

# Brexipiprazole I: In Vitro and In Vivo Characterization of a Novel Serotonin-Dopamine Activity Modulator<sup>§</sup>

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## ABSTRACT

Brexipiprazole (OPC-34712, 7-[4-[4-(1-benzothiophen-4-yl)piperazin-1-yl]butoxy]quinolin-2(1*H*)-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 human serotonin 1A (h5-HT<sub>1A</sub>), h5-HT<sub>2A</sub>, long form of human D<sub>2</sub> (hD<sub>2L</sub>), hα<sub>1B</sub>, and hα<sub>2C</sub>-adrenergic receptors. It displayed partial agonism at h5-HT<sub>1A</sub> and hD<sub>2</sub> receptors in cloned receptor systems and potent antagonism of h5-HT<sub>2A</sub> receptors and hα<sub>1B/2C</sub>-adrenoceptors. Brexipiprazole also had affinity ( $K_i < 5$  nM) for hD<sub>3</sub>, h5-HT<sub>2B</sub>, h5-HT<sub>7</sub>, hα<sub>1A</sub>, and hα<sub>1D</sub>-adrenergic receptors, moderate affinity for hH<sub>1</sub> ( $K_i = 19$  nM), and low affinity for hM<sub>1</sub> receptors ( $K_i > 1000$  nM). Brexipiprazole potently bound to rat 5-HT<sub>2A</sub> and D<sub>2</sub> receptors in vivo, and ex vivo binding studies further confirmed high 5-HT<sub>1A</sub> receptor binding potency. Brexipiprazole inhibited DOI (2,5-dimethoxy-4-iodoamphetamine)-induced

head twitches in rats, suggestive of 5-HT<sub>2A</sub> antagonism. Furthermore, in vivo D<sub>2</sub> partial agonist activity of brexipiprazole was confirmed by its inhibitory effect on reserpine-induced DOPA accumulation in rats. In rat microdialysis studies, brexipiprazole slightly reduced extracellular dopamine in nucleus accumbens but not in prefrontal cortex, whereas moderate increases of the dopamine metabolites, homovanillic acid and DOPAC (3,4-dihydroxy-phenyl-acetic acid), in these areas also suggested in vivo D<sub>2</sub> partial agonist activity. In particular, based on a lower intrinsic activity at D<sub>2</sub> receptors and higher binding affinities for 5-HT<sub>1A/2A</sub> receptors than aripiprazole, brexipiprazole would have a favorable antipsychotic potential without D<sub>2</sub> receptor agonist- and antagonist-related adverse effects. In conclusion, brexipiprazole 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.

## Introduction

The main strategy for treatment of schizophrenia is based on antagonizing dopamine D<sub>2</sub> receptors. In addition, most second-generation antipsychotics are antagonists of serotonin 5-HT<sub>2A</sub> receptors and α<sub>1</sub>-adrenoceptors, and individual compounds have a variety of effects on other monoamine

receptors, such as 5-HT<sub>1A</sub> 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).

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However, because of tolerability issues, treatment with D<sub>2</sub> receptor antagonists is not considered to be the optimal strategy to modulate dopaminergic activity, and the discovery and development of D<sub>2</sub> receptor partial agonists has provided a well tolerated treatment with stabilizing effects on dopamine function (Stahl, 2001; Citrome, 2013). So far, only one D<sub>2</sub> partial agonist, aripiprazole, with moderate D<sub>2</sub> intrinsic activity, has reached the market (Burriss et al., 2002; Potkin et al., 2003), whereas other compounds with higher D<sub>2</sub> 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 D<sub>2</sub> partial agonists is to ascertain how much intrinsic activity (or relative efficacy) is ideal in leading

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to optimal stabilization of dopaminergic transmission. If the D<sub>2</sub> intrinsic activity is too high, this can lead to lack of robust antipsychotic activity as well as pronounced adverse effects related to increased D<sub>2</sub> 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), whereas D<sub>2</sub> antagonist activity 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 D<sub>2</sub> receptors, but with lower intrinsic activity.

In addition to the issue of optimal D<sub>2</sub> 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% D<sub>2</sub> receptor occupancy, aripiprazole modulates a limited number of additional target receptors, the primary effect being partial agonist activity at 5-HT<sub>1A</sub> receptors with lower potency than at D<sub>2</sub> receptors (Mamo et al., 2007; Dahan et al., 2009). In addition, human 5-HT<sub>2A</sub> 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  $\alpha$ -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. (Tokyo, Japan) and is being developed in collaboration with H. Lundbeck A/S (Valby, Denmark). It combines 5-HT<sub>1A</sub> receptor partial agonism and low-intrinsic activity D<sub>2</sub> receptor partial agonism with antagonist activity on a variety of 5-HT and  $\alpha$ -adrenergic receptors. The main focus of this article is the receptors that are most influenced by brexpiprazole at clinically and pharmacologically relevant plasma exposures (more detailed profiling data can be found in Supplemental Figs. 1–3 and Supplemental Tables 1–4). The pharmacological effects of brexpiprazole in test

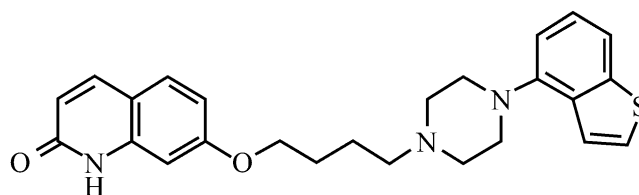


Fig. 1. Chemical structure of brexpiprazole (OPC-34712).

models of positive symptoms of schizophrenia and of cognitive impairment are described in an accompanying paper (Maeda et al., 2014).

## Materials and Methods

### Subjects

Male Wistar rats [in vitro rat binding, L-DOPA, and DOI (2,5-dimethoxy-4-iodoamphetamine) test; 145–200 g; Japan SLC Inc., Shizuoka, Japan] and Charles River (in vivo binding; 200–225 g; Koln, Germany) and male Sprague-Dawley rats from Charles River (dopamine and metabolite microdialysis; 220–375 g; Wilmington, MA), Charles River (dopamine, noradrenaline, and serotonin microdialysis; 275–300 g; Koln, Germany), and Harlan (300–360 g; Horst, The Netherlands) (histamine and acetylcholine microdialysis) have been used. All animals were maintained on a 12-hour 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 November 23, 2007, on Animal Testing, and with National Institutes of Health guide for the Care and Use of Laboratory Animals.

### Drugs

Brexpiprazole, aripiprazole, and risperidone were synthesized in our laboratory (Otsuka Pharmaceutical Co., Ltd.). Buspirone hydrochloride, butaclamol hydrochloride, DOI, DL-isoproterenol, dopamine, DOPA, DOPAC (3,4-dihydroxy-phenyl-acetic acid), homovanillic acid (HVA), NSD-1015 (3-hydroxybenzyl-hydrazine dihydrochloride), noradrenaline, (–)-raclopride, serotonin, and WAY-100635 (*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate) were obtained from Sigma-Aldrich (St. Louis, MO). Reserpine (Apoplone 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 l-octanesulfonate was obtained from

**ABBREVIATIONS:** 8-OH-DPAT, 2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene; aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; CHO, Chinese hamster ovary; cIC<sub>50</sub>, corrected IC<sub>50</sub> value; CNS, central nervous system; D, dopamine; D<sub>2L</sub>, long form of human D<sub>2</sub> receptor; DMSO, dimethylsulfoxide; DOI, 2,5-dimethoxy-4-iodoamphetamine; DOPAC, 3,4-dihydroxy-phenyl-acetic acid; EPS, extrapyramidal symptoms; [<sup>35</sup>S]GTP $\gamma$ S, guanosine 5'-O-(3-[<sup>35</sup>S]thio)-triphosphate; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; 5-HT, serotonin; HVA, homovanillic acid; IBMX, 3-isobutyl-1-methylxanthine; IMDM, Iscove's modified Dulbecco's medium; IP<sub>1</sub>, inositol monophosphate; Lu AE60157, 8-(4-methylpiperazin-1-yl)-3-(phenylsulfonyl)quinoline; M100907, (*R*)-(2,3-dimethoxyphenyl)[1-(4-fluorophenethyl)piperidin-4-yl]methanol; mPFC, medial prefrontal cortex; MS/MS, tandem mass spectrometry; NKH 477, (3*R*,4*aR*,5*S*,6*S*,6*aS*,10*S*,10*aR*,10*bS*)-5-acetoxy-10,10b-dihydroxy-3,4*a*,7,7,10*a*-pentamethyl-1-oxo-3-vinyldodecahydro-1*H*-benzo[*f*]chromen-6-yl 3-(dimethylamino)propanoate hydrochloride; NSD-1015, 3-hydroxybenzylhydrazine dihydrochloride; OPC-34712, 7-[4-[4-(1-benzothiophen-4-yl)piperazin-1-yl]butoxy]quinolin-2(1*H*)-one; RX 821002, 2-(2-methoxy-2,3-dihydrobenzo[*b*][1,4]dioxin-2-yl)-4,5-dihydro-1*H*-imidazole; SB269970, (*R*)-3-[2-[2-(4-methylpiperidin-1-yl)ethyl]pyrrolidin-1-ylsulfonyl]phenol; WAY-100635, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate.

