Pharmacological Characterization of JNJ-40068782, a New Potent, Selective, and Systemically Active Positive Allosteric Modulator of the mGlu2 Receptor and Its Radioligand [3H]JNJ-40068782

Hilde Lavreysen, Xavier Langlois, Abdel Ahnaou, Wilhelmsen Drinkenburg, Paula te Riele, Ilse Biesmans, Ilse Van der Linden, Luc Peeters, Anton Megens, Cindy Wintmolders, Jose Maria Cid, André Trabanco, Jose Ignacio Andrés, Frank M. Dautzenberg, Robert Lütjens, Gregor Macdonald, and John R. Attack


Received March 21, 2013; accepted June 12, 2013

ABSTRACT

Modulation of the metabotropic glutamate type 2 (mGlu2) receptor is considered a promising target for the treatment of central nervous system disorders such as schizophrenia. Here, we describe the pharmacological properties of the novel mGlu2 positive allosteric modulator (PAM) 3-cyano-1-cyclopropylmethyl-4-(4-phenyl-piperidin-1-yl)-pyridine-2(1H)-one (JNJ-40068782) and its radioligand [3H]JNJ-40068782. In guanosine 5′-O-(3′-thiotriphosphate) binding, JNJ-40068782 produced a leftward and upward shift in the glutamate concentration-effect curve at human recombinant mGlu2 receptors. The EC50 of JNJ-40068782 for potentiation of an EC20-equivalent concentration of glutamate was 143 nM. Although JNJ-40068782 did not affect binding of the orthosteric antagonist [3H]2,2-dioxide monohydrate to its radioligand [3H]JNJ-40068782. In guanosine 5′-O-(3′-thiotriphosphate) binding, JNJ-40068782 suggests that PAMs may bind to common determinants within the same site. It is noteworthy that agonists also increased the binding affinity of [3H]JNJ-40068782. JNJ-40068782 influenced rat sleep-wake organization by decreasing rapid eye movement sleep with a lowest active dose of 3 mg/kg PO. In mice, JNJ-40068782 reversed phencyclidine-induced hyperlocomotion with an ED50 of 5.7 mg/kg s.c. Collectively, the present data demonstrate that JNJ-40068782 has utility in investigating the potential of mGlu2 modulation for the treatment of diseases characterized by disturbed glutamatergic signaling and highlight the value of [3H]JNJ-40068782 in exploring allosteric binding.

Introduction

Glutamate is the major excitatory neurotransmitter in the central nervous system of vertebrates and elicits and modulates synaptic responses by activating ionotropic or metabotropic glutamate (mGlu) receptors (Kew and Kemp, 2005). To date,
eight mGlu receptor subtypes have been cloned and classified into three groups based on sequence homology, pharmacological profile, and preferential signal transduction pathway (Niswender and Conn, 2010).

The drug discovery efforts related to group III mGlu receptors have lagged behind (Lavreyesen and Dautzenberg, 2005), and various research groups have focused on the development of ligands acting at group I and II receptors. For example, mGlu1 receptor blockade is thought to have anxiolytic-like properties and to relieve pain (Tatarczynska et al., 2001; Varney and Gereau, 2002; Steckler et al., 2005), whereas inhibitors of the mGlu5 receptor are believed to be alternative treatments for anxiety, depression, and Fragile X and mGlu5 activators may have beneficial effects in schizophrenia (Spooren et al., 2003; Auerbach et al., 2011). Because group II mGlu receptors reduce transmission at glutamatergic synapses in brain regions where excessive glutamatergic transmission may be implicated in the pathophysiology of anxiety and schizophrenia, it is hypothesized that activation of group II mGlu receptors may provide anxiolytic and/or antipsychotic effects (Swanson et al., 2005).

