Fleischhacker, 2005; Newman-Tancredi et al., 2007; Casey et al., 2008; Stip and Tourjman, 2010), while D₂ antagonist activity

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leads to an increased risk of EPS and increased prolactin secretion (Casey, 1996). Although aripiprazole has offered a new approach to stabilizing the dopaminergic system, an improvement could potentially be made by developing a novel compound that maintains significant partial agonist activity at D₂ receptors, but with lower intrinsic activity.

In addition to the issue of optimal D₂ intrinsic activity, optimization of the pharmacological profile by a combination of additional target effects is a well-known strategy to improve the clinical efficacy and tolerability of antipsychotics. At the clinically equivalent dose range leading to 80–90% D₂ receptor occupancy, aripiprazole modulates a limited number of additional target receptors, the primary effect being partial agonist activity at 5-HT_{1A} receptors with lower potency than at D₂ receptors (Mamo et al., 2007; Dahan et al., 2009). In addition, human 5-HT_{2A} receptor occupancy is significantly lower at clinically relevant dosages (Mamo et al., 2007). Accordingly, an optimized target profile may lead to improvements in both clinical efficacy and adverse effect profile in the treatment of schizophrenia. Furthermore, a broader pharmacological profile (e.g. on selected 5-HT receptors and α -adrenoceptor subtypes) could potentially provide opportunities for the treatment of a variety of other central nervous system (CNS) disorders and symptoms, such as depression and anxiety, as well as stress and impulse control disorders (Drouin et al., 2002; Roth et al., 2004; Arnt et al., 2008; Wong et al., 2008; Sallinen et al., 2013).

The present paper describes the basic pharmacological *in vitro* and *in vivo* characterization of a novel serotonin-dopamine activity modulator, brexpiprazole (OPC-34712; 7-{4-[4-(1-benzothiophen-4-yl)piperazin-1-yl]butoxy}quinolin-2(1H)-one; Fig.1). Brexpiprazole was discovered by Otsuka Pharmaceutical Co Ltd., and is being developed in collaboration with H. Lundbeck A/S. It combines 5-HT_{1A} receptor partial agonism and low-intrinsic activity D₂ receptor partial agonism with antagonist activity on a variety of 5-HT and α -adrenergic receptors.

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The main focus of this article is the receptors that are most influenced by brexpiprazole at clinically and pharmacologically relevant plasma exposures, while more detailed profiling data can be found in the Supplementary Material. The pharmacological effects of brexpiprazole in test models of positive symptoms of schizophrenia and of cognitive impairment are described in an accompanying paper (Maeda et al., submitted).

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5. Methods

Subjects

Male Wistar rats (Japan SLC Inc., Shizuoka, Japan [*in vitro* rat binding, L-DOPA and 2,5-dimethoxy-4-iodoamphetamine (DOI) test; 145–200 g] and Charles River, Germany [*in vivo* binding; 200–225 g]) and male Sprague-Dawley (SD) rats from Charles River, Wilmington, MA, USA (dopamine and metabolite microdialysis; 220–375 g), Charles River, Germany (dopamine, noradrenaline, and serotonin microdialysis; 275–300 g) and Harlan, Horst, Netherlands; 300–360 g (histamine and acetylcholine [ACh] microdialysis) have been used. All animals were maintained on a 12 h light/dark cycle (lights on at 6:00 or 7:00 AM) in controlled environmental conditions. Rats were group-housed with food and water available ad libitum.

The care and handling of rats was conducted in accordance with Guidelines for Animal Care and Use in Otsuka Pharmaceutical Co, Ltd; Revised on 01 Apr 2004, the Guide for the Care and Use of Laboratory Animals, The Animal Welfare Act, Code of Federal Regulations Title 9, Chapter 1, Subchapter A, or Danish Executive Order No. 1306 of 23 November 2007 on Animal Testing, and with National Institutes of Health guide for the Care and Use of Laboratory Animals.

Drugs

Brexpirazole, aripiprazole and risperidone were synthesized in our laboratory (Otsuka pharmaceutical Co., Ltd, Tokushima, Japan). Buspirone hydrochloride, butaclamol hydrochloride, DOI, DL-isoproterenol, dopamine, DOPA, 3,4-dihydroxy-phenyl-acetic acid (DOPAC), homovanillic acid (HVA), 3-hydroxybenzyl-hydrazine dihydrochloride (NSD-1015), noradrenaline, (-)-raclopride, serotonin, and WAY-100635 maleate were obtained from Sigma-

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Aldrich (St. Louis, MO). Reserpine (Apoplon[®] Injection) was obtained from Daiichi Sankyo Pharmaceutical Co., Ltd. (Tokyo, Japan). Rauwolscine was obtained from Tocris (Bristol, UK). Perchloric acid, EDTA 2Na, citric acid, and sodium acetate were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Sodium I-octanesulfonate was obtained from Nacal Tesque (Kyoto, Japan). The radioligands for *in vitro* studies, [³H]-raclopride, [³H]-(+)-8-OH-DPAT, [³H]-ketanserin, [³H]-prazosin, [¹²⁵I]-LSD, [³H]-methylnscopolamine, and [³H]-GTPγS were purchased from PerkinElmer (Waltham, MA). [³H]-(+)-7-OH-DPAT and [³H]-doxepin were purchased from American Biosciences (Piscataway, NJ) and American Radiolabeled Chemicals Inc. (St. Louis, MO), respectively. [³H]-RX 821002 and [³H]-prazosin were purchased from PerkinElmer (Waltham, MA), and [¹²⁵I](±)DOI from Chelatec (Saint-Herblain, France). For *in vivo* and *ex vivo* binding studies the binding ligands were (supplier in parentheses): [³H]-raclopride (Perkin-Elmer), [³H]-M100907 (volinanserin; Amersham Bioscience, UK), [³H]-Lu AE60157 (compound synthesized at Lundbeck A/S and tritiated by Red Glead Discovery, Sweden), [³H]-8-OH-DPAT (Perkin-Elmer) and [³H]-SB269970 (Amersham).

Brexpiprazole, aripiprazole and risperidone and other reference compounds (buspirone hydrochloride, (-)-raclopride, WAY-100635) were dissolved in dimethyl sulfoxide (DMSO) for *in vitro* studies, or suspended in 5% gum arabic-distilled water solution for *in vivo* studies, and diluted with the same solution. For *in vivo* binding studies and microdialysis studies (dopamine, noradrenaline, serotonin, histamine, and acetylcholine experiments in prefrontal cortex and ventral hippocampus) brexpiprazole was dissolved in minimum amounts of 1 mM methanesulphonic acid and 10% 2-hydroxypropyl-beta-cyclodextrin solution, adjusted to pH 5 with sufficient amount of 0.1 mM NaOH. Doses of salts are expressed as free bases. Drugs for *in*

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in vivo studies were administered p.o. using a volume of 5 ml/kg. NSD-1015 was dissolved in saline and DOI was dissolved in distilled water.

Cell Lines and Culture

D_{2L} receptor: The establishment of the clonal Chinese hamster ovary (CHO) cell line lacking dehydrofolate reductase (DHFR) and stably expressing a high density of human recombinant D_{2L} receptors has previously been reported as CHO-hD_{2L}-high cells (Tadori et al., 2005). The cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) + L-glutamine, 25 mM HEPES supplemented with 10% fetal bovine serum, 50 units/mL penicillin, 50 µg/ml streptomycin, 200 µg/ml G418, 0.1 mM sodium hypoxanthine, and 16 µM thymidine in a humidified 5% CO₂ incubator maintained at 37°C.

D₃ receptor: CHO cells stably co-expressing human recombinant D₃ receptors with rat adenylate cyclase V were established as follows. Firstly, CHO-K1 cells were incubated with the pEF1-V5 HisA vector (Invitrogen, Grand Island, NY) inserted with a hD₃ receptor (Ser9 type) gene (amplified by polymerase chain reaction [PCR] using human cDNA library; Clontech, Palo Alto, CA) in culture medium supplemented with Lipofectamine™ 2000 (Invitrogen), and a single cell line was isolated by limiting dilution. Secondly, in order to increase sensitivity to stimuli at D₃ receptors (Robinson and Caron, 1997), rat adenylate cyclase V was co-transfected in hD₃ receptor expressing CHO cells. The cells were incubated with the pcDNA3.1/Hygro(+) vector (Invitrogen) inserted with a rat adenylate cyclase V gene (amplified by PCR using rat liver cDNA library; BD Bioscience, San Jose, CA) in serum-free F-12 Ham's medium supplemented with 2 mM L-glutamine, 400 µg/ml hygromycin B, 200 µg/ml G418, and Lipofectamine™ 2000 (Invitrogen), and a single cell line was isolated by limiting dilution. The cells were cultured in F-

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12 Ham's medium supplemented with 2 mM L-glutamine, 400 µg/ml hygromycin B, 200 µg/ml G418, and 10% fetal bovine serum in a humidified 5% CO₂ incubator maintained at 37°C.

5-HT_{1A} receptor: Human cervical epithelial adenocarcinoma (HeLa) cells expressing h5-HT_{1A} receptors were established as follows. HeLa cells were incubated with the pEF1-V5 HisA vector (Invitrogen) inserted with a h5-HT_{1A} receptor gene (Origene, Rockville, MD) in serum-free minimum essential medium (MEM) supplemented with 0.3% G418 and Lipofectamine™ 2000. Then, a single cell line was isolated by limiting dilution. The cells were cultured in MEM medium supplemented with 0.3% G418, 10% fetal bovine serum in a humidified 5% CO₂ incubator maintained at 37°C.

5-HT_{2B} receptor: A CHO cell line expressing human recombinant 5-HT_{2B} receptors was constructed by Cerep S.A. (Celle l'Evescault, France). The cells were cultured in F-12 Ham's medium supplemented with 1.2% G418, 1% non-essential amino acids, 10% fetal bovine serum dialyzed in a humidified 5% CO₂ incubator maintained at 37°C.

α_{1B}- and α_{2C}-adrenoceptor: CHO cell lines expressing human recombinant α_{1B}- and α_{2C}-adrenoceptors were constructed by Cerep S.A. (Celle l'Evescault, France).

Cell Membrane Preparation

D_{2L} receptor: hD_{2L} receptor expressing cells were harvested, washed twice with ice-cold 50 mM Tris-HCl buffer (pH 7.4), and homogenized. The homogenate was centrifuged at 53,900g for 20 min at 4°C. The pellets were re-suspended with ice-cold assay buffer 50 mM Tris-HCl (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and stored at -80 °C until use.

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D₃ receptor: hD₃ receptor expressing cells were harvested, washed twice with ice-cold Dulbecco's phosphate buffered saline (Invitrogen), and homogenized. The homogenate was centrifuged at 48,000g for 20 min at 4°C. The pellet was re-suspended with 50 mM Tris-HCl (pH 7.4) containing 10 mM MgSO₄, 0.5 mM EDTA, and 0.1% ascorbic acid, and stored at -135°C until use.

5-HT_{1A} receptor: h5-HT_{1A} receptor expressing cells were harvested, washed twice with ice-cold 20 mM HEPES/NaHEPES and 10 mM EDTA (pH 7.4) containing 0.1% protease inhibitor cocktail (Sigma-Aldrich), and homogenized. The homogenate was centrifuged at 40,000g for 10 min at 4°C. The pellet was re-suspended with the same buffer and centrifuged twice. The pellet was re-suspended in ice-cold 20 mM HEPES/NaHEPES and 0.1 mM EDTA (pH 7.4), containing 1% bovine serum albumin, and stored at -135°C until use.