Nacalai Tesque (Kyoto, Japan). The radioligands for *in vitro* studies, [ $^3\text{H}$ ]raclopride, [ $^3\text{H}$ ](+)-8-OH-DPAT (2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene), [ $^3\text{H}$ ]ketanserin, [ $^3\text{H}$ ]prazosin, [ $^{125}\text{I}$ ]lysergic acid diethylamide, [ $^3\text{H}$ ]methylscopolamine, and [ $^3\text{H}$ ]GTP $\gamma\text{S}$  (guanosine 5'-*O*-(3- $^{35}\text{S}$ )thio)-triphosphate) were purchased from PerkinElmer (Waltham, MA). [ $^3\text{H}$ ](+)-7-Hydroxy-2-(di-*n*-propylamino)tetralin and [ $^3\text{H}$ ]doxepin were purchased from American Biosciences (Piscataway, NJ) and American Radiolabeled Chemicals Inc. (St. Louis, MO), respectively. [ $^3\text{H}$ ]RX 821002 [2-(2-methoxy-2,3-dihydrobenzo[*b*][1,4]dioxin-2-yl)-4,5-dihydro-1*H*-imidazole] and [ $^3\text{H}$ ]prazosin were purchased from PerkinElmer and [ $^{125}\text{I}$ ]( $\pm$ )DOI from Chelatec (Saint-Herblain, France). For *in vivo* and *ex vivo* binding studies, the binding ligands were: [ $^3\text{H}$ ]raclopride (PerkinElmer), [ $^3\text{H}$ ]M100907 (volinanserin, (*R*)-2,3-dimethoxyphenyl)[1-(4-fluorophenethyl)piperidin-4-yl]methanol; Amersham Bioscience, Buckinghamshire, UK), [ $^3\text{H}$ ]Lu AE60157 [8-(4-methylpiperazin-1-yl)-3-(phenylsulfonyl)quinoline]; compound synthesized at Lundbeck A/S and tritiated by Red Glead Discovery, Lund, Sweden, [ $^3\text{H}$ ]8-OH-DPAT (PerkinElmer), and [ $^3\text{H}$ ]SB269970 (*R*)-3-[2-(4-methylpiperidin-1-yl)ethyl]pyrrolidin-1-ylsulfonylphenol; Amersham Bioscience).

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 methanesulfonic 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 vivo* studies were administered by mouth 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<sub>2L</sub> Receptor.** The establishment of the clonal Chinese hamster ovary (CHO) cell line lacking dehydrofolate reductase and stably expressing a high density of human recombinant D<sub>2L</sub> receptors was previously reported as CHO-hD<sub>2L</sub>-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  $\mu\text{g}/\text{ml}$  streptomycin, 200  $\mu\text{g}/\text{ml}$  G418, 0.1 mM sodium hypoxanthine, and 16  $\mu\text{M}$  thymidine in a humidified 5% CO<sub>2</sub> incubator maintained at 37°C.

**D<sub>3</sub> Receptor.** CHO cells stably coexpressing human recombinant D<sub>3</sub> receptors with rat adenylate cyclase V were established as follows. First, CHO-K1 cells were incubated with the pEF1-V5 HisA vector (Invitrogen, Grand Island, NY) inserted with a hD<sub>3</sub> receptor (Ser9 type) gene (amplified by polymerase chain reaction 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. Second, to increase sensitivity to stimuli at D<sub>3</sub> receptors (Robinson and Caron, 1997), rat adenylate cyclase V was cotransfected in hD<sub>3</sub> 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 polymerase chain reaction 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  $\mu\text{g}/\text{ml}$  hygromycin B, 200  $\mu\text{g}/\text{ml}$  G418, and Lipofectamine 2000, and a single cell line was isolated by limiting dilution. The cells were cultured in F-12 Ham's medium supplemented with 2 mM L-glutamine, 400  $\mu\text{g}/\text{ml}$  hygromycin B, 200  $\mu\text{g}/\text{ml}$  G418, and 10% fetal bovine serum in a humidified 5% CO<sub>2</sub> incubator maintained at 37°C.

**5-HT<sub>1A</sub> Receptor.** Human cervical epithelial adenocarcinoma cells expressing h5-HT<sub>1A</sub> receptors were established as follows. Human cervical epithelial adenocarcinoma cells were incubated with the pEF1-V5 HisA vector (Invitrogen) inserted with a h5-HT<sub>1A</sub> receptor

gene (Origene, Rockville, MD) in serum-free minimum essential medium supplemented with 0.3% G418 and Lipofectamine 2000. Then, a single cell line was isolated by limiting dilution. The cells were cultured in minimum essential medium supplemented with 0.3% G418, 10% fetal bovine serum in a humidified 5% CO<sub>2</sub> incubator maintained at 37°C.

**5-HT<sub>2B</sub> Receptor.** A CHO cell line expressing human recombinant 5-HT<sub>2B</sub> 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% nonessential amino acids, 10% fetal bovine serum dialyzed in a humidified 5% CO<sub>2</sub> incubator maintained at 37°C.

**$\alpha_{1B}$ - and  $\alpha_{2C}$ -Adrenoceptor.** CHO cell lines expressing human recombinant  $\alpha_{1B}$ - and  $\alpha_{2C}$ -adrenoceptors were constructed by Cerep S.A.

## Cell Membrane Preparation

**D<sub>2L</sub> Receptor.** hD<sub>2L</sub> 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 minutes at 4°C. The pellets were resuspended with ice-cold assay buffer 50 mM Tris-HCl (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and stored at -80°C until use.

**D<sub>3</sub> Receptor.** hD<sub>3</sub> 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 minutes at 4°C. The pellet was resuspended with 50 mM Tris-HCl (pH 7.4) containing 10 mM MgSO<sub>4</sub>, 0.5 mM EDTA, and 0.1% ascorbic acid, and stored at -135°C until use.

**5-HT<sub>1A</sub> Receptor.** h5-HT<sub>1A</sub> 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 minutes at 4°C. The pellet was resuspended with the same buffer and centrifuged twice. The pellet was resuspended 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<sub>2A</sub>, 5-HT<sub>2B</sub>,  $\alpha_{1A}$ -adrenergic, histamine H<sub>1</sub>, and muscarinic M<sub>1</sub> receptors were purchased from PerkinElmer.

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

## Rat Brain Membrane Preparation

Wistar rats for D<sub>2</sub>, 5-HT<sub>2</sub>, and  $\alpha_1$ -adrenergic receptors and Sprague-Dawley rats for 5-HT<sub>1A</sub> receptors were decapitated and the brains were removed and placed on ice.

**D<sub>2</sub> 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, Lucerne, Switzerland) in ice-cold 50 mM Tris-HCl buffer (pH 7.4), and centrifuged at 48,000g for 10 minutes at 4°C. The pellet was suspended in the same buffer, incubated at 37°C for 10 minutes, and centrifuged under the same conditions once more. The final pellet was resuspended in ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>, and stored at -80°C until use.

**5-HT<sub>1A</sub> 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 1000g for 5 minutes at 4°C. The supernatant was stored on ice. The pellet was rehomogenized 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 minutes at 4°C. The pellet was suspended in the same buffer and centrifuged at 27,000g for 20 minutes at 4°C. The final pellet was suspended in the same buffer and stored at -135°C until use.

**5-HT<sub>2</sub> 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 1000g for 10 minutes at 4°C. The supernatant was stored on ice. The pellet was rehomogenized 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 minutes 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.

**$\alpha_1$ -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 minutes at 4°C. The pellet was suspended in the same buffer. After incubation for 10 minutes at 37°C, the suspension was centrifuged under the same conditions. The same process of suspension and 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<sub>2B</sub>, h $\alpha_{1B}$ , h $\alpha_{2C}$ , and hM<sub>1</sub> Receptors).** The reaction mixture was transferred to the filter plate (UniFilter-96 GF/B; PerkinElmer) or Whatman GF/B filter (Sigma-Aldrich) using the harvester (FilterMate; PerkinElmer) or Brandel Harvester (Brandel Biomedical Research and 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) 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) to calculate the counting efficacy of the radioactivity.

**5-HT<sub>2B</sub> 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 minutes

using a gamma counter (COBRA; PerkinElmer). These studies were performed at Sekisui Medical Co., Ltd. (Ibaraki, Japan).

**$\alpha_{1B}$ - and  $\alpha_{2C}$ -Adrenoceptor.** The incubation mixtures were filtered through glass fiber filters (GF/B; Packard, Meriden, CT) presoaked with 0.3% polyethylenimine and rinsed four times with ice-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.

**M<sub>1</sub> 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) for 2 minutes. This study was performed at Sekisui Medical Co., Ltd.

### Forskolin-Induced cAMP Accumulation at hD<sub>2L</sub> and hD<sub>3</sub> Receptors

**D<sub>2L</sub> Receptor.** hD<sub>2L</sub> receptor expressing cells were seeded at 10<sup>4</sup> cells per well in poly-L-lysine-coated 96-well plates and grown for 2 days. The cells were washed with IMDM supplemented with 0.1 mM sodium hypoxanthine and 16  $\mu$ M thymidine. Cells were preincubated with test compounds for 20 minutes at 37°C in IMDM medium supplemented with 0.1% sodium ascorbate, 0.1 mM sodium hypoxanthine, and 16  $\mu$ M thymidine. Then, 10  $\mu$ M forskolin and 500  $\mu$ M IBMX (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 minutes 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.

**D<sub>3</sub> Receptor.** A Flashplate adenyl cyclase activation assay system (PerkinElmer) was used to determine the intracellular cAMP concentration in each well. hD<sub>3</sub> receptor expressing cells (4  $\times$  10<sup>5</sup> cells/well) were incubated with test compounds in a Flashplate well containing 0.1  $\mu$ M forskolin for 1 hour at 25°C. Subsequent steps of the assay procedure were conducted according to the manufacturer's instructions. Final concentration of DMSO was 1%.