Accordingly, mGlu2/3 receptor agonists, such as (−)-(1R,4S,5S,6S)-4-amino-2-sulfonylbicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY404039), are effective in a number of animal models of schizophrenia (Rorick-Kehn et al., 2007) and anxiety (Helton et al., 1998; Tizzano et al., 2002; Swanson et al., 2005; Witkin et al., 2007). These observations have been extended into the clinic where (1R,4S,5S,6S)-2-thiabicyclo[3.1.0]hexane-4,6-dicarboxylic acid, 4-[(2S)-2-amino-4-methyl-thio]-1-oxybutylaminoo, 2,2-dioxide monohydrate (LY2140023), a methionine amide prodrug of LY404039, has been shown to demonstrate efficacy in a phase 2 study in schizophrenia patients (Patil et al., 2007; Mezler et al., 2010), although these observations could not be confirmed in a follow-up trial (Kinon et al., 2011). The structurally related agonist (1S,2S,5R,6S)-(−)-2-aminoxybicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740) was able to reduce anxiety in healthy volunteers in a fear-potentiated startle model (Grillon et al., 2003) and attenuated CO2-induced panic in subjects with panic disorder (Schoepf et al., 2003). These anxiolytic-like properties were reproduced in patients with generalized anxiety disorder using LY354740 (Michelson et al., 2005) as well as its prodrug (1S,2S,5R,6S)-(2S'(−)-2-amino-propionyl)aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid hydrochloride (LY544344) (Dunayevich et al., 2008).

The use of knockout mice lacking either mGlu2 or mGlu3 receptors has further refined our understanding of the mechanism of action of mGlu2/3 receptor agonists. For example, the anxiolytic actions of LY354740 in the elevated plus maze test require the expression of one or both of these receptors (Linden et al., 2005). On the other hand, mGlu2 rather than mGlu3 receptors appear to be responsible for the antipsychotic-like properties of LY404039 (Fell et al., 2008), (−)-2-oxa-4-amino-bicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268) (Woolley et al., 2008), and LY314582, which is the racemate of LY354740 (Spooren et al., 2000). On the basis of the collective data using mGlu2/3-selective agonists as well as mice lacking either the mGlu2 or mGlu3 receptor, the mGlu2 receptor would appear to be an attractive potential therapeutic target (Niswender and Conn, 2010).

mGlu2-positive allosteric modulators (PAMs) that bind at a receptor site that is different (i.e., allosteric) from glutamate offer an alternative approach for enhancing mGlu2 activity. mGlu2 PAMs have minimal effects on their own but increase the response of glutamate. As a consequence, mGlu2 receptor PAMs offer an opportunity to enhance mGlu2 receptor signaling by maintaining activity based on transient release of glutamate but have a lower risk of tolerance development (Conn et al., 2008, 2009). 2,2,2-Trifluoro-N-[4-(2-methoxyphenoxo)phenyl]-N-[3-pyridinylmethyl]-ethanesulfonamide (LY487379) was identified as the first mGlu2 receptor−specific PAM (Johnson et al., 2003) and was active in animal models predictive of anxiolytic or antipsychotic activity (Johnson et al., 2003, 2005; Galici et al., 2005), providing preclinical proof of concept that PAMs can mimic aspects of the pharmacology of agonists. Minimal efficacy at sufficiently low doses as well as short duration of action, however, limited its use for further development. Pinkerton et al. (2005) reported an alternative series of mGlu2 PAMs, of which the prototypic example biphenyl-indanone A (BINA) demonstrates long-lasting activity in some animal models used to predict potential antipsychotic and anxiolytic activity (Galici et al., 2006). Recently, the structurally novel allosteric potentiator N-(4-((2-trifluoromethyl)-3-hydroxy-4-(isobutryl)phenoxo)methoxybenzyl)-1-methyl-1H-imidazo-4-carboxamide (THIC; also known as LY2607540), which seems to have activity in models predicting either anxiolytic or antidepressant effects, was reported (Fell et al., 2011). Although preclinical data hold promise for mGlu2 PAMs as novel therapeutics, clinical proof of concept is awaited with more drug-like compounds compared with the first-generation PAMs.

Here, we describe the in vitro and in vivo properties of the novel PAM 3-cyano-1-cyclopropylmethyl-4-(4-phenyl-piperidin-1-yl)-pyridine-2(1H)-one (JNJ-40068782) (Imogai et al., 2007; Cid et al., 2008). We furthermore describe [3H]JNJ-40068782 as the first high-affinity, selective mGlu2 PAM radioligand and show that this is an excellent tool to study allosteric mGlu2 interactions.