Cell membranes of other cell lines including human 5-HT_{2A}, 5-HT_{2B}, adrenergic α_{1A} , histamine H₁, and muscarinic M₁ receptors were purchased from PerkinElmer.

Cell membrane homogenates of cell lines expressing human α_{1B} -adrenoceptor or human α_{2C} -adrenoceptor were produced by Cerep S.A.

Rat Brain Membrane Preparation

Wistar rats for D₂, 5-HT₂, and α_1 -adrenergic receptors, and SD rats for 5-HT_{1A} receptors were decapitated and the brains were removed and placed on ice.

D₂ receptor binding assay: The striatum was dissected from the brains on ice (Kohler et al., 1985), homogenized using a Polytron™ Model PT 10-35 homogenizer (Kinematica) in ice-cold 50 mM Tris-HCl buffer (pH 7.4), and centrifuged at 48,000g for 10 min at 4°C. The pellet was suspended in the same buffer, incubated at 37°C for 10 min, and centrifuged under the same

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conditions once more. The final pellet was re-suspended in ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, and 1 mM MgCl₂, and stored at -80°C until use.

5-HT_{1A} receptor binding assay: The hippocampus was dissected from the brains on ice, homogenized in ice-cold 50 mM Tris-HCl (pH 7.4) containing 1 mM dithiothreitol and 1 mM EGTA, and centrifuged at 1,000g for 5 min at 4°C. The supernatant was stored on ice. The pellet was re-homogenized in the same buffer and centrifuged under the same conditions. The supernatant was mixed with the first supernatant, and centrifuged at 11,000g for 20 min at 4°C. The pellet was suspended in the same buffer and centrifuged at 27,000g for 20 min at 4°C. The final pellet was suspended in the same buffer, and stored at -135°C until use.

5-HT₂ receptor binding assay: The frontal cortex was dissected from the brains on ice (Leysen et al., 1982), homogenized in ice-cold 0.25 M sucrose and centrifuged at 1,000g for 10 min at 4°C. The supernatant was stored on ice. The pellet was re-homogenized in ice-cold 0.25 M sucrose and centrifuged under the same conditions. The two supernatants were pooled, diluted in ice-cold 50 mM Tris-HCl buffer (pH 7.4) and centrifuged at 35,000g for 10 min at 4°C. The pellet was suspended in the same buffer and centrifuged under the same conditions. The pellet was suspended in the same buffer, and stored at -80°C until use.

α₁-adrenoceptor binding assay: The cerebral cortex was dissected from the brains on ice (Gross et al., 1987). Cerebral cortex tissue was homogenized in ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl and 2 mM EDTA, and centrifuged at 80,000g for 20 min at 4°C. The pellet was suspended in the same buffer. After incubation for 10 min at 37°C, the suspension was centrifuged under the same conditions. The same process of suspension and

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centrifugation was repeated. The final pellet was suspended in 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, and stored at -80°C until use.

Receptor Binding Assays

The binding assays for human and rat receptors were carried out under incubation condition as summarized in Table 1. The final concentration of DMSO was 1%.

All human and rat receptor binding assays (except h5-HT_{2B}, hα_{1B}, hα_{2C}, and hM₁ receptors):

The reaction mixture was transferred to the filter plate (Unifilter[®] 96GF/B, PerkinElmer) or Whatman[®] GF/B filter (Sigma-Aldrich) using the harvester (FilterMate[™], PerkinElmer) or Brandel Harvester (Brandel, Biomedical Research & Development Laboratories, Gaithersburg, MD). The filter plate was rinsed with ice-cold 50 mM Tris-HCl buffer (pH 7.4) and dried up. The radioactivity was measured by TopCount (TopCount NXT[™], PerkinElmer) or Tri-Carb 2900 TR (PerkinElmer Life and Analytical Sciences, Inc., Shelton, CT) with a liquid scintillation cocktail MicroScint[™]-O (PerkinElmer). The radioactivity of radioligands was measured by both TopCount and liquid scintillation counter LSC-5101 (Aloka, Tokyo, Japan) using Aquasol[™]-2 (PerkinElmer) in order to calculate the counting efficacy of the radioactivity.

5-HT_{2B} receptor: The incubation mixtures were filtered through filter paper using a cell harvester (Brandel) and the filter paper was rinsed three times with 50 mM Tris-HCl (pH 7.4). The filter paper was placed in a tube, and the radioactivity was counted for 2 min using a gamma counter (COBRA, PerkinElmer Life and Analytical Sciences, Inc.). These studies were performed at Sekisui Medical Co., Ltd. (Ibaraki, Japan).

α_{1B}- and α_{2C}-adrenoceptor: The incubation mixtures were filtered through glass fiber filters (GF/B, Packard) presoaked with 0.3% polyethylenimine (PEI) and rinsed four times with ice-

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cold 50 mM Tris-HCl using a cell harvester (Unifilter, Packard). The filters were dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). These studies were performed at Cerep S.A.

M₁ receptor: The incubation mixtures were filtered through filter paper using a Brandel harvester, and the filter paper was rinsed three times with 50 mM Tris-HCl (pH 7.4). The filter paper was placed in a vial with liquid scintillator (Atomlight, PerkinElmer), and the radioactivity was counted with a liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Inc.) for 2 min. This study was performed at Sekisui Medical Co., Ltd.

Forskolin-induced cAMP Accumulation at hD_{2L} and hD₃ Receptors

D_{2L} receptor: hD_{2L} receptor expressing cells were seeded at 10⁴ cells per well in poly-L-lysine coated 96 well plates, and grown for 2 days. The cells were washed with Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 0.1 mM sodium hypoxanthine and 16 μM thymidine. Cells were pre-incubated with test compounds for 20 min at 37°C in IMDM medium supplemented with 0.1% sodium ascorbate, 0.1 mM sodium hypoxanthine, and 16 μM thymidine. Then 10 μM forskolin and 500 μM 3-isobutyl-1-methylxanthine (inhibitor of adenosine 3',5'-cyclic monophosphate phosphodiesterase) were added and incubation in the presence of test compounds was continued for an additional 10 min at 37°C. The final concentration of DMSO was 0.7%. The intracellular cAMP concentration in each well was determined using the cAMP Biotrak™ enzyme immunoassay system (GE Healthcare Bioscience, Buckinghamshire, UK) according to the manufacturer's instructions.

D₃ receptor: A Flashplate® Adenylyl Cyclase Activation Assay System (PerkinElmer Life and Analytical Sciences, Inc.) was used to determine the intracellular cAMP concentration in

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each well. hD₃ receptor expressing cells (4×10⁵ cells/well) were incubated with test compounds in a Flashplate[®] well containing 0.1 μM forskolin for 1 h at 25°C. Subsequent steps of the assay procedure were conducted according to the manufacturer's instructions. Final concentration of DMSO was 1%.

h5-HT_{1A} Receptor GTPγS Binding Assay

The agonistic activities of test compounds were evaluated using [³⁵S]-GTPγS binding to h5-HT_{1A} receptor-expressing cell membranes. The membrane preparation was incubated in 25 mM Tris-HCl (pH 7.4) buffer containing 50 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA, 0.55 μM GDP, and 0.03 nM [³⁵S]-GTPγS with test compounds for 1 h at room temperature. After the reaction was terminated by cooling on ice, the membrane was filtered through a Whatman[®] GF/B filter (Sigma Aldrich) and the radioactivity was counted using a Tri-Carb 2900TR Liquid Scintillation Analyzer (PerkinElmer). Final concentration of DMSO was 1%.

h5HT_{2A} Receptor IP₁-HTRF Functional Assay

Cells were suspended in buffer containing 10 mM HEPES/NaOH (pH 7.4), 4.2 mM KCl, 146 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 5.5 mM glucose, and 50 mM LiCl, then distributed in microplates at a density of 1.5×10⁴ cells/well. For agonist functional assays, the cells were incubated for 30 min at 37°C in the presence of buffer (basal control), test compound, or reference agonist. For stimulated control measurement, separate assay wells contained 10 μM serotonin. For antagonist functional assays, the cells were pre-incubated for 5 min at room temperature in the presence of buffer (basal control), test compound, or reference antagonist. Thereafter, the reference agonist, serotonin, was added at a final concentration of 100 nM. For

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basal control measurements, separate assay wells without serotonin were used. Cells were further incubated for 30 min.

Following incubation, the cells were lysed and the fluorescence acceptor (d2-labeled IP₁) and fluorescence donor (anti-IP₁ antibody labeled with europium cryptate) were added. After 60 min at room temperature, the fluorescence transfer was measured at $\lambda_{ex}=337$ nm and $\lambda_{em}=620$ and 665 nm using a microplate reader (Rubystar, BMG). The IP₁ concentration was determined by dividing the signal measured at 665 nm by that measured at 620 nm (ratio). The results were expressed as a percent of the control response to 10 μ M serotonin. These studies were performed at Cerep S.A.

h5-HT_{2B} Receptor IP₁-HTRF Functional Assay

The cells were suspended in a buffer containing 10 mM HEPES/NaOH (pH 7.4), 4.2 mM KCl, 146 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 5.5 mM glucose, and 50 mM LiCl, then distributed in microplates at a density of 4×10^4 cells/well and incubated for 30 min at 37°C in the presence of buffer (basal control), test compound, or reference agonist. For agonist functional assays, separate assay wells containing 1 μ M serotonin were used as stimulated control. For antagonist functional assays, the reference agonist, serotonin, was added at a final concentration of 30 nM. Separate assay wells without serotonin were used as basal control. Following incubation, the cells were lysed and the fluorescence acceptor (d2-labeled IP₁) and fluorescence donor (anti-IP₁ antibody labeled with europium cryptate) were added. After 60 min at room temperature, the fluorescence transfer was measured at $\lambda_{ex}=337$ nm and $\lambda_{em}=620$ and 665 nm using a microplate reader (Rubystar). The IP₁ concentration was determined by dividing the signal measured at 665 nm by that measured at 620 nm (ratio). The agonism results were expressed as a percent of the

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control response to 1 μM serotonin; the antagonism results were expressed as a percent inhibition of the control response to 30 nM serotonin. These studies were performed at Cerep S.A.

$\text{h}\alpha_{1\text{B}}$ -adrenoceptor cAMP-HTRF Functional Assay

The cells were suspended in Hank's Balanced Salt Solution (HBSS) buffer (Invitrogen) complemented with 20 mM HEPES (pH 7.4) and 500 μM IBMX, then distributed in microplates at a density of 2×10^4 cells/well and incubated for 30 min at 37°C in the presence of HBSS (basal control), the test compound or the reference agonist. For agonist functional assays, separate assay wells containing 10 μM adrenaline were used as functional control. For antagonist functional assays, the reference agonist, adrenaline, was added at a final concentration of 3 μM . Separate assay wells without adrenaline were used as basal control. Following incubation, the cells were lysed and the fluorescence acceptor (d2-labeled cAMP) and fluorescence donor (anti-cAMP antibody labeled with europium cryptate) were added. After 60 min at room temperature, the fluorescence transfer was measured at $\lambda_{\text{ex}}=337$ nm and $\lambda_{\text{em}}=620$ and 665 nm using a microplate reader (Rubystar). The cAMP concentration was determined by dividing the signal measured at 665 nm by that measured at 620 nm (ratio). The results were expressed as a percent of the control response to 10 μM adrenaline. These studies were performed at Cerep S.A.