TABLE 1  
Summary of binding assay condition for human and rat receptor assays in vitro

Receptors	Source	Radioligand ( $K_d$ Value)	Cold Ligand	Incubation Buffer	Incubation Time and Temperature
		<i>nM</i>		<i>mM concentration</i>	
hD <sub>2L</sub>	CHO/DHFR(-)	[ <sup>3</sup> H]Raclopride (2.2)	Butaclamol	50 Tris-HCl (pH 7.4), 120 NaCl, 5 KCl, 2 CaCl <sub>2</sub> , 1 MgCl <sub>2</sub>	60 minutes, 25°C
rD <sub>2</sub>	Striatum	[ <sup>3</sup> H]Raclopride (2.1)	Butaclamol	50 Tris-HCl (pH 7.4), 120 NaCl, 5 KCl, 2 CaCl <sub>2</sub> , 1 MgCl <sub>2</sub>	60 minutes, 25°C
hD <sub>3</sub>	CHO-K1	[ <sup>3</sup> H](+)-7-OH-DPAT (2.3)	GR103691	50 Tris-HCl (pH 7.4), 120 NaCl, 5 KCl, 2 CaCl <sub>2</sub> , 1 MgCl <sub>2</sub>	60 minutes, 25°C
h5-HT <sub>1A</sub>	HeLa	[ <sup>3</sup> H](+)-8-OH-DPAT(0.49)	(+)-8-OH-DPAT	50 Tris-HCl (pH 7.4), 10 MgSO <sub>4</sub> , 0.5 EDTA, 0.1% ascorbate	60 minutes, 25°C
r5-HT <sub>1A</sub>	Hippocampus	[ <sup>3</sup> H](+)-8-OH-DPAT(0.46)	WAY-100635	50 Tris-HCl (pH 7.4), 10 MgSO <sub>4</sub> , 0.5 EDTA	60 minutes, 25°C
h5-HT <sub>2A</sub>	CHO-K1	[ <sup>3</sup> H]Ketanserin (0.70)	Siperone	50 Tris-HCl (pH 7.4)	20 minutes, 37°C
r5-HT <sub>2</sub>	Frontal cortex	[ <sup>3</sup> H]Ketanserin (1.1)	Siperone	50 Tris-HCl (pH 7.4)	20 minutes, 37°C
h5-HT <sub>2B</sub>	CHO cells	[ <sup>125</sup> I]LSD (0.41)	Serotonin	50 Tris-HCl (pH 7.4), 4 CaCl <sub>2</sub>	30 minutes, 37°C
h $\alpha_{1B}$ -Adrenergic	CHO cells	[ <sup>3</sup> H]Prazosin (0.15)	Phentolamine	50 Tris-HCl (pH 7.4), 0.5 EDTA	60 minutes, 22°C
h $\alpha_{2C}$ -Adrenergic	CHO cells	[ <sup>3</sup> H]RX 821002 (2.0)	(-)-Adrenaline	50 Tris-HCl (pH 7.4), 2 MgCl <sub>2</sub> , 1 EDTA	60 minutes, 22°C
r $\alpha_1$ -Adrenergic	Cortex	[ <sup>3</sup> H]Prazosin (0.30)	Phentolamine	50 Tris-HCl (pH 7.4), 1 EDTA	45 minutes, 30°C
hH <sub>1</sub>	CHO-K1	[ <sup>3</sup> H]Doxepine (0.49)	Pyrilamine	50 Tris-HCl (pH 7.4), 5 MgCl <sub>2</sub>	90 minutes, 28°C
hM <sub>1</sub>	CHO-K1	[ <sup>3</sup> H]Methylscopolamine (0.4)	Atropine	50 Tris-HCl (pH 7.4), 10 MgCl <sub>2</sub> , 1 EDTA	60 minutes, 25°C

7-OH-DPAT, 7-hydroxy-2-(di-*n*-propylamino)tetralin; DHFR, dehydrofolate reductase; HeLa, Human cervical epithelial adenocarcinoma; LSD, lysergic acid diethylamide.

### h5-HT<sub>1A</sub> Receptor GTP $\gamma$ S Binding Assay

The agonistic activities of test compounds were evaluated using [<sup>35</sup>S]GTP $\gamma$ S binding to h5-HT<sub>1A</sub> receptor-expressing cell membranes. The membrane preparation was incubated in 25 mM Tris-HCl (pH 7.4) buffer containing 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 0.55  $\mu$ M GDP, and 0.03 nM [<sup>35</sup>S]GTP $\gamma$ S with test compounds for 1 hour 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<sub>2A</sub> Receptor Inositol Monophosphate-Homogeneous Time-Resolved Fluorescence Functional Assay

Cells were suspended in buffer containing 10 mM HEPES/NaOH (pH 7.4), 4.2 mM KCl, 146 mM NaCl, 1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 5.5 mM glucose, and 50 mM LiCl and then distributed in microplates at a density of  $1.5 \times 10^4$  cells/well. For agonist functional assays, the cells were incubated for 30 minutes at 37°C in the presence of buffer (basal control), test compound, or reference agonist. For stimulated control measurement, separate assay wells contained 10  $\mu$ M serotonin. For antagonist functional assays, the cells were preincubated for 5 minutes 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 basal control measurements, separate assay wells without serotonin were used. Cells were further incubated for 30 minutes.

After incubation, the cells were lysed and the fluorescence acceptor (d2-labeled inositol monophosphate [IP<sub>1</sub>]) and fluorescence donor (anti-IP<sub>1</sub> antibody labeled with europium cryptate) were added. After 60 minutes 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 Labtech, Cary, NC). The IP<sub>1</sub> 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  $\mu$ M serotonin. These studies were performed at Cerep S.A.

### h5-HT<sub>2B</sub> Receptor IP<sub>1</sub>-Homogeneous Time-Resolved Fluorescence 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 CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 5.5 mM glucose, and 50 mM LiCl and then distributed in microplates at a density of  $4 \times 10^4$  cells/well and incubated for 30 minutes at 37°C in the presence of buffer (basal control), test compound, or reference agonist. For agonist functional assays, separate assay wells containing 1  $\mu$ 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. After incubation, the cells were lysed and the fluorescence acceptor (d2-labeled IP<sub>1</sub>) and fluorescence donor (anti-IP<sub>1</sub> antibody labeled with europium cryptate) were added. After 60 minutes 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 IP<sub>1</sub> 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 control response to 1  $\mu$ M 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.

### h $\alpha$ <sub>1B</sub>-Adrenoceptor cAMP-Homogeneous Time-Resolved Fluorescence Functional Assay

The cells were suspended in Hanks' balanced salt solution (HBSS) buffer (Invitrogen) complemented with 20 mM HEPES (pH 7.4) and

500  $\mu$ M IBMX, then distributed in microplates at a density of  $2 \times 10^4$  cells/well and incubated for 30 minutes 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  $\mu$ M adrenaline were used as functional control. For antagonist functional assays, the reference agonist adrenaline was added at a final concentration of 3  $\mu$ M. Separate assay wells without adrenaline were used as basal control. After 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 minutes 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 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  $\mu$ M adrenaline. These studies were performed at Cerep S.A.

### h $\alpha$ <sub>2C</sub>-Adrenoceptor cAMP-Homogeneous Time-Resolved Fluorescence Functional Assay

The cells were suspended in HBSS buffer (Invitrogen) complemented with 20 mM HEPES (pH 7.4) and 500  $\mu$ M IBMX and 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 adrenaline at 1  $\mu$ M or various concentrations (EC<sub>50</sub> determination), or the test compounds. Next, the adenylyl cyclase activator NKH 477 [(3R,4aR,5S,6S,6aS,10S,10aR,10bS)-5-acetoxy-10,10b-dihydroxy-3,4a,7,7,10a-pentamethyl-1-oxo-3-vinyldecahydro-1H-benzof[1]chromen-6-yl 3-(dimethylamino)propanoate hydrochloride] was added at a final concentration of 5  $\mu$ M. For antagonism measurement, the cells were incubated with HBSS (stimulated control), the reference antagonist rauwolscine at 10  $\mu$ M (basal control) or various concentrations (IC<sub>50</sub> determination), or the test compounds. Next, the reference agonist adrenaline (100 nM) and the adenylyl cyclase activator NKH 477 (5  $\mu$ M) were added. For basal control measurements, adrenaline was omitted from the wells containing 3  $\mu$ M rauwolscine.

After 10-minute 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 minutes 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 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  $\mu$ M 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<sub>50</sub>, EC<sub>50</sub>, and  $E_{max}$  values were calculated by nonlinear 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,  $K_i = IC_{50}/[1 + (\text{radioligand concentration})/K_d]$  (Cheng and Prusoff, 1973). In functional assays, cIC<sub>50</sub> 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<sub>2</sub>, r5-HT<sub>2A</sub>, and r5-HT<sub>6</sub> Receptors in Rat Brain

Occupancy for the D<sub>2</sub>, 5-HT<sub>2A</sub>, and 5-HT<sub>6</sub> receptors was measured by in vivo binding with [<sup>3</sup>H]raclopride (D<sub>2</sub> receptor binding), [<sup>3</sup>H]M100907 (5-HT<sub>2A</sub> binding), or [<sup>3</sup>H]Lu AE60157 (5-HT<sub>6</sub> binding), respectively (Idris et al., 2010). Briefly, 10  $\mu$ Ci (D<sub>2</sub> and 5-HT<sub>6</sub> binding)

or 15  $\mu\text{Ci}$  (5-HT<sub>2A</sub> binding) was injected intravenously in the tail vein. Fifteen minutes after the injection, the animals were killed by a blow to the head and decapitated, and the striatum (D<sub>2</sub> and 5-HT<sub>6</sub> binding) or cortex (5-HT<sub>2A</sub> binding) was dissected. The tissue was homogenized in ice-cold buffer [50 mM KPO<sub>4</sub>, pH 7.4 (for D<sub>2</sub>); 50 mM Tris, pH 7.7 (for 5-HT<sub>2A</sub> and 5-HT<sub>6</sub>)], samples were filtered through Whatman GF/C filters, and the filters were washed with 2 × 5 ml ice-cold buffer. Filtration was completed 60–120 seconds after death. 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 bicinchoninic acid–enzyme-linked immunosorbant and was subsequently used for normalization. Cerebellum was dissected from vehicle-treated animals and used to determine nonspecific binding.

Data are presented as percentage (%) receptor occupancy  $\pm$  S.E.M. for each receptor subtype investigated. ED<sub>50</sub> values were calculated using nonlinear regression analysis (GraphPad Prism).