**Materials and Methods**

BINA, LY487379, THIC (LY2607540), 1-butyl-4-[4(2-methylpyridin-4-yl)oxy]phenyl]-2-oxo-1,2-dihydropyridine-3-carbonitrile (JNJ-40264796), JNJ-40068782, and [3H]JNJ-40068782 (Fig. 1) were synthesized at Janssen Research and Development. Both [3H]JNJ-40068782 and [3H]2S-2-amino-2-(1S,2S)-2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl) propanoic acid ([3H]LY341495) were obtained from American Radiolabeled Chemicals (St. Louis, MO). Guanosine 5′-(O-3-[35S]thiotriphosphate (35S[GTPγS]) (specific activity, 1250 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). GDP, GDP...
HEPES, EGTA, bovine serum albumin (BSA), probenecid, and glutamate were purchased from Sigma-Aldrich (St. Louis, MO); MgCl₂ was purchased from VWR Prolabo (Belgium), saponin was from Calbiochem (San Diego, CA), and Hank’s balanced salt solution (HBSS) and fluo-4-AM were from Invitrogen (Carlsbad, CA), and phenylmethanesulfonyl fluoride from Fluka (Buchs, Switzerland). All other reagents were obtained from Merck (Darmstadt, Germany). For in vitro experiments, concentration-response curves of JNJ-40068782 were prepared in 100% dimethylsulfoxide and further diluted in assay buffer with final assay concentrations reaching 1% dimethylsulfoxide. For in vivo studies, JNJ-40068782 solutions were prepared in 20% hydroxypropyl-β-cyclodextrine.

**Tritiation of JNJ-40068782**

The brominated precursor 4-(4-(4-bromophenyl)piperidin-1-yl)-1-(cyclopropylmethyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile was dissolved in tetrahydrofuran. N,N-Diisopropylethylamine and palladium 10% on carbon then were added. The reaction mixture was added to a tritium manifold (RC Tritic, Teufen, Switzerland), and 183 megabars of tritium gas was placed on top of the reaction mixture and was stirred for 1 hour at room temperature. The remaining tritium gas was trapped on a uranium bed, and all volatile compounds were lyophilized to a waste ampoule. The crude reaction mixture was rinsed with methanol, dissolved in ethanol, and filtered over an Acrodisc filter, yielding 16.4 GBq. A small portion of this material was further purified and analyzed by high-performance liquid chromatography to obtain the tritiated compound 4-(4-(4-tritiumphenyl)piperidin-1-yl)-1(cyclopropylmethyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (radiochemical purity of >98%; 865 GBq/mmol).

**Functional mGlu₂ Assays**

**Ca²⁺ Assay.** Intracellular Ca²⁺ levels were measured using the Functional Drug Screening System (FDSS 6000; Hamamatsu Photonics, Hamamatsu City, Japan). Human mGlu2 receptor—expressing Glu₄-HEK293 cells were seeded at 10,000 cells/well in 384-well black/clear-bottom poly-lysine-coated plates 24 hours before the experiment. On the day of the experiment, medium was removed by brief centrifugation, and 20 μl of a fluo-4-AM solution (HBSS buffer containing 20 mM HEPES, 2.5 mM probenecid, and ~2 μM fluo-4-AM, pH 7.4) was added. After an incubation period of 1 hour at room temperature in the dark, 40 μl buffer was added, and cells were kept at room temperature for another 10 minutes before measurement. Fluorescence was recorded upon the addition of compound (20 μl), and the ratio of the peak over basal fluorescence was measured and used for further calculation. For the measurement of intrinsic agonism, compound was added alone (i.e., in the absence of glutamate); for measurement of PAM activity, compound was added together with an EC₅₀-equivalent concentration of glutamate (0.3 μM).

**[^8]SGTPyS Binding.** Chinese hamster ovary (CHO) cells expressing the rat or human mGlu2 receptor were grown until 80% confluence, washed in ice-cold phosphate-buffered saline, and stored at −20°C until membrane preparation. After thawing, cells were suspended in 50 mM Tris-HCl, pH 7.4, and collected through centrifugation for 10 minutes at 23,500g at 4°C. Cells were lysed in 5 mM hypotonic Tris-HCl, pH 7.4; and after recentrifugation for 20 minutes at 30,000g at 4°C, the pellet was homogenized with an Ultra Turrax homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany) in 50 mM Tris-HCl, pH 7.4. Protein concentrations were measured by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) using BSA as standard.