$\text{h}\alpha_{2\text{C}}$ -adrenoceptor cAMP-HTRF Functional Assay

The cells were suspended in HBSS buffer (Invitrogen) complemented with 20 mM HEPES (pH 7.4) and 500 μM IBMX, then distributed in microplates at a density of 10^4 cells/well. For agonism measurement, the cells were incubated with HBSS (basal control), the reference agonist

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adrenaline at 1 μM or various concentrations (EC_{50} determination), or the test compounds. Next, the adenylyl cyclase activator NKH 477 was added at a final concentration of 5 μM . For antagonism measurement, the cells were incubated with HBSS (stimulated control), the reference antagonist, rauwolscine, at 10 μM (basal control) or various concentrations (IC_{50} determination), or the test compounds. Next, the reference agonist adrenaline (100 nM) and the adenylyl cyclase activator NKH 477 (5 μM) were added. For basal control measurements, adrenaline was omitted from the wells containing 3 μM rauwolscine.

Following 10 min incubation at 37°C, the cells were lysed and the fluorescence acceptor (d2-labeled cAMP) and fluorescence donor (anti-cAMP antibody labeled with europium cryptate) were added. After 60 min at room temperature, the fluorescence transfer was measured at $\lambda_{\text{ex}}=337$ nm and $\lambda_{\text{em}}=620$ and 665 nm using a microplate reader (Rubystar). The cAMP concentration was determined by dividing the signal measured at 665 nm by that measured at 620 nm (ratio). The results were expressed as a percent of the control response to 1 μM adrenaline for agonism and as a percent inhibition of the control response to 30 nM adrenaline for antagonism. These studies were performed at Cerep S.A.

***In vitro* Data Analysis**

In *in vitro* assays, the concentration-response curves, K_d , IC_{50} , EC_{50} , and E_{max} values were calculated by non-linear regression analysis using GraphPad Prism® software (Version 3.00, 4.02, and 5.02, GraphPad Software Inc., San Diego, CA) or XLfit (Version 5.3.1.3, ID Business Solutions Ltd., Guildford, UK). In receptor binding assays, K_i values were calculated using Cheng-Prusoff equation, $K_i = \text{IC}_{50} / [1 + (\text{radioligand concentration}) / K_d]$ (Cheng and Prusoff,

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1973). In functional assays, cIC_{50} values were calculated using Cheng-Prusoff equation, $cIC_{50} = IC_{50} / [1 + (\text{agonist concentration}) / EC_{50}]$ (Cheng and Prusoff, 1973).

***In Vivo* Binding to rD_2 , $r5-HT_{2A}$ and $r5-HT_6$ Receptors in Rat Brain**

Occupancy for the D_2 , $5-HT_{2A}$ and $5-HT_6$ receptors was measured by *in vivo* binding with [3H]-raclopride (D_2 receptor binding), [3H]-M100907 ($5-HT_{2A}$ binding) or [3H]-Lu AE60157 ($5-HT_6$ binding), respectively (Idris et al., 2010). Briefly, 10 μ Ci (D_2 and $5-HT_6$ binding) or 15 μ Ci ($5-HT_{2A}$ binding) was injected intravenously in the tail vein. Fifteen minutes after the injection, the animals were sacrificed by a blow to the head, decapitated and the striatum (D_2 and $5-HT_6$ binding) or cortex ($5-HT_{2A}$ binding) was dissected. The tissue was homogenized in ice-cold buffer [50 mM KPO_4 , pH 7.4 (for D_2); 50 mM Tris, pH 7.7 (for $5-HT_{2A}$ and $5-HT_6$)], samples were filtered through Whatman GF/C filters, and the filters were washed with 2 x 5 ml ice-cold buffer. Filtration was completed 60–120 s after sacrifice. Filters were counted in a scintillation counter. Trunk blood together with remaining brain tissue was collected during the experiment from each animal for compound exposure analysis. Protein content was measured in all brain samples according to Pierces method by BCA-ELISA assay, and was subsequently used for normalization. Cerebellum was dissected from vehicle-treated animals and used to determine non-specific binding.

Data are presented as percentage (%) receptor occupancy \pm S.E.M. for each receptor subtype investigated. ED_{50} values were calculated using non-linear regression analysis (GraphPad Prism).

***Ex Vivo* Binding to $r5-HT_{1A}$ and $r5-HT_7$ Receptors in Rat Brain**

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Blood and brains were collected 2 h after p.o. administration of brexpiprazole, aripiprazole, or vehicle. Rats were anesthetized using CO₂ gas and sacrificed by decapitation. Trunk blood was collected in vacutainers containing EDTA and gently mixed for 30 s before placing on ice. Later, blood was centrifuged at 3000 rpm for 15 min at 4°C. The plasma layer was collected and frozen at -20°C until use. Brains were dissected from the skull, flash frozen on powdered dry ice, and stored at -20°C.

Tissue sectioning: Brains were sectioned coronally at 20 µm thickness using a cryostat and mounted on slides. Slices were collected beginning at approximately -4.5 mm posterior to bregma for the 5-HT_{1A} receptor assay, and -2.1 mm posterior from bregma for the 5-HT₇ receptor assay. Three replicate slices were taken from each brain for each experiment, and slides were stored at -20°C.

Autoradiography: Boxes containing slides were defrosted under a constant stream of air for 30 min. In some cases, slides were briefly pre-incubated in the appropriate buffer (see below), and placed under constant air flow until completely dried. Subsequently, slides were incubated in an assay buffer that included the appropriate tritiated radioligand. Non-specific binding was determined by incubating slices from a vehicle-treated animal in assay buffer that contained the appropriate radioligand and a high concentration of a non-radioactive competitor for the target receptor. After incubation, slides were washed twice for 5 min in cold (4°C) assay buffer and dried under constant airflow for 1 h before being transferred to a vacuum dessicator for at least 1 h. Finally, slides were exposed using a Beta-imager (Biospace Labs, Paris, France) for 20–24 h. Specific details for each autoradiography assay are listed below.

5-HT_{1A} receptor occupancy: 5-HT_{1A} receptor occupancy was conducted as previously reported (du Jardin et al. 2013), with minor modifications. Briefly, slides were incubated for 1 h

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in assay buffer consisting of 170 mM Tris-HCl, 4 mM CaCl₂, 0.1% L-ascorbic acid and 10 μM of pargyline (pH 7.4) and containing 3 nM of the radioligand [³H]-8-OH-DPAT. Non-specific binding was determined by including 1 μM of the 5-HT_{1A} receptor antagonist WAY100635 in the incubation medium. Surface radioactivity was quantified in the hippocampus.

5-HT₇ receptor occupancy: Defrosted slides were pre-incubated for 3 min at room temperature in an assay buffer containing 170 mM Tris HCl, 4 mM CaCl₂, and 0.5 uM L-ascorbic acid (pH7.4). After drying, slides were incubated for 1 h in the assay buffer described above with 100 μM of pargyline and 5.9 nM of the 5-HT₇-selective radioligand [³H]-SB269970. Non-specific binding was determined by the inclusion of 1 μM non-radioactive SB269970. Subsequently, slides were rinsed, dried, and exposed in a Beta imager as described above. Surface radioactivity for the 5-HT₇ receptor occupancy assay was quantified from medial thalamic nuclei such as the paraventricular thalamic nucleus and interanteromedial thalamic nucleus.

Data analysis: *Ex vivo* autoradiography data were quantified as described previously (du Jardin et al. 2013). Results are expressed as mean ± S.E.M. Where appropriate, ED₅₀ / EC₅₀ values and 95% confidence intervals were determined using Graphpad Prism. Doses or exposure levels were log transformed and a non-linear regression procedure was performed on occupancy values using a sigmoidal dose response curve. The top and bottom values were constrained to 100 and 0, respectively, while the Hill coefficient was not constrained.

Exposure Analysis

Blood and brain samples were drawn from the animals upon completion of the *in vivo* or *ex vivo* binding procedures. Brain homogenate was prepared by homogenizing the brain 1:4 (v/v)

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with water:2-propanol:DMSO (50:30:20 v/v/v) followed by centrifugation and collection of the supernatant. Plasma and brain supernatant samples were frozen at -80°C until analysis.

Brexiprazole or aripiprazole concentrations were determined using UltraPerformance liquid chromatography followed by tandem mass spectrometry (MS/MS) detection in positive-ion electrospray ionisation mode. The peak area correlated linearly with the plasma and brain concentration of both analytes in the range of 1–1000 ng/ml plasma and 5–4000 ng/g brain (corrected for dilution).

Reserpine-induced DOPA Accumulation Assay in Rat Striatum

Agonist effect on dopamine autoreceptor was evaluated by measuring inhibitory effect on DOPA accumulation after treatment with reserpine and 3-hydroxybenzylhydrazine dihydrochloride (NSD-1015, a DOPA decarboxylase inhibitor) (Hjorth et al., 1981; Yasuda et al., 1988). Reserpine (1 mg/kg s.c.) was injected, after which rats were fasted for 18 h before sacrifice. Brexiprazole or aripiprazole were administered p.o. 1 and 2 h before sacrifice, respectively. Next, NSD-1015 (100 mg/kg, i.p.) was injected 30 min before sacrifice. Each rat was sacrificed by exposure to head-focused microwave in a microwave applicator (power; 5.5, 2.1 s, Model TMW-6402C, Muromachi Kikai, Tokyo, Japan). The striatum was dissected out of the whole brain on ice. Each striatum was weighed and homogenized individually by sonication in a buffer (1:10; w/v) containing 1.2% perchloric acid, 100 µM EDTA 2Na, and 0.1 µg/ml DL-isoproterenol (which is used as an internal standard substance). After incubation for 1 h on ice, the homogenates were centrifuged at 20,000g for 15 min at 4°C. The supernatants were filtered through a disposable syringe filter unit (DISMIC-3cp, cellulose acetate, 0.45 µm, Advantec, Tokyo, Japan). The amount of DOPA in each supernatant was measured by an HPLC system

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(Eicom, Kyoto, Japan) equipped with an electrochemical detector (ECD-300, Eicom) using a separation column (SC-5ODS, diameter 3.0 mm × length 150 mm, Eicom). The mobile phase consisted of 70.8 mM citric acid and 14.2 mM sodium acetate buffer (pH 2.7) containing 0.5 mM sodium l-octanesulfonate, 14.9 μM EDTA 2Na, and 15% (v/v) methanol, and the flow rate was 0.5 ml/min. For DOPA detection, the electrode potential was set at +0.75 V against an Ag/AgCl reference electrode.

The difference between the reserpine-untreated (normal) group and the reserpine-treated (control) groups was assessed by a two-tailed t-test. Differences between compound-treated groups were analyzed by two-way analysis of variance (ANOVA). Differences between the control group and each compound-treated group were assessed by a two-tailed Dunnett's test. ED₂₅ values were calculated by nonlinear regression analysis using SAS software (SAS institute Japan, Tokyo, Japan).