### Ex Vivo Binding to r5-HT<sub>1A</sub> and r5-HT<sub>7</sub> Receptors in Rat Brain

Blood and brains were collected 2 hours after p.o. administration of brexpiprazole, aripiprazole, or vehicle. Rats were anesthetized using CO<sub>2</sub> gas and killed by decapitation. Trunk blood was collected in vacutainers containing EDTA and gently mixed for 30 seconds before being placed on ice. Later, blood was centrifuged at 3000g for 15 minutes 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  $\mu\text{m}$  thickness using a cryostat and mounted on slides. Slices were collected beginning at approximately –4.5 mm posterior to bregma for the 5-HT<sub>1A</sub> receptor assay and –2.1 mm posterior from bregma for the 5-HT<sub>7</sub> 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 minutes. In some cases, slides were briefly preincubated in the appropriate buffer (see below) and placed under constant airflow until completely dried. Subsequently, slides were incubated in an assay buffer that included the appropriate tritiated radioligand. Nonspecific 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 nonradioactive competitor for the target receptor. After incubation, slides were washed twice for 5 minutes in cold (4°C) assay buffer and dried under constant airflow for 1 hour before being transferred to a vacuum dessicator for at least 1 hour. Finally, slides were exposed using a Beta-imager (Biospace Laboratories, Paris, France) for 20–24 hours. Specific details for each autoradiography assay are listed below.

**5-HT<sub>1A</sub> Receptor Occupancy.** 5-HT<sub>1A</sub> receptor occupancy was conducted as previously reported (du Jardin et al., 2014), with minor modifications. Briefly, slides were incubated for 1 hour in assay buffer consisting of 170 mM Tris-HCl, 4 mM CaCl<sub>2</sub>, 0.1% L-ascorbic acid, and 10  $\mu\text{M}$  of pargyline (pH 7.4) and containing 3 nM of the radioligand [<sup>3</sup>H]8-OH-DPAT. Nonspecific binding was determined by including 1  $\mu\text{M}$  of the 5-HT<sub>1A</sub> receptor antagonist WAY-100635 in the incubation medium. Surface radioactivity was quantified in the hippocampus.

**5-HT<sub>7</sub> Receptor Occupancy.** Defrosted slides were preincubated for 3 minutes at room temperature in an assay buffer containing 170 mM Tris HCl, 4 mM CaCl<sub>2</sub>, and 0.5  $\mu\text{M}$  L-ascorbic acid (pH 7.4). After drying, slides were incubated for 1 hour in the assay buffer described above with 100  $\mu\text{M}$  of pargyline and 5.9 nM of the 5-HT<sub>7</sub>-selective radioligand [<sup>3</sup>H]SB269970. Nonspecific binding was determined by the inclusion of 1  $\mu\text{M}$  nonradioactive SB269970. Subsequently, slides were rinsed, dried, and exposed in a  $\beta$ -imager as described above. Surface radioactivity for the 5-HT<sub>7</sub> 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., 2014). Results are expressed as mean  $\pm$  S.E.M. Where appropriate, ED<sub>50</sub>/EC<sub>50</sub> values and 95% confidence intervals were determined using GraphPad Prism. Doses or exposure levels were log transformed, and a nonlinear 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) 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. Brexpiprazole or aripiprazole concentrations were determined using ultraperformance liquid chromatography followed by tandem mass spectrometry (MS/MS) detection in positive-ion electrospray ionization 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 hours before death. Brexpiprazole or aripiprazole were administered by mouth 1 and 2 hours before death, respectively. Next, NSD-1015 (100 mg/kg i.p.) was injected 30 minutes before death. Each rat was killed by exposure to head-focused microwave in a microwave applicator (power; 5.5, 2.1 second, 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  $\mu\text{M}$  EDTA 2Na, and 0.1  $\mu\text{g/ml}$  DL-isoproterenol (which is used as an internal standard substance). After incubation for 1 hour on ice, the homogenates were centrifuged at 20,000g for 15 minutes at 4°C. The supernatants were filtered through a disposable syringe filter unit (DISMIC-3cp, cellulose acetate, 0.45  $\mu\text{m}$ ; Advantec, Tokyo, Japan). The amount of DOPA in each supernatant was measured by an high-performance liquid chromatography (HPLC) system (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 1-octanesulfonate, 14.9  $\mu\text{M}$  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) group 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. ED<sub>25</sub> values were calculated by nonlinear regression analysis using SAS software (SAS institute Japan, Tokyo, Japan).

### DOI-Induced Head Twitches in Rats

After systemic injection, DOI induces characteristic head twitches that are mediated by CNS 5-HT<sub>2A</sub> receptors (Sanchez and Arnt, 2000). Wistar rats were fasted for 16 to 22 hours before administration of



test compounds. Brexipiprazole or risperidone were administered by mouth 1 hour before injection of DOI (5 mg/kg s.c.), whereas aripiprazole was administered 2 hours 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 minutes immediately after DOI injection. The mean number of head twitches per 10 minutes in control groups varied between 18 and 21. The experimenter was blinded to the drug and dose to avoid bias. ED<sub>50</sub> values were calculated by nonlinear regression analysis using SAS software.

### 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  $\mu$ l/min) with sterile artificial cerebrospinal fluid (aCSF) (145 mM NaCl, 2.7 mM KCl, 1 mM MgCl<sub>2</sub>, 1.2 mM CaCl<sub>2</sub>, 2 mM Na<sub>2</sub>HPO<sub>4</sub>; pH 7.4, or 1.2 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.27 mM NaH<sub>2</sub>PO<sub>4</sub>, 140 mM NaCl, 3 mM KCl, 2.5 mM CaCl<sub>2</sub>, 7.2 mM glucose; pH 7.29). Microdialysate samples were collected at 30-minute intervals (CMA-142 microfraction collector) into silanized microvials. Vehicle and test/reference compounds were administered 3.5 hours after microdialysis probe implantation, and microdialysate sampling continued for 3 hours thereafter. A dissecting microscope was used to verify correct microdialysis probe placement at the end of each experiment. Isocratic, HPLC-electrochemical detection 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 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 brexipiprazole were revealed using repeated measures of ANOVA followed by two-tailed Dunnett's test. Mean ( $\pm$  S.E.M.) basal concentrations (pg/ $\mu$ l) of dopamine, DOPAC, and HVA detected in microdialysate collected from the nucleus accumbens (without correction for probe recovery) were 0.81  $\pm$  0.08 (*n* = 49), 124  $\pm$  6.2 (*n* = 49), and 66.3  $\pm$  3.6 (*n* = 61), respectively. For the mPFC, basal values of dopamine, DOPAC, and HVA were 0.083  $\pm$  0.006 (*n* = 77), 26.2  $\pm$  1.48 (*n* = 89), and 32.3  $\pm$  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 MgCl<sub>2</sub>, 1.2 mM CaCl<sub>2</sub>) for the duration of the experiment at a constant flow rate of 1.3  $\mu$ l/min. After

180 minutes of stabilization, the experiments were initiated. Dialysates were collected every 30 minutes. After the experiments the animals were killed, 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 analyzed by means of HPLC with electrochemical detection. The monoamines were separated by reverse phase liquid chromatography (ODS 150  $\times$  2 mm column). The mobile phase consisted of 75 mM Li-acetate, 4 mM sodium 1-heptane sulfonate, 100  $\mu$ 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<sub>2</sub> = 250 mV (Coulchem II; ESA, Chelmsford, MA). The absolute basal levels without considering probe recovery were 2.1  $\pm$  0.27 (*n* = 30) and 10.7  $\pm$  0.73 (*n* = 33) fmol/20  $\mu$ l dialysate for cortical serotonin and noradrenaline, respectively. The absolute basal levels without considering probe recovery were 2.7  $\pm$  0.52 (*n* = 21) and 12.8  $\pm$  1.49 (*n* = 22) fmol/20  $\mu$ 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 preinjection 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, Chicago, IL).

**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, Groningen, 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 CaCl<sub>2</sub>, and 1.2 mM MgCl<sub>2</sub> was perfused at a flow rate of 1.5  $\mu$ l/min. After 2-hour prestabilization, microdialysis samples were collected into minivials (Microbiotech/se AB, Stockholm, Sweden; 4001029). All samples were stored at -80°C until offline analyses. The content of acetylcholine 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  $\times$  150 mm, particle size: 4  $\mu$ m; Torrance, CA) and 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 H<sub>2</sub>O) at a flow rate of 0.25 ml/min. A postcolumn make-up 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 (Applied Biosystems, Foster City, CA).

Analysis of histamine was done by HPLC and 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, Bleiswijk, 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 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 preadministration 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 postadministration samples were expressed as a percentage of basal levels within the same subject. Data are expressed as levels relative to

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  $1.93 \pm 0.14$  and  $4.49 \pm 0.26$  nM ( $n = 40$ ), respectively.

## 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 Supplemental Table 1.

**Human Receptors.** Brexpiprazole showed subnanomolar binding affinities ( $K_i$ , nanomolar, indicated in parentheses) for h5-HT<sub>1A</sub> (0.12), hD<sub>2L</sub> (0.30), and h5-HT<sub>2A</sub> (0.47) receptors, as well as for h $\alpha_{1B}$  (0.17)- and h $\alpha_{2C}$ -adrenoceptors (0.59). Brexpiprazole had nanomolar affinity for hD<sub>3</sub> (1.1), h5-HT<sub>2B</sub> (1.9), h5-HT<sub>7</sub> (3.7) receptors, and h $\alpha_{1A}$ -adrenoceptors (3.8) (Table 2; Supplemental Table 2). The affinities of brexpiprazole for hH<sub>1</sub> (19) and for hM<sub>1</sub> (67% binding at 10  $\mu$ M) receptors were moderate and very low, respectively.

A number of other receptor and target affinities have been investigated. Results are listed in Supplemental Table 2, because 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 (Supplemental Table 4), the affinities for human dopamine, noradrenaline, and serotonin transporters were weaker than those for the above mentioned receptors (Supplemental Table 2).