For[^8]SGTPyS measurements, compound and glutamate were diluted in buffer containing 10 mM HEPES acid, 10 mM HEPES salt, pH 7.4, containing 100 mM NaCl, 3 mM MgCl₂, and 10 μM GDP. Membranes were thawed on ice and diluted in the same buffer, supplemented with 14 μg/ml saponin (final assay concentration of 2 μg/ml saponin). Final assay mixtures contained 7 (human mGlu2) or 10 μg (rat mGlu2) of membrane protein and were preincubated with compound alone (determination of agonist effects) or together with an EC₅₀ concentration (4 μM) of glutamate (determination of PAM effects) for 30 minutes at 30°C.[^8]SGTPyS was added at a final concentration of 0.1 nM and incubated for another 30 minutes at 30°C. Reactions were terminated by rapid filtration through Unifilter-96 GF/B filter plates (PerkinElmer Life and Analytical Sciences) using a Unifilter-96 harvester (PerkinElmer Life and Analytical Sciences). Filters were washed three times with ice-cold 10 mM NaH₂PO₄/10 mM Na₂HPO₄, pH 7.4; and filter-bound radioactivity was counted in a Topcount NXT microplate scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences). Typically,[^8]SGTPyS binding in the absence of agonist was in the region of 400 cpm, whereas a maximal glutamate response corresponded to ~2500 cpm.

[^8]SGTPyS Binding to Rat Brain Sections. Autoradiography of agonist-stimulated[^8]SGTPyS binding in brain sections was performed as previously described (Happe et al., 2001). Hence, 20-μm-thick sagittal sections were incubated twice in assay buffer, pH 7.5 (50 mM Tris-HCl, 10 mM MgCl₂, 0.2 mM EGTA, 100 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride, and 1% BSA), for 10 minutes at 25°C and once in assay buffer with 2 mM GDP for 15 minutes at 25°C. Slides were then incubated in assay buffer containing 0.04 nM[^8]SGTPyS in the presence or absence of 10 μM glutamate and increasing concentrations of JNJ-40068782 for 2 hours at 25°C. The incubation was stopped by washing twice for 3 minutes in ice-cold 50 mM Tris-HCl, pH 7.4, and a quick dip in ice-cold distilled water.

Sections were then dried under a cold stream of air and placed in a light-tight cassette together with commercially available radioactive polymer standards and exposed to Kodak Biomax MR film (PerkinElmer Life and Analytical Sciences) for 4 days. Films were developed by standard techniques, digitized, and analyzed using the MCID basic 7.0 system (Imaging Research, St. Catharines, ON, Canada).

Agonist-stimulated activity in brain sections was calculated by subtracting the optical density in basal sections (incubated with GDP alone) from that of agonist-stimulated sections; results were expressed as percentage basal activity.

**Radioligand Binding to Human mGlu2-CHO Membranes or Rat Cortical Membranes**

Membranes from human mGlu2 receptor stably transfected CHO cells were prepared as above (see ‘[^8]SGTPyS Binding’ under Materials and Methods). For cortical membranes, male Wistar rats (150 g) were sacrificed and decapitated. Brains were removed, and cortex tissue was dissected and kept on ice. Tissue was homogenized with an Ultra-Turrax homogenizer in 50 mM Tris-HCl, pH 7.4; and the homogenate centrifuged for 10 minutes at 16,000 rpm. The pellet was resuspended and homogenized again with a glass Teflon homogenizer (Dulbecco, Thomas Scientific, Swedesboro, NJ). After two washing/centrifugation steps, the final pellet was resuspended in 50 mM Tris-HCl, pH 7.4. Aliquots were made and frozen at −80°C until further use. Protein concentration was determined with the Bio-Rad Protein Assay method using BSA as standard.

After thawing, membranes were homogenized using an Ultra Turrax homogenizer and suspended in ice-cold binding buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, and 2 mM CaCl₂.

[^8]HLY341495 Binding. For competition binding studies, assay mixtures were incubated for 60 minutes at room temperature in a volume of 0.5 ml containing 10-μg membrane protein (hmGlu2 CHO), appropriate concentrations of test compound, and 2 nM[^8]HLY341495. Nonspecific binding was determined in the presence of 1000 μM glutamate and was approximately 10% of total binding. The incubation was stopped by rapid filtration using Unifilter-96 GF/B filter plates and a Unifilter-96 harvester.

[^8]HDGC-IV Binding. For saturation experiments, 50–75-μg membrane protein (hmGlu2 CHO), test compound, and increasing concentrations ranging from 2 to 800 nM[^8]HDGC-IV were incubated for 60 minutes at room temperature (in total, 11 or 12 concentrations...
were tested. Nonspecific binding (~30% of total binding) was determined in the presence of 10 μM LY341495. Displacement studies were done with 50 nM [3H]DCG-IV. Filtration was performed using Unifilter-96 GF/C filters presoaked in 0.1% polyethyleneimine and a Unifilter-96 harvester.