DOI-induced Head Twitches in Rats

Following systemic injection, DOI induces characteristic head twitches which are mediated by CNS 5-HT_{2A} receptors (Sanchez and Arnt, 2000). Wistar rats were fasted for 16 to 22 h before administration of test compounds. Brexpiprazole or risperidone were administered p.o. 1 h before injection of DOI (5 mg/kg, s.c.), while aripiprazole was administered 2 h before DOI injection. Each rat was placed individually in an acrylic cylinder (diameter 23 cm; height 30 cm), and the number of head twitches was counted for 10 min immediately after DOI injection. The mean number of head twitches per 10 min in control groups varied between 18 and 21. The experimenter was blinded to the drug and dose to avoid bias. ED₅₀ values were calculated by nonlinear regression analysis using SAS software.

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Microdialysis Studies of Brain Neurotransmitter Release

Dopamine and its metabolites: Extracellular levels of dopamine and metabolites in the rat nucleus accumbens and prefrontal cortex were investigated as described previously (Jordan et al., 2004). Briefly, a CMA-12 guide cannula (CMA Microdialysis, Acton, MA) was implanted above the nucleus accumbens (1.4 mm rostral to bregma; 2 mm lateral; 5.8 mm ventral to dura) or medial prefrontal cortex (mPFC; 3.2 mm rostral to bregma, 0.6 mm lateral; 5.3 mm ventral to dura), using coordinates according to the atlas of Paxinos and Watson (1998). Two days after cannulation, a microdialysis probe (CMA-12, 4 mm tip length) was inserted into the guide cannula and the probe was continuously perfused (1 μ l/min) with sterile artificial cerebrospinal fluid (aCSF) (145 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1.2 mM CaCl₂, 2 mM Na₂HPO₄; pH 7.4, or 1.2 mM Na₂HPO₄, 0.27 mM NaH₂PO₄, 140 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 7.2 mM glucose; pH7.29). Microdialysate samples were collected at 30 min intervals (CMA-142 microfraction collector) into silanized microvials. Vehicle and test/reference compounds were administered 3.5 h after microdialysis probe implantation and microdialysate sampling continued for 3 h thereafter. A dissecting microscope was used to verify correct microdialysis probe placement at the end of each experiment. Isocratic, high-performance liquid chromatography-electrochemical detection (HPLC-ECD) was used to provide simultaneous measurements of dopamine, DOPAC, and HVA in the microdialysate samples as described previously (Jordan et al., 2004). External standards of dopamine, DOPAC, and HVA were dissolved in aCSF and used to determine their retention times and corresponding peaks in microdialysate. A small number of microdialysis samples were spiked with internal standards of dopamine, DOPAC, and HVA to confirm peak identity and retention times. The concentrations of dopamine, DOPAC, and HVA

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in the microdialysate samples were expressed as a percentage of their respective mean basal concentration (i.e., mean of three samples preceding treatment). The data were pooled for each treatment group and statistical analyses were performed using GraphPad Prism and SAS Software. Differences between mean basal analyte concentrations were considered statistically significant when the p-value was less than 0.05. Statistically significant differences between vehicle and individual doses of brexpiprazole were revealed using repeated measures of ANOVA followed by two-tailed Dunnett's test. Mean (\pm S.E.M.) basal concentrations (pg/ μ l) of dopamine, DOPAC, and HVA detected in microdialysate collected from the nucleus accumbens (without correction for probe recovery) were 0.81 ± 0.08 (n = 49), 124 ± 6.2 (n = 49), and 66.3 ± 3.6 (n = 61), respectively. For the mPFC, basal values of dopamine, DOPAC, and HVA were 0.083 ± 0.006 (n = 77), 26.2 ± 1.48 (n = 89), and 32.3 ± 1.44 (n = 88), respectively.

Serotonin and noradrenaline: Extracellular levels in the rat mPFC and ventral hippocampus were investigated as described previously (Mørk et al., 2009). Guide cannulas (CMA-12) were stereotaxically implanted into the brain, aiming to position the dialysis probe tip in the mPFC (3.2 mm anterior to bregma; 0.8 mm lateral; 4.0 mm ventral to dura) or in the ventral hippocampus (5.6 mm posterior to bregma; 4.8 mm lateral; 7.0 mm ventral to dura). On the day of the experiment a microdialysis probe (CMA-12, 0.5 mm diameter, 3 mm length) was inserted through the guide cannula and the microdialysis probe was perfused with filtered aCSF solution (145 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1.2 mM CaCl₂) for the duration of the experiment at a constant flow rate of 1.3 μ l/min. After 180 min of stabilization, the experiments were initiated. Dialysates were collected every 30 min. After the experiments the animals were sacrificed, the brains were removed and the probe placement was verified. Analysis of dialysate for serotonin was performed as described previously (Mørk et al., 2009). The content of noradrenaline was

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analyzed by means of HPLC with electrochemical detection. The monoamines were separated by reverse phase liquid chromatography (ODS 150 x 2 mm column). The mobile phase consisted of 75 mM Li-acetate, 4 mM sodium 1-heptane sulphonate, 100 μ M EDTA, 8.0 % methanol (pH 4.7) at a flow rate of 0.17 ml/min. Electrochemical detection was accomplished using a coulometric detector; potential set at $E_2 = 250$ mV (Coulchem II, ESA). The absolute basal levels without considering probe recovery were 2.1 ± 0.27 (n = 30) and 10.7 ± 0.73 (n = 33) fmol/20 μ l dialysate for cortical serotonin and noradrenaline, respectively. The absolute basal levels without considering probe recovery were 2.7 ± 0.52 (n = 21) and 12.8 ± 1.49 (n = 22) fmol/20 μ l dialysate for hippocampal serotonin and noradrenaline, respectively.

In the data analysis the mean value of two consecutive monoamine samples immediately before drug administration served as the basal level for each experiment, and data were converted to percentage of basal (mean basal pre-injection values normalized to 100%). The data are expressed as group means \pm S.E.M. Data were analyzed by two-way ANOVA with repeated measures by means of SigmaPlot (v 11.0, SPSS Corporation).

Acetylcholine and histamine: Extracellular acetylcholine and histamine levels in the rat mPFC were investigated as described previously (Flik et al., 2011). The study was performed by Brains On-Line B.V. (Groningen, The Netherlands). A microdialysis probe with a 4 mm exposed surface (polyacrylonitril membrane, Brainlink, the Netherlands) was implanted into the prefrontal cortex (3.4 mm rostral anterior to bregma; 0.8 mm lateral; 5.0 mm ventral to dura). Experiments started after 1 day of recovery. aCSF solution containing 147 mM NaCl, 3.0 mM KCl, 1.2 mM CaCl₂, and 1.2 mM MgCl₂ was perfused at a flow rate of 1.5 μ l/min. After 2 h pre-stabilization, microdialysis samples were collected into minivials (Microbiotech/se AB, Sweden; 4001029). All samples were stored at -80°C until off-line analyses. The content of acetylcholine

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in the microdialysis samples was determined by HPLC with MS/MS as described previously (Giorgetti et al., 2010). Chromatographic separation was performed on a reversed phase Phenomenex Synergi Max-RP column (2.0 × 150 mm, particle size: 4 μm), held at a temperature of 50°C. Components were separated using an isocratic flow of eluent (20 mM ammonium acetate, 5 % acetonitrile, and 0.3 % trifluoroacetic acid in ultrapure H₂O) at a flow rate of 0.25 ml/min. A post-column makeup flow of 98% 2-propanol, 1% acetic acid plus 1% formic acid (0.125 ml/min) was added to the column effluent to enhance ionization efficiency. Concentrations were calculated with Analyst™ data system.

Analysis of histamine was done by HPLC and tandem mass spectrometry (MS/MS) detection using d₄-histamine as internal standard as described previously (Mørk et al., 2013). In brief, the MS analyses were performed using an API 4000 MS/MS system consisting of an API 4000 MS/MS detector and a Turbo Ion Spray interface (Applied Biosystems, the Netherlands). The acquisitions on API 4000 were performed in positive ionization mode, with ion spray voltage set at 4 kV. The instrument was operated in multiple-reaction-monitoring (MRM) mode for detection of the compound. Calibration curves were fitted using weighted (1/x) regression, and the sample concentrations were determined using these calibration curves. Accuracy was verified by quality control samples after each sample series. Concentrations were calculated with Analyst™ data system.

Data evaluation: The average of the four pre-administration samples was set to 100%. If relative basal samples were <50% or >150%, they were considered to be outliers and were not used for baseline calculation. For histamine, basal levels higher than 10 nM were also considered to be outliers based on historical data. All post-administration samples were expressed as a percentage of basal levels within the same subject. Data are expressed as levels relative to

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baseline to allow comparison within and between treatment groups. Statistical analyses were performed on the relative data using SigmaPlot (v12.3). Time and treatment effects were evaluated using two-way ANOVA for repeated measurements followed by a Student-Newman-Keuls post-hoc test. Time and treatment were the main factors. The level of statistical significance was defined a priori as $P < 0.05$. Basal levels of acetylcholine and histamine were 1.93 ± 0.14 nM and 4.49 ± 0.26 nM ($n = 40$), respectively.

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6. Results

In Vitro Receptor Binding Profile of Brexpiprazole

The binding affinities of brexpiprazole for selected human and rat receptors are summarized in Tables 2 and 3, respectively. For further information on target profile, see Supplementary Material.

Human receptors: Brexpiprazole showed subnanomolar binding affinities (K_i , nM, indicated in parentheses) for h5-HT_{1A} (0.12), hD_{2L} (0.30), and h5-HT_{2A} (0.47) receptors, as well as for h α _{1B} (0.17) and h α _{2C} adrenoceptors (0.6). Brexpiprazole had nanomolar affinity for hD₃ (1.1), h5-HT_{2B} (1.9), h5-HT₇ (3.7) receptors, and h α _{1A} adrenoceptors (3.8) (Table 2 and Supplementary Material). The affinities of brexpiprazole for hH₁ (19) and for hM₁ (67% binding at 10 μ M) receptors were moderate and very low, respectively.

A number of other receptor and target affinities have been investigated. Results are listed in the Supplementary Material, since they are currently not considered to be important for understanding the pharmacological profile of brexpiprazole because of low affinities. Although brexpiprazole had moderate inhibitory effects on monoamine uptake in rat brain synaptosomes, the affinities for human dopamine, noradrenaline and serotonin transporters were weaker than those for the above mentioned receptors (see Supplementary Material).

Rat receptors: The affinities of brexpiprazole for selected rat receptors are presented in Table 3. The affinities for r5-HT_{1A} (0.09 nM) and rD₂ (0.35 nM) receptors were consistent with the human receptor affinities, while the affinity for r5-HT₂ receptors (3.8 nM) was about 10 times lower than for human 5-HT_{2A} receptors. The α ₁-adrenoceptor affinity (non-selective for subtypes, 18 nM, measured by [³H]-prazosin binding) was lower than that of h α ₁-adrenoceptor subtype affinities (Table 3 and Supplementary Material).