**Rat Receptors.** The affinities of brexpiprazole for selected rat receptors are presented in Table 3. The affinities for r5-HT<sub>1A</sub> (0.09 nM) and rD<sub>2</sub> (0.35 nM) receptors were consistent with the human receptor affinities, whereas the affinity for r5-HT<sub>2</sub> receptors (3.8 nM) was about 10 times lower than for human 5-HT<sub>2A</sub> receptors. The  $\alpha_1$ -adrenoceptor affinity (nonselective for subtypes, 18 nM, measured by [<sup>3</sup>H]prazosin binding) was lower

TABLE 2

Binding affinities for cloned human receptors in vitro  
Data are calculated by nonlinear regression analysis using data from three assays performed in duplicate or triplicate and expressed as mean values.

Human Receptor	Binding Affinity ( $K_i$ )			
	Brexpiprazole	Aripiprazole	Bifeprunox	Risperidone
	<i>nM</i>			
hD <sub>2L</sub>	0.30	0.87	0.41	1.9
hD <sub>3</sub>	1.1	1.6	0.63	13
h5-HT <sub>1A</sub>	0.12	1.3	0.73	420 <sup>a</sup>
h5-HT <sub>2A</sub>	0.47	4.7	NT	0.22
h5-HT <sub>2B</sub>	1.9	0.36 <sup>b</sup>	NT	35 <sup>c</sup>
h $\alpha_{1B}$ -adrenergic	0.17	35 <sup>b</sup>	NT	9 <sup>d</sup>
h $\alpha_{2C}$ -adrenergic	0.59	38 <sup>b</sup>	NT	9.1 <sup>a</sup>
hH <sub>1</sub>	19	18	NT	27 <sup>a</sup>
hM <sub>1</sub>	67% at 10 $\mu$ M	6780 <sup>b</sup>	NT	>10,000 <sup>d</sup>

NT, not tested.

<sup>a</sup>Data from Schotte et al., 1996.

<sup>b</sup>Data from Shapiro et al., 2003.

<sup>c</sup>Data from Kroeze et al., 2003.

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

TABLE 3

Receptor binding affinities for rat receptors in vitro  
 $K_i$  values are calculated by nonlinear regression analysis using data from three assays performed in duplicate or triplicate and expressed as mean values.

	Binding affinity ( $K_i$ )		
	Brexpiprazole	Aripiprazole	Risperidone
	<i>nM</i>		
rD <sub>2</sub>	0.35	1.4	1.8
r5-HT <sub>1A</sub>	0.09	1.2	250 <sup>a</sup>
r5-HT <sub>2</sub>	3.8	180	0.35
r $\alpha_1$ -adrenergic	18	290	1.2

<sup>a</sup>Data from Schotte et al., 1996.

than that of h $\alpha_1$ -adrenoceptor subtype affinities (Table 3; Supplemental Table 1).

### Brexpiprazole Is a h5-HT<sub>1A</sub>, hD<sub>2L</sub>, and hD<sub>3</sub> Receptor Partial Agonist In Vitro

**h5-HT<sub>1A</sub> Receptors.** Partial agonist activities of brexpiprazole and reference compounds were evaluated using [<sup>35</sup>S]GTP $\gamma$ S binding to h5-HT<sub>1A</sub> receptor-expressing cell membranes. Brexpiprazole, aripiprazole, buspirone, and bifeprunox increased [<sup>35</sup>S]GTP $\gamma$ S 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  $\mu$ M serotonin), whereas 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<sub>1A</sub> receptor antagonist WAY-100635 (IC<sub>50</sub> = 0.30 nM; data not shown).

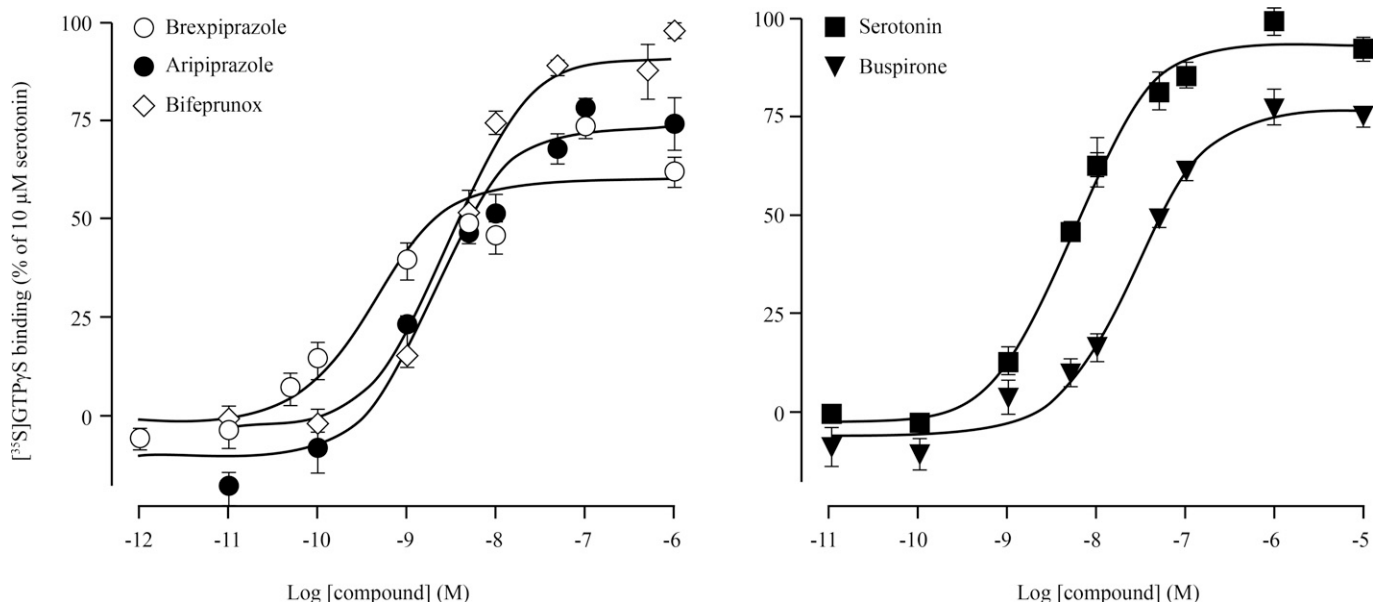
**hD<sub>2L</sub> Receptors.** Partial agonist activities of brexpiprazole and aripiprazole were evaluated using the inhibition of forskolin-induced cAMP accumulation in hD<sub>2L</sub> receptor-expressing cells. Dopamine almost completely inhibited cAMP accumulation in a concentration-dependent manner (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).

**hD<sub>3</sub> Receptors.** Partial agonist activities of brexpiprazole and aripiprazole were evaluated using inhibition of forskolin-induced increase of cAMP accumulation in hD<sub>3</sub> 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 with 10  $\mu$ 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 incubation with (-)-raclopride (1  $\mu$ M; data not shown).

### Brexpiprazole Is an Antagonist of h5-HT<sub>2A</sub> and h5-HT<sub>2B</sub> Receptors and of h $\alpha_{1B}$ - and h $\alpha_{2C}$ -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





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

serotonin on IP<sub>1</sub> production at h5-HT<sub>2A</sub> and h5-HT<sub>2B</sub> receptors, as well as the effect of adrenaline on cAMP production at hα<sub>1B</sub>- and hα<sub>2C</sub>-adrenoceptors. Highest antagonist potency was observed at hα<sub>1B</sub>-adrenoceptors (cIC<sub>50</sub> = 0.66 nM), whereas slightly lower potencies were found at h5-HT<sub>2A/2B</sub> receptors and hα<sub>2C</sub>-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 μM (h5-HT<sub>2A</sub>; hα<sub>1B</sub>) or 10 μM (h5-HT<sub>2B</sub>; hα<sub>2C</sub>), respectively (data not shown).

#### Brexpiprazole Binds to 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> Receptors in Rat CNS Ex Vivo

Brexpiprazole bound ex vivo to 5-HT<sub>1A</sub> receptors in rat hippocampus with an ED<sub>50</sub> of 5.6 mg/kg p.o. In comparison, aripiprazole had lower potency with an ED<sub>50</sub> of about 30 mg/kg

p.o. (Table 5). Finally, the ex vivo binding to r5-HT<sub>7</sub> 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).

#### Brexpiprazole Binds to D<sub>2</sub>, 5-HT<sub>2A</sub>, and 5-HT<sub>6</sub> Receptors in Rat CNS In Vivo

Brexpiprazole dose dependently displaced in vivo [<sup>3</sup>H]raclopride binding to rat striatal D<sub>2</sub> receptors, with an ED<sub>50</sub> value of 2.5 mg/kg p.o. (Table 5). Plasma and brain exposure measurements indicated EC<sub>50</sub> 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 D<sub>2</sub> receptor occupancy between 1 and 4 hours after administration and indicated that D<sub>2</sub> occupancy of brexpiprazole follows

TABLE 4

Functional effects on human receptors in vitro

Agonist activity (*E*<sub>max</sub> and EC<sub>50</sub>) is shown for D<sub>2</sub>, D<sub>3</sub>, and 5-HT<sub>1A</sub> receptors, whereas antagonist activity (cIC<sub>50</sub>) is shown for 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, α<sub>1B</sub>, and α<sub>2C</sub>-adrenergic receptors. No agonist activity of brexpiprazole is observed in the latter assays. *E*<sub>max</sub> and EC<sub>50</sub> (mean ± S.E.M.) in cAMP assays, calculated by nonlinear regression analysis.