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Brexpiprazole is a h5-HT_{1A}, hD_{2L} and hD₃ Receptor Partial Agonist *In Vitro*

h5-HT_{1A} receptors: Partial agonist activities of brexpiprazole and reference compounds were evaluated using [³⁵S]-GTPγS binding to h5-HT_{1A} receptor-expressing cell membranes. Brexpiprazole, aripiprazole, bupirone and bifeprunox increased [³⁵S]-GTPγS binding in a concentration-dependent manner (Fig. 2, Table 4). The maximum effect (E_{max}) values of the three first compounds were relatively similar (60%, 73%, and 78%, respectively, expressed relative to the maximum effect of 10 μM serotonin), while bifeprunox had a significantly higher E_{max} value (90%; *P* < 0.05) than brexpiprazole and aripiprazole. The agonist effect of brexpiprazole (100 nM) was blocked by the selective 5-HT_{1A} receptor antagonist WAY-100635 (IC₅₀ 0.30 nM; data not shown).

hD_{2L} receptors: Partial agonist activities of brexpiprazole and aripiprazole were evaluated using the inhibition of forskolin-induced cAMP accumulation in hD_{2L} receptor-expressing cells. Dopamine almost completely inhibited cAMP accumulation in a concentration-dependent manner (Fig. 3A) and bifeprunox also had high E_{max} value in this assay. In contrast, brexpiprazole and aripiprazole only partially inhibited cAMP accumulation, with potencies consistent with their receptor binding affinities (Fig. 3A; Table 4). The maximum inhibitory effect (E_{max}) of brexpiprazole (43%) was significantly (*P* < 0.05) lower than that of aripiprazole (61%), expressed relative to the maximum inhibition induced by dopamine (Table 4).

hD₃ receptors: Partial agonist activities of brexpiprazole and aripiprazole were evaluated using inhibition of forskolin-induced increase of cAMP accumulation in hD₃ receptor-expressing cells. Brexpiprazole and aripiprazole partially inhibited cAMP accumulation in a concentration-dependent manner (Fig. 3B, Table 4). In comparison with the maximum inhibition measured

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with 10 μ M dopamine, brexpiprazole had a significantly lower E_{\max} value than aripiprazole in this assay (15% and 28%, respectively, $P < 0.05$). The agonist effect of brexpiprazole (500 nM) was blocked by co-incubation with (-)-raclopride (1 μ M; data not shown).

Brexpiprazole is an Antagonist of h5-HT_{2A} and h5-HT_{2B} Receptors, and of h α _{1B}- and h α _{2C}-adrenoceptors *In Vitro*

Functional effects of brexpiprazole were explored for the other receptors for which subnanomolar binding affinities have been observed. Brexpiprazole inhibited the effect of serotonin on IP₁ production at h5-HT_{2A} and h5-HT_{2B} receptors, as well as the effect of adrenaline on cAMP production at h α _{1B}- and h α _{2C}-adrenoceptors. Highest antagonist potency was observed at h α _{1B}-adrenoceptors (cIC₅₀ 0.66 nM), while slightly lower potencies were found at h5-HT_{2A/2B} receptors and h α _{2C}-adrenoceptors (Table 4).

Brexpiprazole had no agonist activity in the absence of agonist stimulation in these assays after incubation with concentrations of up to 1 μ M (h5-HT_{2A}; h α _{1B}) or 10 μ M (h5-HT_{2B}; h α _{2C}), respectively (data not shown).

Brexpiprazole Binds to 5-HT_{1A} and 5-HT₇ Receptors in Rat CNS *Ex Vivo*

Brexpiprazole bound *ex vivo* to 5-HT_{1A} receptors in rat hippocampus with an ED₅₀ of 5.6 mg/kg, p.o. In comparison, aripiprazole had lower potency with an ED₅₀ of about 30 mg/kg, p.o. (Table 5). Finally, the *ex vivo* binding to r5-HT₇ receptors in paraventricular nucleus of thalamus was insignificant for both brexpiprazole and aripiprazole over the dose range used in this study, showing a maximum of about 40% receptor occupancy at 30 mg/kg, p.o. (Table 5).

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Brexpiprazole Binds to D₂, 5-HT_{2A}, and 5-HT₆ Receptors in Rat CNS *In Vivo*

Brexpiprazole dose-dependently displaced *in vivo* [³H]-raclopride binding to rat striatal D₂ receptors, with an ED₅₀ value of 2.5 mg/kg, p.o. (Table 5). Plasma and brain exposure measurements indicated EC₅₀ values of 49 ng/ml and 20 ng/g, respectively (Table 5). A separate time-course experiment with brexpiprazole 3 mg/kg p.o. showed more than 60% striatal D₂ receptor occupancy between 1–4 h after administration, and indicated that D₂ occupancy of brexpiprazole follows plasma and brain exposure in rats (Fig 4). Plasma exposures were between 44 and 146 ng/ml, 1–4 h after administration (Fig. 4A), while brain levels were slightly lower (12–46 ng/g; Fig. 4B).

Brexpiprazole also potently displaced the binding of the 5-HT_{2A} receptor ligand [³H]-M100907 in cortex (ED₅₀ 4.6 mg/kg, p.o.). The binding potency at 5-HT_{2A} receptors was 26 times higher than that of aripiprazole (ED₅₀ 120 mg/kg, p.o.) based on the ED₅₀ values (Table 5).

Brexpiprazole had moderate potency in displacing binding of the 5-HT₆ ligand [³H]-Lu AE60157 in rat striatum (ED₅₀ 17 mg/kg, p.o.), whereas the potency of aripiprazole was very low (Table 5).

An overview of the plasma exposure-receptor occupancy relationship of brexpiprazole for the *in vivo* and *ex vivo* binding studies in rats illustrates the rank order of binding potencies at the different receptors, as described above (Fig. 5).

Brexpiprazole is a D₂ Partial Agonist *In Vivo*: Inhibitory Effects on Reserpine-induced DOPA Accumulation in Rat Striatum

Reserpine (1 mg/kg, s.c., 18 h before sacrifice) increased DOPA accumulation about 4-fold in rat striatum (See Supplementary Material). Brexpiprazole and aripiprazole both showed D₂ agonist

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effects by inhibiting the reserpine-induced increase in DOPA accumulation, but had different maximum inhibitory responses (Table 6). They had similar potencies based on ED₂₅ values, while the maximum inhibitory effect of brexpiprazole was lower (55%) than that of aripiprazole (89%). Two-way ANOVA indicated a significant effect of drug (main effect; $P < 0.01$) and a drug \times dose interaction ($P < 0.05$).

Brexpiprazole is a 5-HT_{2A} Antagonist *In Vivo*: DOI-induced Head Twitches in Rats

DOI induces frequent head twitches, with on average 19–21 episodes for 10 min after injection of 5 mg/kg s.c. (data not shown). Brexpiprazole and aripiprazole dose-dependently inhibited DOI-induced head twitches (Table 6), brexpiprazole (ED₅₀ 4.7 mg/kg, p.o.) being more potent than aripiprazole (ED₅₀ 21 mg/kg, p.o.). As expected, risperidone potently blocked DOI-induced head twitches (ED₅₀ 0.096 mg/kg, p.o.; data not shown).

Microdialysis Studies of Extracellular Monoamines in Freely-moving Rats

A series of experiments have been performed to explore the effects of brexpiprazole on extracellular dopamine, noradrenaline, serotonin, acetylcholine, and histamine levels in rats.

Dopamine and metabolites in nucleus accumbens: Brexpiprazole (10 mg/kg, p.o.) slightly, but significantly, reduced extracellular dopamine levels (Fig. 6A). Maximum decrease was $74 \pm 6.5\%$ of baseline levels, in comparison with the vehicle group ($87 \pm 5.8\%$ of baseline), and was observed 120–150 min after drug administration. The higher dose (20 mg/kg) induced a non-significant decrease. In contrast, brexpiprazole slightly increased the extracellular levels of the dopamine metabolites HVA and DOPAC (Fig. 6B and 6C, respectively). Maximum increases of HVA and DOPAC were 30 and 20% above baseline levels, respectively, after 10 and 20 mg/kg,

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occurring 150–180 min (HVA) and 60–180 min (DOPAC) after drug administration. In the vehicle group slight decreases (4 and 8%, respectively) were seen, compared with baseline levels.

Dopamine and metabolites in prefrontal cortex: Brexpiprazole had no effect on extracellular dopamine levels after administration of 1, 3, and 10 mg/kg, p.o. (Fig. 7A), while dopamine metabolite levels significantly increased at the 3 and 10 mg/kg doses. Maximum increases in comparison with baseline levels at the two doses were 41 and 96% for HVA (Fig. 7B); 44 and 110% for DOPAC (Fig. 7C), while levels in the vehicle group increased non-significantly by 12 and 6%, respectively, for HVA and DOPAC. Maximum effects occurred 150–180 min after drug administration.

Serotonin and noradrenaline in prefrontal cortex and ventral hippocampus: Brexpiprazole had no effect on extracellular levels of serotonin or noradrenaline in either brain region after administration of doses up to 30 mg/kg, p.o. (Supplementary Material).

Histamine and acetylcholine in prefrontal cortex: Brexpiprazole increased extracellular histamine levels after administration of 10, and in particular 30 mg/kg, p.o. (Fig. 8), while no changes in extracellular acetylcholine were observed at any dose (Supplementary Material). Histamine levels also increased after vehicle administration, but the effect was short-lasting, while the increase after brexpiprazole (30 mg/kg) was stable for at least 3 h.

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7. Discussion

Brexpiprazole had a balanced and unique activity on several monoaminergic receptors. Its major effects at clinically relevant concentrations consisted of partial agonist activity on h5-HT_{1A} receptors and hD_{2L} and hD₃ receptors, combined with antagonist activity on h5-HT_{2A} receptors and h α _{1B}- and h α _{2C}-adrenoceptors.

Serotonin-dopamine-noradrenergic Binding *In Vitro* and *In Vivo*

Brexpiprazole displayed almost equal subnanomolar affinities for several cloned human receptors, including h5-HT_{1A}, hD_{2L}, and 5-hHT_{2A} receptors, as well as h α _{1B}- and h α _{2C}-adrenoceptors. The affinity constants (K_i) were in the range 0.12–0.6 nM. Consequently, the occupancies of these receptors after brexpiprazole treatment will likely be similar *in vivo*. While both brexpiprazole and aripiprazole showed high affinities for h5-HT_{1A} and hD₂ receptors, brexpiprazole had a slightly higher affinity for h5-HT_{1A} receptors than hD₂ receptors, whereas the reverse was true for aripiprazole (Table 2). Furthermore, brexpiprazole bound with about ten-times higher affinity to h5-HT_{1A} and h5-HT_{2A} receptors and much higher affinity to h α _{1B}- and h α _{2C}-adrenoceptors than aripiprazole (Table 2). The only available method to measure human receptor occupancy is PET imaging, and D₂ antagonist antipsychotics are usually dosed to obtain 60–80% occupancy (e.g. Mamo et al., 2007). At the recommended doses of aripiprazole human D₂/D₃ receptor occupancies are higher, up to 90% (Yokoi et al., 2002; Kegeles et al., 2008). Consistent with its low h5-HT_{2A} affinity, it has been shown that h5-HT_{2A} receptor occupancy of aripiprazole is lower (54–60%; Mamo et al., 2007), compared with second generation antipsychotics, e.g. olanzapine, which almost saturates 5-HT_{2A} receptors (Kapur et al., 1998). No

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PET ligands for measuring α -adrenoceptor subtype occupancies are presently available, so only relative *in vitro* affinities can predict human occupancies.

In order to support translation from animal pharmacology to human efficacy, we have included rat receptor affinity data for selected receptors (D_2 , 5-HT_{1A} , 5-HT_2 , and α_1 -adrenergic receptors). While there was consistency in the affinity (K_i values) between humans and rats for D_2 and 5-HT_{1A} receptors, $r5\text{-HT}_2$ receptor affinity was slightly lower than that for $h5\text{-HT}_{2A}$ receptors. A similar comparison could not be made for α_1 -adrenoceptors, due to assay limitations and lack of selective ligands.

To further improve the predictive validity of animal data for human pharmacology, *in vivo* and *ex vivo* binding potencies to rat CNS receptors have been included for brexpiprazole and aripiprazole. These results confirmed the equipotency of brexpiprazole at 5-HT_{1A} , $D_{2/3}$, and 5-HT_{2A} receptors, whereas the relative potencies of aripiprazole were largely different. In contrast, the low *in vivo* and *ex vivo* binding potencies of both compounds at 5-HT_6 and 5-HT_7 receptors suggest that these targets do not contribute to their pharmacological profiles in rats, although the $h5\text{-HT}_7$ receptor affinity was rather high for brexpiprazole (K_i 3.7 nM; Supplementary Material) and may contribute to the pharmacological profile in humans.

***In Vitro* and *In Vivo* Modulation of Serotonin-dopamine Activity**

Receptor binding affinity profiling is insufficient to characterize the functional effects of brexpiprazole, which displays partial agonist and antagonist activities at different receptor subtypes. Accordingly, functional characterization was performed at several human receptors *in vitro*, and at rat D_2 and 5-HT_{2A} receptors *in vivo*. Brexpiprazole showed partial agonist activity at hD_2 and hD_3 receptors in cloned cell assays, with lower intrinsic activity than aripiprazole. In

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accordance with this, brexpiprazole had lower maximum agonist effect than aripiprazole in an *in vivo* model of D₂ autoreceptor activity, inhibition of reserpine-induced L-DOPA accumulation, confirming a lower intrinsic activity at the D₂ receptor *in vivo*. D₂ autoreceptors develop increased agonist sensitivity following monoamine depletion after reserpine treatment (Hjorth et al., 1988), and consequently aripiprazole, but not brexpiprazole, had marked efficacy. In contrast, D₂ partial agonists, including aripiprazole and brexpiprazole, have functional D₂ antagonist activity in most tests of postsynaptic D₂ receptor-mediated effects, as described earlier (Arnt and Hyttel, 1990) and in the accompanying paper (Maeda et al., submitted).

In vitro functional studies at h5-HT_{1A} receptors suggest similar efficacies for brexpiprazole, aripiprazole and bupropion, although *in vivo* functional data in rats are not yet available. Brexpiprazole and aripiprazole showed antagonist activity *in vitro* at other serotonin receptor subtypes. For 5-HT_{2A} receptors, the *in vitro* profiling of brexpiprazole is supported by *in vivo* results showing potent inhibition of DOI-induced head twitches in rats that is consistent with its *in vivo* binding potency. Antagonism of 5-HT_{2A} receptors is the primary mechanism involved in head twitching, but 5-HT_{1A} agonists are also inhibitory (Arnt and Hyttel, 1989; Schreiber et al., 1995). Therefore, the precise mechanism involved in the effect of brexpiprazole is uncertain and needs further exploration. Brexpiprazole is a potent antagonist with no agonist activity at 5-HT_{2B} receptors, which is important for avoiding an increased risk of serious cardiac valvulopathy (Elangbam, 2010). Effects of brexpiprazole on other serotonin receptor subtypes in rats were weaker and are unlikely to be of importance at clinically relevant doses (Supplementary Material).

In acute microdialysis studies the functional effects of brexpiprazole on extracellular neurotransmitter levels *in vivo* have been explored in nucleus accumbens, prefrontal cortex, and

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ventral hippocampus. The results showed limited changes at the doses studied, suggesting that in an uncompromised system brexpiprazole has neutral effects as measured by microdialysis techniques. A small decrease in dopamine levels in nucleus accumbens paralleled with slight increases in the metabolites HVA and DOPAC may indicate D₂ partial agonism, as previously reported in the striatum (Jordan et al., 2004). In the prefrontal cortex the regulation of neurotransmitter levels is different from nucleus accumbens; while selective D₂ antagonists alone have no effect, D₂ antagonism in conjunction with, e.g. 5-HT_{2A} antagonism or 5-HT_{1A} partial agonism, may increase extracellular dopamine and acetylcholine (Mørk et al., 2009; Meltzer, 1999; 2012). Accordingly, brexpiprazole would be expected to increase dopamine and acetylcholine in the prefrontal cortex as well, which did not happen despite slight increases in levels of HVA and DOPAC. Similar results were seen with aripiprazole and they are likely due to D₂ partial agonism (Jordan et al., 2004; Li et al., 2004). Finally, high doses of brexpiprazole increased extracellular levels of histamine in the prefrontal cortex. This may be a consequence of its moderate affinity and antagonist activity on hH₁ receptors as shown *in vitro* (Table 2 and Supplementary Material, respectively; Fell et al., 2012). Accordingly, the risk for weight gain and metabolic adverse effects after brexpiprazole treatment is limited (Nasrallah, 2008).

Relevance of Target Profile for Clinical Efficacy and Side Effect Potential

As mentioned, brexpiprazole displayed significantly lower intrinsic activity at hD₂ and rD₂ receptors compared with aripiprazole. This may improve the tolerability of brexpiprazole with respect to D₂ agonist-mediated adverse effects, e.g. nausea, insomnia and akathisia (Fleischhacker, 2005). Also, the potential for D₂ antagonist-like adverse effects (e.g. EPS and hyperprolactinemia) may be lower than experienced with D₂ antagonist antipsychotics (Casey,

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1996; Maeda et al., submitted; Supplementary Material). The optimum balance between D₂ agonism and antagonism is not known, but the weak effects of brexpiprazole on extracellular monoamine levels in normal rats suggest that its low intrinsic activity stabilizes dopaminergic function. However, it remains to be documented whether these differences can be extended to the clinical profiles of the compounds.

The fact that the antagonist activity at 5-HT_{2A} and activity at D₂ receptors occurred at similar doses of brexpiprazole, whereas aripiprazole had a lower 5-HT_{2A} receptor occupancy at any given D₂ receptor occupancy may lead to clinical advantages, as 5-HT_{2A} antagonism is thought to contribute to antipsychotic activity, reduced akathisia potential, improvement of cognitive performance and sleep patterns, as well as effects on affective states, (Monti et al., 2010; Snigdha et al., 2010; Ebdrup et al., 2011; Laoutidis and Luckhaus, 2013).

The effects of brexpiprazole and aripiprazole on h5-HT_{1A} and hD₃ receptors are important for their pharmacological profiles. Partial agonism at 5-HT_{1A} and D₃ receptors is involved in the antipsychotic and procognitive profiles as well as effects on affective states (Newman-Tancredi and Kleven, 2011; Gross and Drescher, 2012). The combined 5-HT_{1A} and 5-HT_{2A} effects are of particular importance for the procognitive effect of brexpiprazole (see Maeda et al., submitted).

The consequences of the high affinities of brexpiprazole for (and antagonist effects on) α_{1B} - and α_{2C} -adrenoceptors are more difficult to predict, due to a lack of selective compounds for studying the functional importance of these receptors. Results using genetically modified mice suggest that α_{1B} -antagonism may contribute to antipsychotic-like activity and effects on stimulant-induced reward (Drouin et al., 2002). Inhibition of peripheral α_{1A} -adrenoceptors (for which brexpiprazole and aripiprazole have moderate affinities) are thought to be important for regulation of blood pressure, but may not contribute to the overall effects of brexpiprazole

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(Docherty et al., 2010). All α_1 -adrenoceptor subtypes are co-expressed with 5-HT_{2A} receptors in frontal cortex, but their relative functional importance is unknown (Santana et al., 2013).

Similarly, the α_{2C} -adrenoceptor field is immature, but antagonism has been proposed to contribute to antidepressant-like and procognitive activity (Quaglia et al., 2011; Sallinen et al., 2013). Clearly, more studies are necessary to explore the role of α -adrenoceptor antagonism for the profile of brexpiprazole.

In conclusion, brexpiprazole is a serotonin-dopamine activity modulator with potential for treatment of several CNS disorders. Brexpiprazole is in advanced clinical development.

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9. Authorship Contributions.

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12. Legends to figures.

Fig. 1. Chemical structure of brexpiprazole (OPC-34712).

Fig. 2. Partial agonist activity of brexpiprazole and reference drugs on human cloned 5-HT_{1A} receptors *in vitro*. Concentration-response curves are shown for brexpiprazole and reference compounds on [³⁵S]-GTPγS binding to human 5-HT_{1A} receptor-expressing HeLa cell membrane. Data are mean ± S.E.M. of three assays performed in triplicate each. [³⁵S]-GTPγS binding was normalized to the effect of 10 μM serotonin-induced [³⁵S]-GTPγS binding (set at 100 %).

Fig. 3. Partial agonist activity of brexpiprazole and reference drugs on human cloned D_{2L} (A) or D₃ receptors *in vitro* (B). Concentration-response curves are shown for brexpiprazole and reference compounds on forskolin-induced cAMP accumulation in hD_{2L} and hD₃ receptor-expressing CHO cells. Data are mean ± S.D. of three assays performed in duplicate (A) or triplicate (B). Cyclic AMP accumulation was normalized to the percentage of forskolin-induced cAMP accumulation (set at 100 %).

Fig. 4. Time-course of striatal dopamine D₂ receptor occupancy in rats following oral brexpiprazole (3 mg/kg) Abscissa indicates time in h, while left ordinate indicates receptor occupancy. Right ordinate indicates exposure in plasma (A) and brain (B). Each value represents the mean ± S.E.M. of results from three rats.

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Fig. 5. Relationship between plasma level and receptor occupancies in rats after brexpiprazole treatment. Brexpiprazole is administered p.o., 2 h before sacrifice, and *in vivo* binding measured in the brain for D₂ (n=5-6), 5-HT_{2A} (n=6), and 5-HT₆ (n=6) receptors and by *ex vivo* receptor autoradiography for 5-HT_{1A} (n=4) and 5-HT₇ (n=4) receptors. Mean ± S.E.M. of results from 4-6 rats. For ligands, see Methods section. Concentration-response curves are calculated by curve fitting. Abscissa shows average plasma exposure (ng/ml) at a given dose, and ordinate shows corresponding receptor occupancy of the indicated receptors. E_{max} was fixed to 100% occupancy during all regression analyses.

Fig. 6. Effect of brexpiprazole on extracellular levels of dopamine in rat nucleus accumbens, measured by microdialysis in awake rats. Brexpiprazole (1, 10, or 20 mg/kg, p.o.) or vehicle is administered at time 0, indicated by arrow. The ordinate shows extracellular levels of dopamine (A), HVA (B) and DOPAC (C), expressed in percentage of baseline levels. Mean ± S.E.M. of results from 8-16 rats. ****P* < 0.01 significant difference from vehicle group.

Fig. 7. Effect of brexpiprazole on extracellular levels of dopamine and metabolites in rat prefrontal cortex, measured by microdialysis in awake rats. Brexpiprazole (1, 3, or 10 mg/kg, p.o.) or vehicle is administered at time 0, indicated by arrow. The ordinate shows extracellular levels of dopamine (A), HVA (B) and DOPAC (C), expressed in percentage of baseline levels. Mean ± S.E.M. of results from 6-9 rats. **P* < 0.05 and ****P* < 0.01 significant difference from vehicle group.