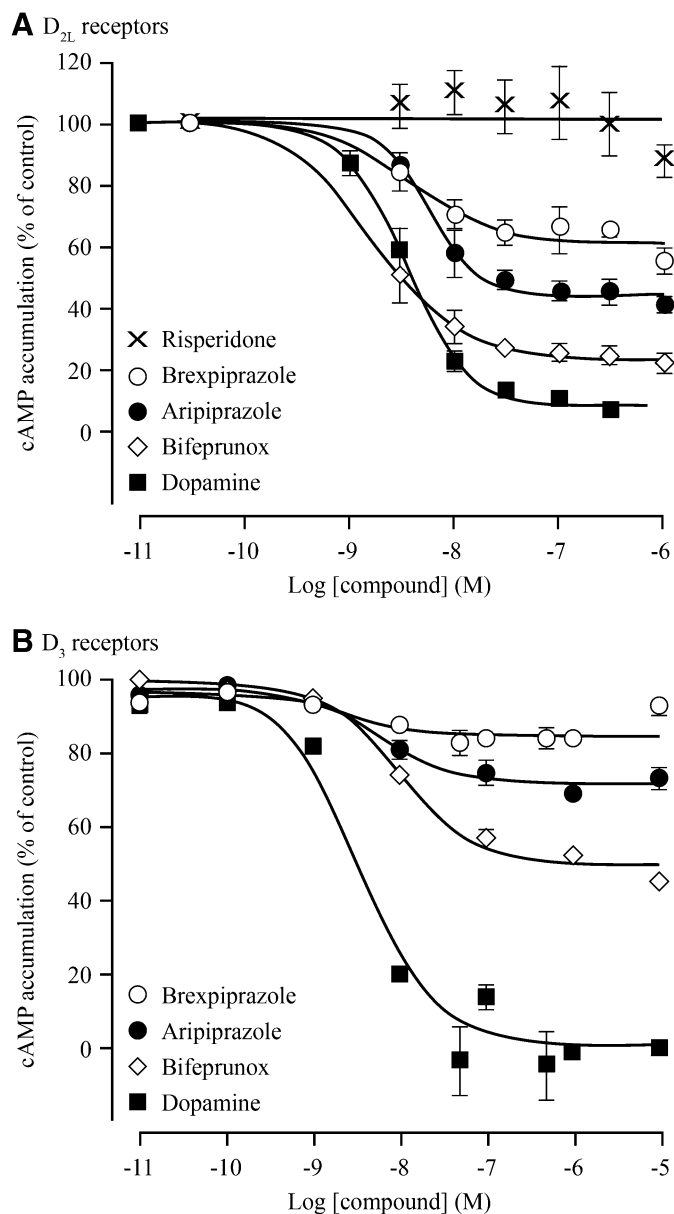
Compounds	h5-HT <sub>1A</sub>		hD <sub>2L</sub>		hD <sub>3</sub>		h5-HT <sub>2A</sub>	h5-HT <sub>2B</sub>	hα <sub>1B</sub>	hα <sub>2C</sub>
	[ <sup>35</sup> S]GTPγS Binding		cAMP Assay		cAMP Assay		IP <sub>1</sub>	IP <sub>1</sub>	cAMP	cAMP
	<i>E</i> <sub>max</sub> <sup>a</sup>	EC <sub>50</sub>	<i>E</i> <sub>max</sub> <sup>b</sup>	EC <sub>50</sub>	<i>E</i> <sub>max</sub> <sup>c</sup>	EC <sub>50</sub>			cIC <sub>50</sub>	
	%	nM	%	nM	%	nM			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

cIC<sub>50</sub>, corrected IC<sub>50</sub>, calculated using the Cheng-Prusoff equation for functional assay: cIC<sub>50</sub> = IC<sub>50</sub>/(1+[Ago]/EC<sub>50</sub>); NT, not tested.

<sup>a</sup>*E*<sub>max</sub>, expressed as percentage of the effect of 10 μM serotonin.

<sup>b</sup>*E*<sub>max</sub>, expressed as percentage of maximum inhibition of dopamine.

<sup>c</sup>*E*<sub>max</sub>, expressed as percentage of the effect of 10 μM dopamine.



**Fig. 3.** Partial agonist activity of brexpiprazole and reference drugs on human cloned D<sub>2L</sub> (A) or D<sub>3</sub> receptors in vitro (B). Concentration-response curves are shown for brexpiprazole and reference compounds on forskolin-induced cAMP accumulation in hD<sub>2L</sub> and hD<sub>3</sub> receptor-expressing CHO cells. Data are mean  $\pm$  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%).

plasma and brain exposure in rats (Fig. 4). Plasma exposures were between 44 and 146 ng/ml, 1–4 hours after administration (Fig. 4A), whereas brain levels were slightly lower (12–46 ng/g; Fig. 4B).

Brexpiprazole also potently displaced the binding of the 5-HT<sub>2A</sub> receptor ligand [<sup>3</sup>H]M100907 in cortex (ED<sub>50</sub> = 4.6 mg/kg p.o.). The binding potency at 5-HT<sub>2A</sub> receptors was 26 times higher than that of aripiprazole (ED<sub>50</sub> = 120 mg/kg p.o.) based on the ED<sub>50</sub> values (Table 5).

Brexpiprazole had moderate potency in displacing binding of the 5-HT<sub>6</sub> ligand [<sup>3</sup>H]Lu AE60157 in rat striatum (ED<sub>50</sub> = 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 D<sub>2</sub> Partial Agonist In Vivo: Inhibitory Effects on Reserpine-Induced DOPA Accumulation in Rat Striatum

Reserpine (1 mg/kg s.c., 18 hours before death) increased DOPA accumulation ~4-fold in rat striatum (Supplemental Fig. 1). Brexpiprazole and aripiprazole both showed D<sub>2</sub> agonist effects by inhibiting the reserpine-induced increase in DOPA accumulation but had different maximum inhibitory responses (Table 6). They had similar potencies based on ED<sub>25</sub> values, whereas 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<sub>2A</sub> Antagonist In Vivo: DOI-Induced Head Twitches in Rats

DOI induces frequent head twitches, with on average 19–21 episodes for 10 minutes after injection of 5 mg/kg s.c. (data not shown). Brexpiprazole and aripiprazole dose dependently inhibited DOI-induced head twitches (Table 6), brexpiprazole (ED<sub>50</sub> = 4.7 mg/kg p.o.) being more potent than aripiprazole (ED<sub>50</sub> = 21 mg/kg p.o.). As expected, risperidone potently blocked DOI-induced head twitches (ED<sub>50</sub> = 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 minutes after drug administration. The higher dose (20 mg/kg) induced a nonsignificant decrease. In contrast, brexpiprazole slightly increased the extracellular levels of the dopamine metabolites HVA and DOPAC (Fig. 6, B and C, respectively). Maximum increases of HVA and DOPAC were 30 and 20% above baseline levels, respectively, after 10 and 20 mg/kg, occurring 150–180 minutes (HVA) and 60–180 minutes (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), whereas 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) and 44 and 110% for DOPAC (Fig. 7C), whereas levels in the vehicle group increased nonsignificantly by 12 and 6%, respectively, for HVA and DOPAC. Maximum effects occurred 150–180 minutes after drug administration.

TABLE 5

In vivo and ex vivo binding effects of brexpiprazole and aripiprazole on D<sub>2</sub> and 5-HT receptor subtypes in rat brain

Data are expressed as ED<sub>50</sub> (mg/kg p.o., 2 hours after drug administration) and EC<sub>50</sub> values in plasma and brain (ng/ml, 2 hours after drug administration), both with 95% confidence intervals in parentheses. For receptors where maximum binding is too low to calculate ED<sub>50</sub> and IC<sub>50</sub> values, the percent specific binding at the highest dose or concentration is indicated as a footnote.

Receptor	Brain Region	Brexpiprazole			Aripiprazole		
		ED <sub>50</sub>	EC <sub>50</sub> plasma	EC <sub>50</sub> brain	ED <sub>50</sub>	EC <sub>50</sub> plasma	EC <sub>50</sub> brain
		mg/kg	ng/ml	ng/g	mg/kg	ng/ml	ng/g
<b>In vivo binding</b>							
rD <sub>2</sub>	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 <sub>2A</sub>	Cortex	4.6 (3.6–6.0)	91 (70–120)	35 (26–48)	120 (49–300)	>520 <sup>a</sup>	>3000 <sup>b</sup>
r5-HT <sub>6</sub>	Striatum	17 (13–21)	1200 (940–1500)	410 (280–590)	>100 <sup>c</sup>	>520 <sup>a</sup>	>3000 <sup>b</sup>
<b>Ex vivo binding</b>							
r5-HT <sub>1A</sub>	Hippocampus	5.6 (3.8–8.5)	320 (210–500)	N.D.	≈30 <sup>d</sup>	≈600	N.D.
r5-HT <sub>7</sub>	Paraventricular thalamus	>30 <sup>e</sup>	>1600	N.D.	>30 <sup>f</sup>	>610 <sup>g</sup>	N.D.

N.D., not determined.

<sup>a</sup>520 ng/ml at 100 mg/kg.

<sup>b</sup>3000 ng/ml at 100 mg/kg.

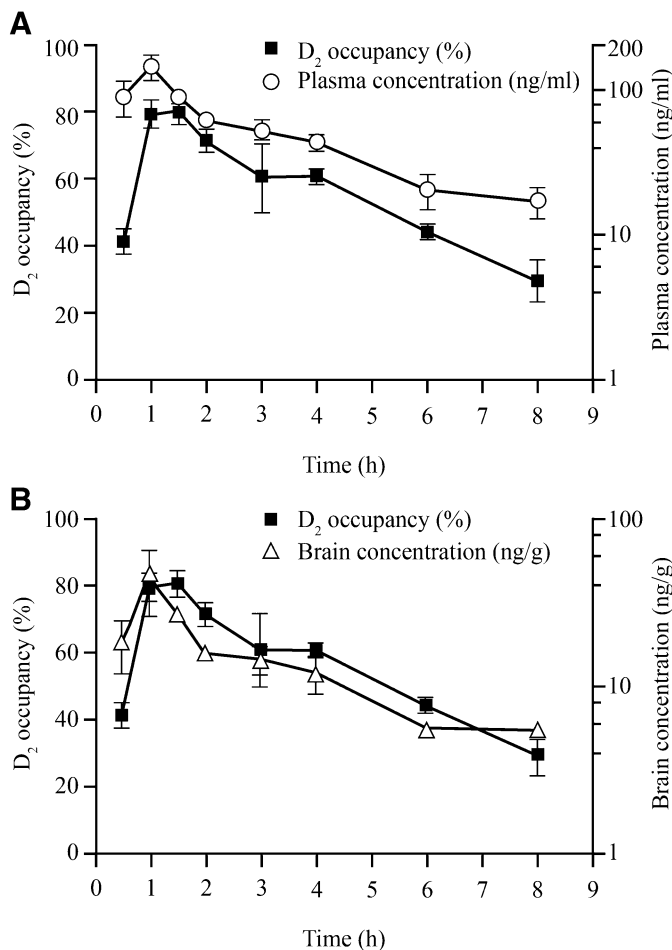
<sup>c</sup>22% occupancy at 100 mg/kg.