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Fig. 8. Effect of brexpiprazole on extracellular levels of histamine in rat prefrontal cortex, measured by microdialysis in awake rats. Brexpiprazole (1, 3, 10, or 30 mg/kg, p.o.) or vehicle is administered at time 0, indicated by arrow. The ordinate shows extracellular levels of histamine, expressed in percentage of baseline levels. Mean \pm S.E.M. of results from 8 rats. * $P < 0.05$ significant difference from vehicle group.

13. Tables.

Table 1. Summary of binding assay condition for human and rat receptor assays *in vitro*.

Receptors	Source	Radioligand (K _d value, nM)	Cold ligand	Incubation buffer (mM conc's)	Incubation time and temperature
hD _{2L}	CHO/DHFR(-)	[³ H]-raclopride (2.2)	Butaclamol	50Tris-HCl (pH7.4), 120NaCl, 5KCl, 2CaCl ₂ , 1MgCl ₂	60 min, 25°C
rD ₂	Striatum	[³ H]-raclopride (2.1)	Butaclamol	50Tris-HCl (pH7.4), 120NaCl, 5KCl, 2CaCl ₂ , 1MgCl ₂	60 min, 25°C
hD ₃	CHO-K1	[³ H](+)-7-OH-DPAT (2.3)	GR103691	50Tris-HCl (pH7.4), 120NaCl, 5KCl, 2CaCl ₂ , 1MgCl ₂	60 min, 25°C
h5-HT _{1A}	HeLa	[³ H](+)-8-OH-DPAT(0.49)	(+)-8-OH-DPAT	50Tris-HCl (pH7.4), 10MgSO ₄ , 0.5EDTA, 0.1% ascorbate	60 min, 25°C
r5-HT _{1A}	Hippocampus	[³ H](+)-8-OH-DPAT(0.46)	WAY-100635	50Tris-HCl (pH7.4), 10MgSO ₄ , 0.5EDTA	60 min, 25°C
h5-HT _{2A}	CHO-K1	[³ H]-ketanserin (0.70)	Spiperone	50Tris-HCl (pH7.4)	20 min, 37°C
r5-HT _{2A}	Frontal cortex	[³ H]-ketanserin (1.1)	Spiperone	50Tris-HCl (pH7.4)	20 min, 37°C
h5-HT _{2B}	CHO cells	[¹²⁵ I]-LSD (0.41)	Serotonin	50Tris-HCl (pH7.4), 4CaCl ₂	30 min, 37°C
hα _{1B} adrenergic	CHO cells	[³ H]-prazosin (0.15)	Phentolamine	50 Tris-HCl (pH 7.4), 0.5EDTA	60 min, 22°C
hα _{2C} adrenergic	CHO cells	[³ H]-RX 821002 (2.0)	(-)-Adrenaline	50Tris-HCl (pH 7.4), 2MgCl ₂ , 1EDTA	60 min, 22°C
rα ₁ adrenergic	Cortex	[³ H]-prazosin (0.30)	Phentolamine	50Tris-HCl (pH7.4), 1EDTA	45 min, 30°C
hH ₁	CHO-K1	[³ H]-doxepine (0.49)	Pyrilamine	50Tris-HCl (pH7.4), 5MgCl ₂	90 min, 28°C
hM ₁	CHO-K1	[³ H]-methylscopolamine (0.4)	Atropine	50Tris-HCl (pH7.4), 10MgCl ₂ , 1EDTA	60 min, 25°C

Table 2. Binding affinities for cloned human receptors *in vitro*.

Human Receptor	Binding affinity (K _i , nM)			
	Brexipiprazole	Aripiprazole	Bifeprunox	Risperidone
hD _{2L}	0.30	0.87	0.41	1.9
hD ₃	1.1	1.6	0.63	13
h5-HT _{1A}	0.12	1.3	0.73	420 ^a
h5-HT _{2A}	0.47	4.7	NT	0.22
h5-HT _{2B}	1.9	0.36 ^d	NT	35 ^b
hα _{1B} adrenergic	0.17	35 ^d	NT	9 ^c
hα _{2C} adrenergic	0.59	38 ^d	NT	9.1 ^a
hH ₁	19	18	NT	27 ^a
hM ₁	67% at 10 μM	6780 ^d	NT	>10,000 ^c

Data are calculated by nonlinear regression analysis using data from three assay performed in duplicate or triplicate and expressed as mean values. Some values are taken from literature:

a) Schotte et al., 1996

b) Kroeze et al., 2003

c) PDSP certified data (<http://pdsp.med.unc.edu/pdsp>)

d) Shapiro et al., 2003

NT, not tested.

Table 3. Receptor binding affinities for rat receptors *in vitro*.

	Binding affinity (K_i , nM)		
	Brexiprazole	Aripiprazole	Risperidone
rD ₂	0.35	1.4	1.8
r5-HT _{1A}	0.09	1.2	250 ^a
r5-HT ₂	3.8	180	0.35
r α_1 adrenergic	18	290	1.2

K_i values are calculated by nonlinear regression analysis using data from 3 assay performed in duplicate or triplicate and expressed as mean values.

a) Data from Schotte et al., 1996

Table 4. Functional effects on human receptors *in vitro*. Agonist activity (E_{max} and EC_{50}) is shown for D_2 , D_3 , and 5-HT_{1A} receptors, while antagonist activity (cIC_{50}) is shown for 5-HT_{2A} , 5-HT_{2B} , α_{1B} - and α_{2C} -adrenergic receptors. No agonist activity of brexpiprazole is observed in the latter assays.

Compounds	h5-HT _{1A}		hD _{2L}		hD ₃		h5-HT _{2A}	h5-HT _{2B}	h α_{1B}	h α_{2C}
	[³⁵ S]GTP γ S binding		cAMP assay		cAMP assay		IP1	IP1	cAMP	cAMP
	E_{max} (%) ^c	EC_{50} (nM)	E_{max} (%) ^a	EC_{50} (nM)	E_{max} (%) ^b	EC_{50} (nM)	cIC_{50} (nM)			
Brexpiprazole	60 ± 4.7	0.49	43 ± 2.4	4.0	15 ± 1.2	2.8	6.5	14	0.66	63
Aripiprazole	73 ± 3.7	2.1	61 ± 1.4	5.6	28 ± 1.4	5.9	NT	NT	NT	NT
Bifeprunox	90 ± 0.85	3.2	84 ± 1.8	1.6	50 ± 1.2	11	NT	NT	NT	NT
Dopamine	NT	NT	100 ± 1.5	3.4	99 ± 1.5	3.5	NT	NT	NT	NT
Buspirone	78 ± 2.6	24	NT	NT	NT	NT	NT	NT	NT	NT
Serotonin	94 ± 1.8	5.1	NT	NT	NT	NT	NT	NT	NT	NT

E_{max} and EC_{50} (mean ± S.E.M.) in cAMP assays, calculated by nonlinear regression analysis.

cIC_{50} , corrected IC_{50} , calculated using the Cheng-Prusoff equation for functional assay: $cIC_{50} = IC_{50}/(1+[Ago]/EC_{50})$

a) E_{max} , expressed as percentage of maximum inhibition of dopamine.

b) E_{max} , expressed as percentage of the effect of 10 μ M dopamine.

c) E_{max} , expressed as percentage of the effect of 10 μ M serotonin.

NT, not tested.

Table 5. *In vivo* and *ex vivo* binding effects of brexpiprazole and aripiprazole on D₂ and 5-HT receptor subtypes in rat brain. Data are expressed as ED₅₀ (mg/kg, p.o., 2 h after drug administration) and EC₅₀ values in plasma and brain (ng/ml, 2 h after drug administration), both with 95% confidence intervals (CI) in parentheses. For receptors where maximum binding is too low to calculate ED₅₀ and IC₅₀ values, the percent specific binding at the highest dose or concentration is indicated as footnote.

Receptor	Brain Region	Brexpiprazole			Aripiprazole		
		ED ₅₀ (mg/kg)	EC ₅₀ plasma (ng/ml)	EC ₅₀ brain (ng/g)	ED ₅₀ (mg/kg)	EC ₅₀ plasma (ng/ml)	EC ₅₀ brain (ng/g)
<i>In vivo binding</i>							
rD ₂	Striatum	2.5 (2.2–2.8)	49 (41–57)	20 (17–23)	5.9 (5.1–6.7)	18 (15–22)	16 (14–19)
r5-HT _{2A}	Cortex	4.6 (3.6–6.0)	91 (70–120)	35 (26–48)	120 (49–300)	>520 ^e	>3000 ^g
r5-HT ₆	Striatum	17 (13–21)	1200 (940–1500)	410 (280–590)	>100 ^b	>520 ^e	>3000 ^g
<i>Ex vivo binding</i>							
r5-HT _{1A}	Hippocampus	5.6 (3.8–8.5)	320 (210–500)	ND	≈ 30 ^c	≈ 600	ND
r5-HT ₇	Paraventricular thalamus	>30 ^a	>1600	ND	>30 ^d	>610 ^f	ND

a) 41% occupancy at 30 mg/kg; b) 22% occupancy at 100 mg/kg; c) 53% occupancy at 30 mg/kg; d) 38% occupancy at 30 mg/kg; e) 520 ng/ml at 100 mg/kg

f) 610 ng/ml at 30 mg/kg; g) 3000 ng/ml at 100 mg/kg; ND, not determined; ≈, almost equal to.

Table 6. Functional effects of brexpiprazole and aripiprazole on rat D₂ and 5-HT_{2A} receptors *in vivo*. ED₂₅ or ED₅₀ values (mg/kg, p.o.) are indicated with 95 % confidence intervals (CI) in parentheses. Brexpiprazole and aripiprazole are administered 1 and 2 h before sacrifice (DOPA test) or DOI injection, respectively. For further methodology, see Methods section.

Test model (receptor)	Brexpiprazole		Aripiprazole	
	ED ₂₅ or ED ₅₀ (mg/kg) (95% CI)	Maximum response (%) (Mean ± S.E.M.)	ED ₂₅ or ED ₅₀ (mg/kg) (95% CI)	Maximum response (%) (Mean ± S.E.M.)
Reserpine-induced DOPA accumulation (D ₂)	4.4 (2.3–8.0) ^a	55 ± 3.7	3.7 (2.2–5.9) ^a	89 ± 2.4
DOI-induced head twitches (5-HT _{2A})	4.7 (3.2– 6.6) ^b	99 ± 0.9	21 (7.2–47) ^b	91 ± 3.6