<sup>d</sup>53% occupancy at 30 mg/kg.

<sup>e</sup>41% occupancy at 30 mg/kg.

<sup>f</sup>38% occupancy at 30 mg/kg.

<sup>g</sup>610 ng/ml at 30 mg/kg.



**Fig. 4.** Time course of striatal dopamine D<sub>2</sub> receptor occupancy in rats after oral brexpiprazole (3 mg/kg). Abscissa indicates time in hours, whereas 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.

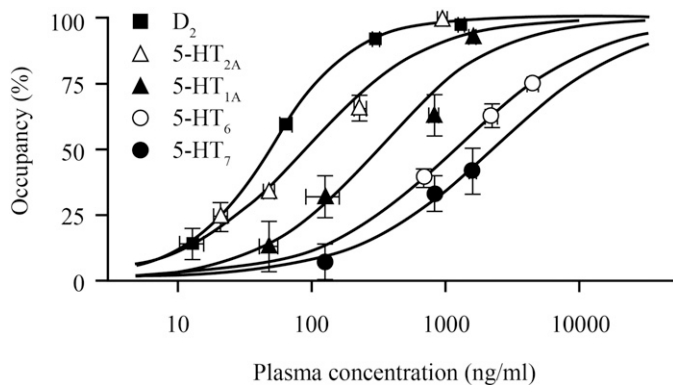
**Serotonin and Noradrenaline in Prefrontal Cortex and Ventral Hippocampus.** Brexpiprazole had no effect on extracellular levels of serotonin or noradrenaline in either brain region after administration of doses up to 30 mg/kg p.o. (Supplemental Fig. 3, A and B).

**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), whereas no changes in extracellular acetylcholine were observed at any dose (Supplemental Fig. 3C). Histamine levels also increased after vehicle administration but the effect was short lasting, whereas the increase after brexpiprazole (30 mg/kg) was stable for at least 3 hours.

## 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<sub>1A</sub> receptors and hD<sub>2L</sub> and hD<sub>3</sub> receptors combined with antagonist activity on h5-HT<sub>2A</sub> receptors and hα<sub>1B</sub>- and hα<sub>2C</sub>-adrenoceptors.

**Serotonin-Dopamine-Noradrenergic Binding In Vitro and In Vivo.** Brexpiprazole displayed almost equal subnanomolar affinities for several cloned human receptors, including h5-HT<sub>1A</sub>, hD<sub>2L</sub>, and 5-hHT<sub>2A</sub> receptors, as well as hα<sub>1B</sub>- and hα<sub>2C</sub>-adrenoceptors. The affinity constants (K<sub>i</sub>) were in the range 0.12–0.59 nM. Consequently, the occupancies of these receptors after brexpiprazole treatment will likely be similar in vivo. Although both brexpiprazole and aripiprazole showed high affinities for h-5HT<sub>1A</sub> and hD<sub>2</sub> receptors, brexpiprazole had a slightly higher affinity for h5-HT<sub>1A</sub> receptors than hD<sub>2</sub> receptors, whereas the reverse was true for aripiprazole (Table 2). Furthermore, brexpiprazole bound with about ten times higher affinity to h5-HT<sub>1A</sub> and h5-HT<sub>2A</sub> receptors and much higher affinity to hα<sub>1B</sub>- and hα<sub>2C</sub>-adrenoceptors than aripiprazole (Table 2). The only available method to measure human receptor occupancy is



**Fig. 5.** Relationship between plasma level and receptor occupancies in rats after brexpiprazole treatment. Brexpiprazole is administered p.o., 2 hours before death, and in vivo binding measured in the brain for  $D_2$  ( $n = 5-6$ ),  $5-HT_{2A}$  ( $n = 6$ ), and  $5-HT_6$  ( $n = 6$ ) receptors and by ex vivo receptor autoradiography for  $5-HT_{1A}$  ( $n = 4$ ) and  $5-HT_7$  ( $n = 4$ ) receptors. Mean  $\pm$  S.E.M. of results from 4–6 rats. For ligands, see *Materials and Methods*. Concentration-response curves are calculated by curve fitting. Abscissa shows average plasma exposure (nanogram per milliliter) 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.

positron emission tomography imaging, and  $D_2$  antagonist antipsychotics are usually dosed to obtain 60–80% occupancy (e.g., Mamo et al., 2007). At the recommended doses of aripiprazole human  $D_2/D_3$  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 positron emission tomography ligands for measuring  $\alpha$ -adrenoceptor subtype occupancies are presently available, so only relative in vitro affinities can predict human occupancies.

To support translation from animal pharmacology to human efficacy, we included rat receptor affinity data for selected receptors ( $D_2$ ,  $5-HT_{1A}$ ,  $5-HT_2$ , and  $\alpha_1$ -adrenergic receptors). Although there was consistency in the affinity ( $K_i$  values) between humans and rats for  $D_2$  and  $5-HT_{1A}$  receptors,  $r5-HT_2$  receptor affinity was slightly lower than that for  $h5-HT_{2A}$  receptors. A similar comparison could not be made for  $\alpha_1$ -adrenoceptors because of 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  $h5-HT_7$  receptor affinity was rather high for brexpiprazole ( $K_i$  3.7 nM; Supplemental Table 1) 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 accordance with this, brexpiprazole had lower maximum agonist effect than aripiprazole in an in vivo model of  $D_2$  autoreceptor activity, inhibition of reserpine-induced L-DOPA accumulation (Supplemental Figs. 1 and 2), confirming a lower intrinsic activity at the  $D_2$  receptor in vivo.  $D_2$  autoreceptors develop increased agonist sensitivity after monoamine depletion after reserpine treatment (Hjorth et al., 1988), and consequently aripiprazole, but not brexpiprazole, had marked efficacy. In contrast,  $D_2$  partial agonists, including aripiprazole and brexpiprazole, have functional  $D_2$  antagonist activity in most tests of postsynaptic  $D_2$  receptor-mediated effects, as described earlier (Arnt and Hyttel, 1990) and in the accompanying paper (Maeda et al., 2014).

In vitro functional studies at  $h5-HT_{1A}$  receptors suggest similar efficacies for brexpiprazole, aripiprazole, and buspirone, 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

TABLE 6

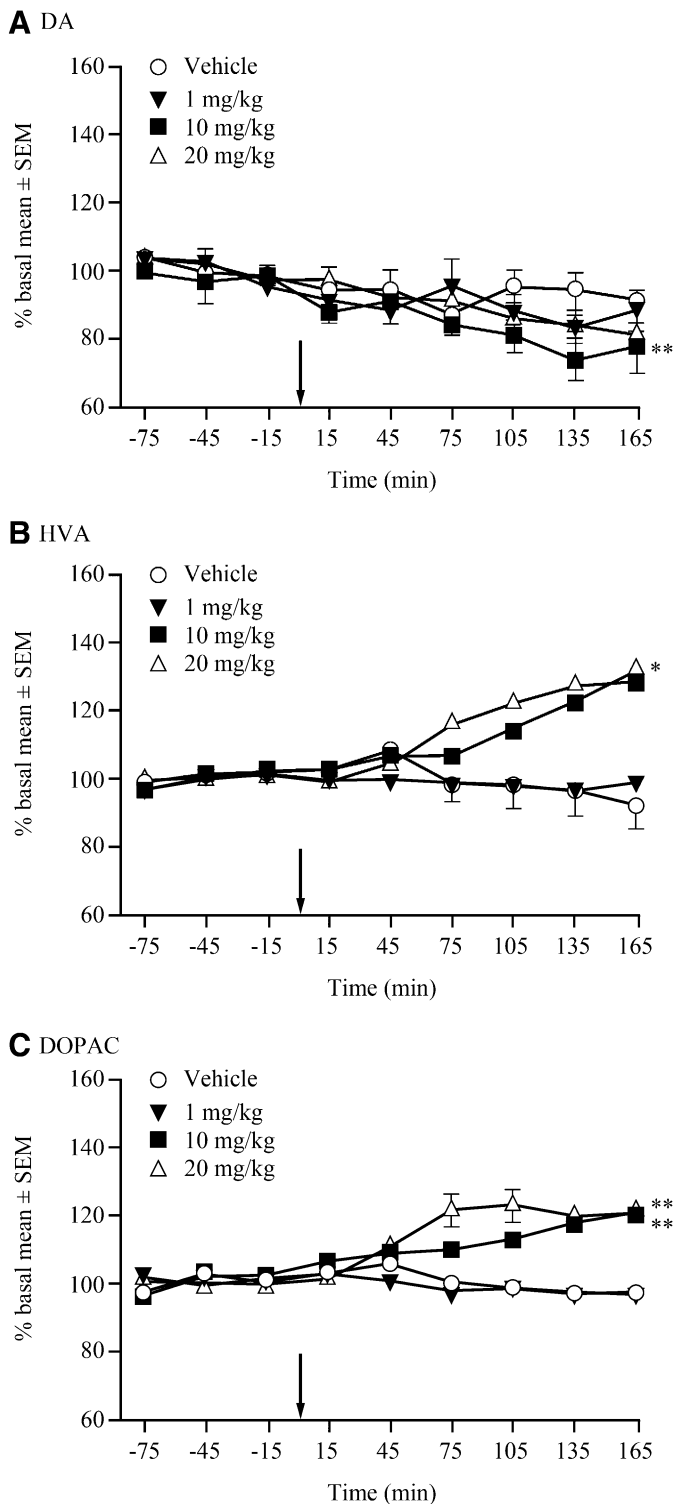
Functional effects of brexpiprazole and aripiprazole on rat  $D_2$  and  $5-HT_{2A}$  receptors in vivo.  $ED_{25}$  or  $ED_{50}$  values (mg/kg p.o.) are indicated with 95% confidence intervals (CI) in parentheses

Brexpiprazole and aripiprazole are administered 1 and 2 hours before death (DOPA test) or DOI injection, respectively. For further methodology, see *Materials and Methods*.