a) ED₂₅ value

b) ED₅₀ value

Figure 1

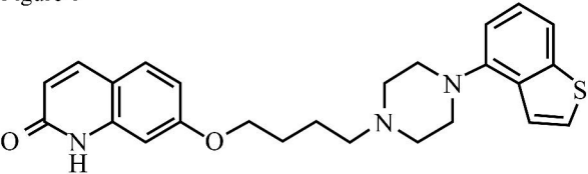


Figure 2

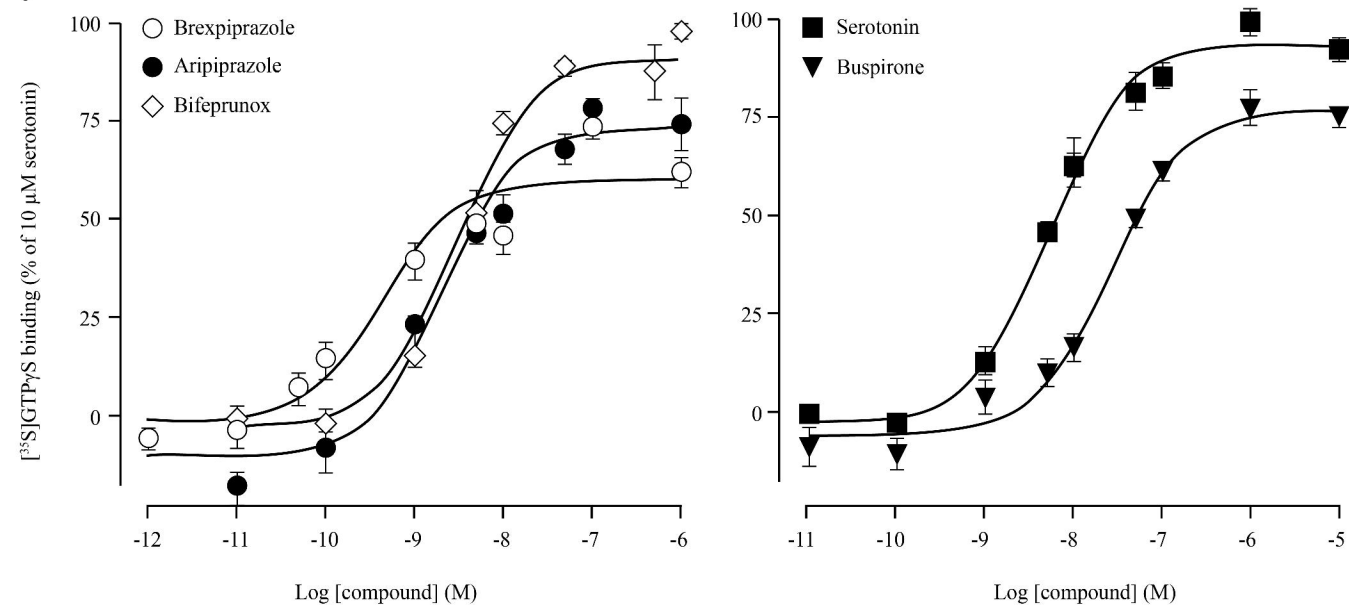
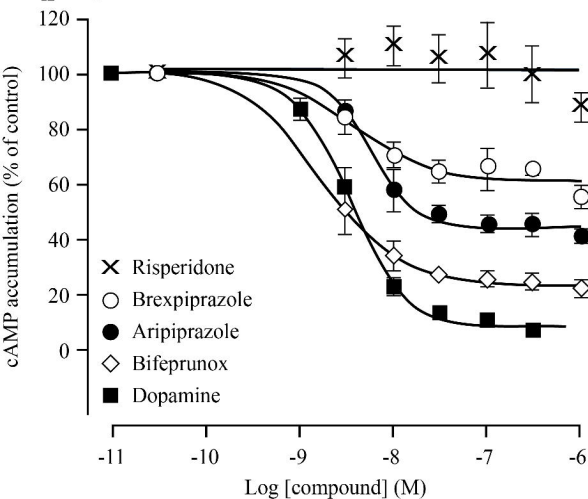


Figure 3

A. D_{2L} receptors



B. D_3 receptors

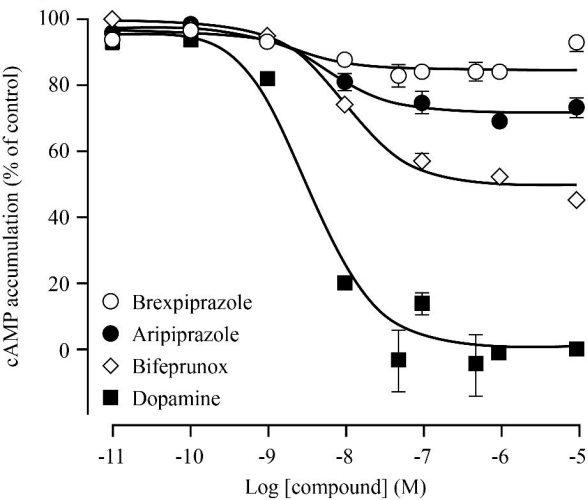
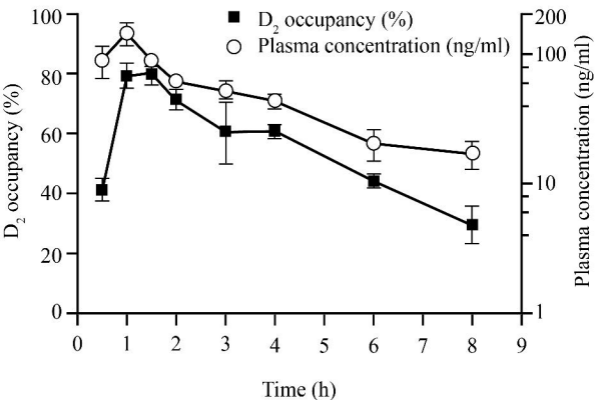


Figure 4

A



B

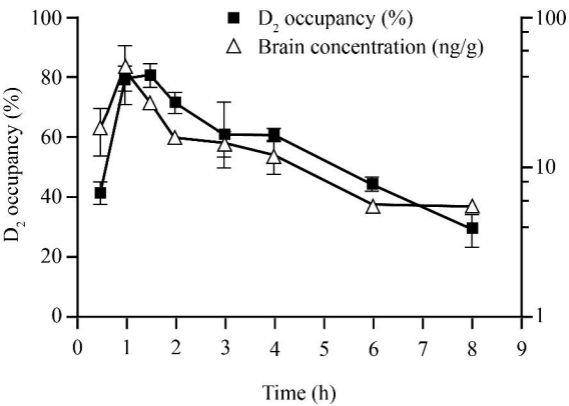


Figure 5

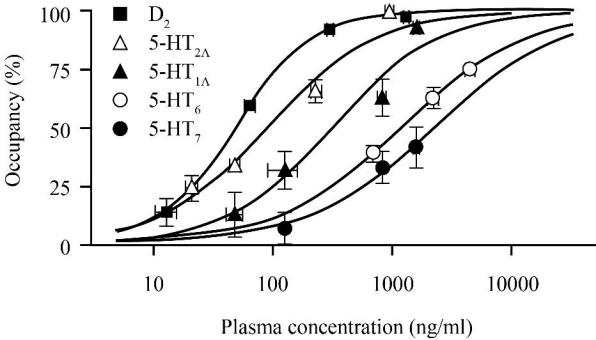
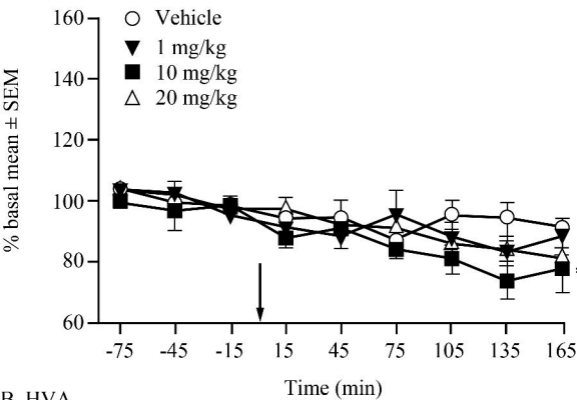
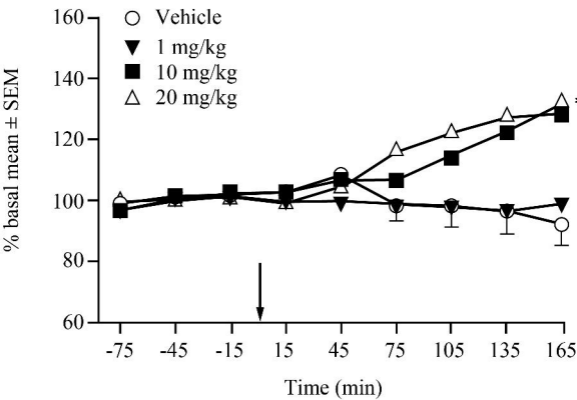


Figure 6

A. DA



B. HVA



C. DOPAC

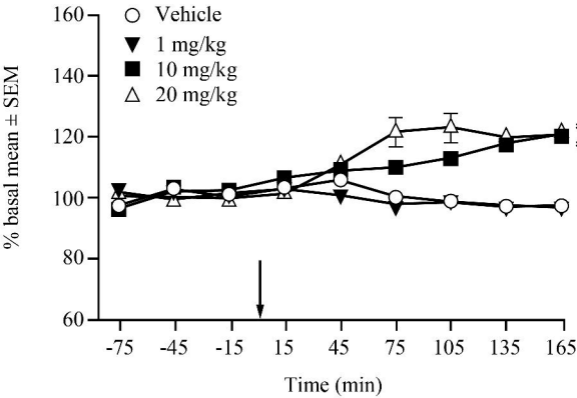
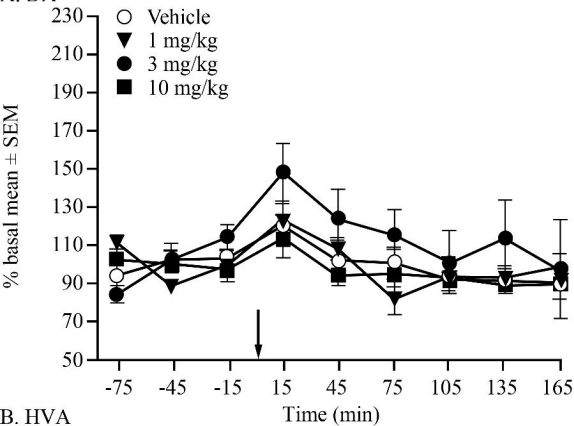
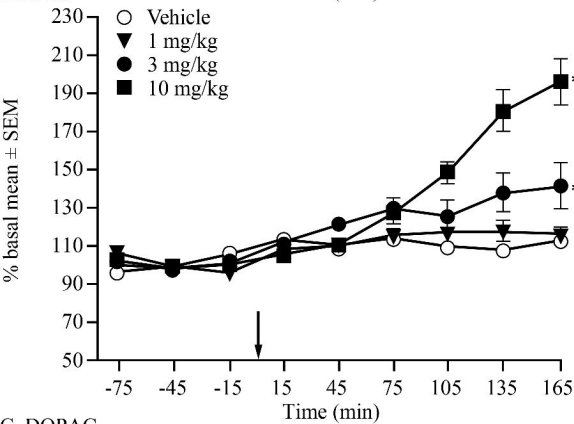


Figure 7

A. DA



B. HVA



C. DOPAC

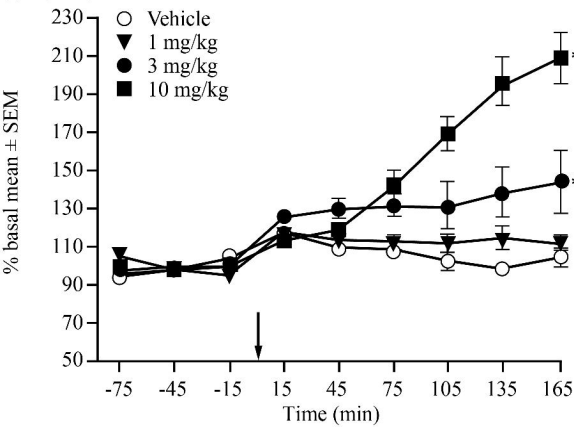


Figure 8