Test Model (Receptor)	Brexpiprazole		Aripiprazole	
	$ED_{25}$ or $ED_{50}$ (95% CI)	Maximum response (Mean $\pm$ S.E.M.)	$ED_{25}$ or $ED_{50}$ (95% CI)	Maximum response (Mean $\pm$ S.E.M.)
	mg/kg	%	mg/kg	%
Reserpine-induced DOPA accumulation ( $D_2$ )	4.4 (2.3–8.0) <sup>a</sup>	55 $\pm$ 3.7	3.7 (2.2–5.9) <sup>a</sup>	89 $\pm$ 2.4
DOI-induced head twitches ( $5-HT_{2A}$ )	4.7 (3.2–6.6) <sup>b</sup>	99 $\pm$ 0.9	21 (7.2–47) <sup>b</sup>	91 $\pm$ 3.6

<sup>a</sup> $ED_{25}$  value.

<sup>b</sup> $ED_{50}$  value.



**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  $\pm$  S.E.M. of results from 8–16 rats. \* $P < 0.05$ ; \*\* $P < 0.01$  significant difference from vehicle group.

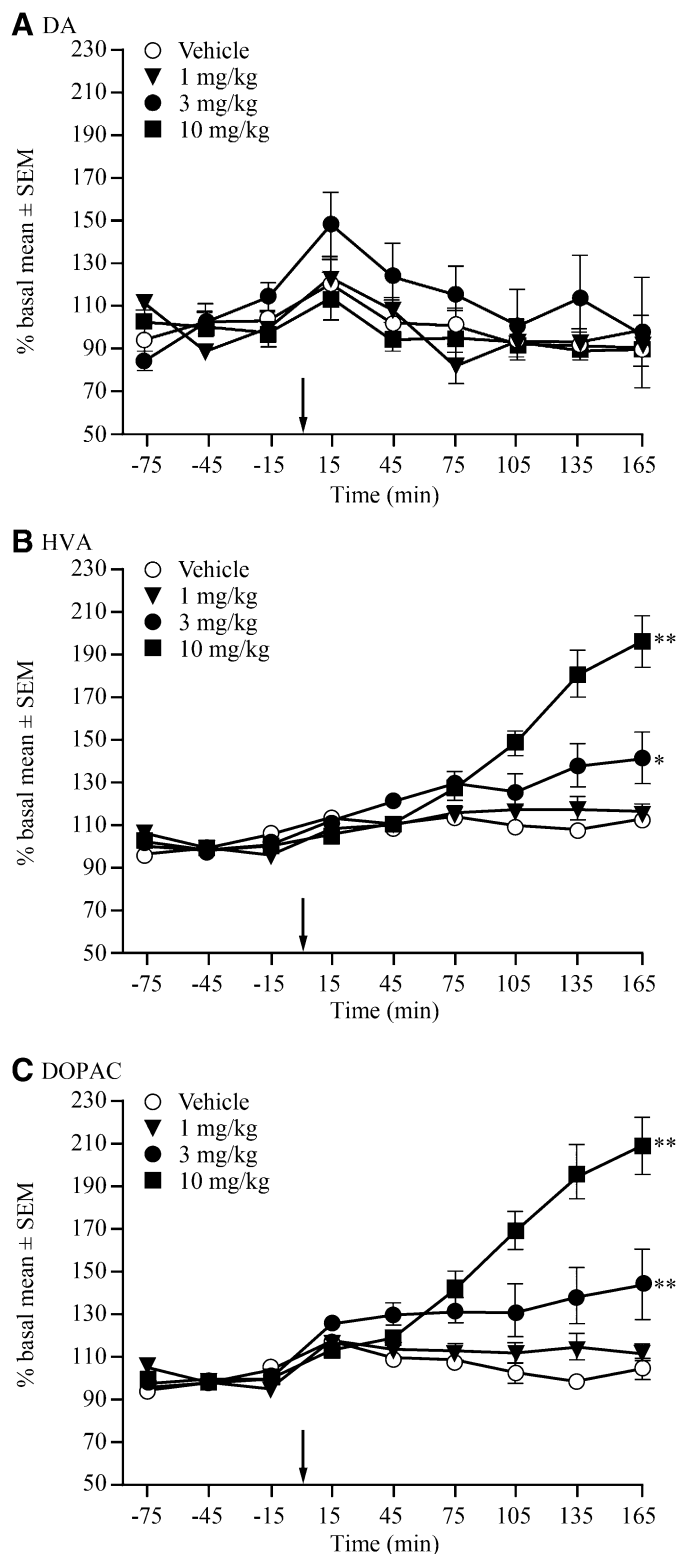
brexpiprazole on other serotonin receptor subtypes in humans were weaker and are unlikely to be of importance at clinically relevant doses (Supplemental Table 3).

In acute microdialysis studies the functional effects of brexpiprazole on extracellular neurotransmitter levels in vivo have been explored in nucleus accumbens, prefrontal cortex, and 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  $D_2$  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; whereas selective  $D_2$  antagonists alone have no effect,  $D_2$  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  $D_2$  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 hH $_1$  receptors as shown in vitro (Table 2 and Supplemental Table 3, respectively; Fell et al., 2012). Accordingly, the risk for weight gain and metabolic adverse effects after brexpiprazole treatment is limited (Nasrallah, 2008).

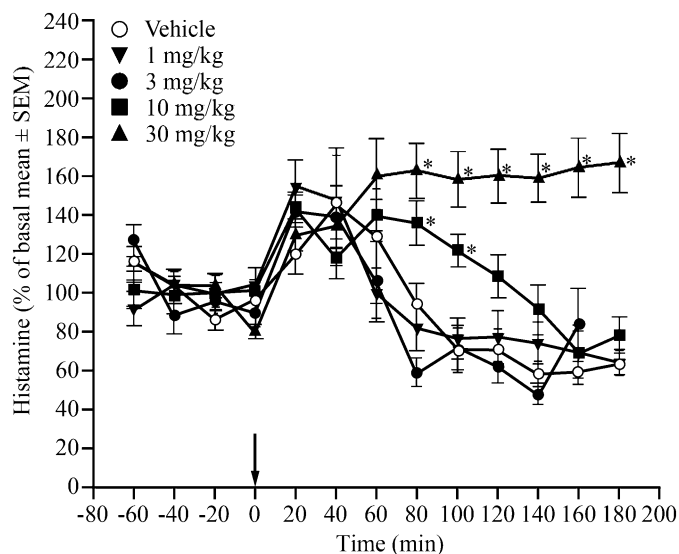
**Relevance of Target Profile for Clinical Efficacy and Side Effect Potential.** As mentioned, brexpiprazole displayed significantly lower intrinsic activity at hD $_2$  and rD $_2$  receptors compared with aripiprazole. This may improve the tolerability of brexpiprazole with respect to  $D_2$  agonist-mediated adverse effects, e.g., nausea, insomnia, and akathisia (Fleischhacker, 2005). Also, the potential for  $D_2$  antagonist-like adverse effects (e.g., EPS and hyperprolactinemia) may be lower than experienced with  $D_2$  antagonist antipsychotics (Casey, 1996; Maeda et al., 2014; Supplemental Figs. 1–3; Supplemental Tables 1–4). The optimum balance between  $D_2$  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  $D_2$  receptors occurred at similar doses of brexpiprazole, whereas aripiprazole had a lower 5-HT $_{2A}$  receptor occupancy at any given  $D_2$  receptor occupancy, may lead to clinical advantages, because 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, 2010; Snigdha et al., 2010; Ebdrup et al., 2011; Laoutidis and Luckhaas, 2013).

The effects of brexpiprazole and aripiprazole on h5-HT $_{1A}$  and hD $_3$  receptors are important for their pharmacological profiles. Partial agonism at 5-HT $_{1A}$  and  $D_3$  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 (Maeda et al., 2014).



**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  $\pm$  S.E.M. of results from 6–9 rats. \* $P < 0.05$ ; \*\* $P < 0.01$  significant difference from vehicle group.



**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.

The consequences of the high affinities of brexpiprazole for (and antagonist effects on)  $\alpha_{1B}$ - and  $\alpha_{2C}$ -adrenoceptors are more difficult to predict because of a lack of selective compounds for studying the functional importance of these receptors. Results using genetically modified mice suggest that  $\alpha_{1B}$ -antagonism may contribute to antipsychotic-like activity and effects on stimulant-induced reward (Drouin et al., 2002). Inhibition of peripheral  $\alpha_{1A}$ -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 (Docherty, 2010). All  $\alpha_1$ -adrenoceptor subtypes are coexpressed with 5-HT<sub>2A</sub> receptors in frontal cortex, but their relative functional importance is unknown (Santana et al., 2013). Similarly, the  $\alpha_{2C}$ -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  $\alpha$ -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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*Conducted experiments:* Maeda, Sugino, Akazawa, Amada, Shimada, Futamura, Mørk, Pehrson, Nielsen, Bundgaard.

*Contributed new reagents or analytic tools:* Yamashita, Ito.

*Performed data analysis:* Maeda, Sugino, Akazawa, Amada, Shimada, Futamura, Mørk, Pehrson, Hentzer, Bundgaard.

*Wrote or contributed to the writing of the manuscript:* Maeda, Mørk, Pehrson, Bundgaard, Arnt, Stensbøl.

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