[3H]JNJ-40068782 Binding. For saturation experiments, 75 μg (hmGlu2 CHO) or 75–125 μg (cortex) of membrane protein, test compound, and increasing concentrations (concentrations in assay ranged from 0.5 to 200 nM, and a total of 12 concentrations were tested) of [3H]JNJ-40068782 were incubated for 60 minutes at room temperature in a total volume of 0.5 ml. Nonspecific binding (~30% of total binding) was determined in the presence of 10 μM JNJ-40068782, a structurally related molecule. Displacement studies were performed using 10 nM radioligand. Filtration was performed using Unifilter-96 GF/C filters presoaked in 0.1% polyethyleneimine and a 40-well manifold or 96-well Brandel harvester 96 (Brandel, Inc., Gaithersburg, MD).

For all radioligands, [3H]LY341495, [3H]DCG-IV, and [3H]JNJ-40068782, assay conditions, including radioligand concentrations, were chosen such that <10% of the free radioligand concentration was receptor-bound. After the addition of scintillation liquid, radioactivity on the filters was counted in a Microplate scintillation and luminescence reader. The sections were dried under a stream of cold air. Rat brain sections were prepared as stated above. The sections were then stored at 20°C until sectioning. Sagittal sections (20 μm) were cut using a Leica C3050 cryostat microtome (Leica Microsystems, Wetzlar, Germany) and thaw-mounted on SuperFrost Plus microscope slides (Menzel-Glaser GmbH, Braunschweig, Germany). The sections were then kept at 20°C until use. Male wild-type C57BL/6 and mGlu2 knockout mice (Deltagen, Inc., San Mateo, CA) weighing 25–30 g were killed by decapitation, and their brains were prepared as stated above.

Receptor Autoradiography. Sections were thawed and dried under a stream of cold air and preincubated (2 × 10 minutes) at room temperature in incubation buffer (50 mM Tris-HCl, 2 mM MgCl2, and 2 mM CaCl2, pH 7.4). Sections were then incubated for 60 minutes at room temperature in buffer containing 10 nM [3H]JNJ-40068782 with nonspecific binding determined by addition of 10 μM JNJ-40068782. After incubation, the excess of radioligand was washed off (2 × 10 minutes) in ice-cold buffer, followed by a rapid dip in cold distilled water. The sections were dried under a stream of cold air. Rat brain sections were exposed to [3H]Hyperlum (Amersham Biosciences, Buckinghamshire, UK) for 6 weeks at room temperature. The films were developed manually in Kodak D19 and fixed with Kodak Readymatic (Eastman Kodak Co., Rochester, NY). Mouse brain sections were exposed for 6 days to a PhosphorImage screen. Digital autoradiograms were obtained using a PhosphorImage FLA7000 (Fuji, Tokyo, Japan).

Selectivity Panel

Ca2+ Assays with Human mGlu1, -3, -5, -7, or -8 Receptor-Expressing HEK293 Cells. Measurement of intracellular Ca2+ mobilization in HEK293 cells stably transfected with the human mGlu1a receptor proceeded as follows. Cells were seeded at 10,000 cells/well in a 384-well plate. Twenty-four hours after seeding, FLIPR Ca2+ assay kit (Molecular Devices, Sunnyvale, CA) dissolved in phosphate-buffered saline supplemented with 5 mM probenecid, pH 7.4 (c.f. 2.5 mM probenecid, as loading buffer was added on the cell layer with no removal of medium) was added, and measurements were initiated 90 minutes thereafter.

Ca2+ assays with human mGlu3 receptor–expressing Go4H-HEK293 cells were performed similarly as for human mGlu2–expressing cells, with the following exception: cells were seeded at a density of 10,000 cells/well, and cell incubation with fluo-4-AM took place at 37°C instead of room temperature.

For the mGlu5 receptor, cells were seeded at 40,000 cells/well in poly-D-lysine-coated 384-well plates, and a day later, medium was removed before addition of a fluo-4-AM solution (HBSS, 2.5 mM probenecid, and 1 μM fluo-4-AM; pH 7.4). Before measurement, cells were incubated for 90 minutes at 37°C and 5% CO2.

For the human mGlu7 and -8 receptor, stably expressing Go4L-HEK cells were seeded at a density of 15,000 cells/well (poly-D-lysine-coated 384-well plates) and incubated with FLIPR Calcium 4 assay kit (dissolved in HBSS, 20 mM HEPES, and 2.5 mM probenecid, pH 7.4) for 90 minutes at 25°C before measurement.

The ratio of peak versus basal fluorescence signals was used for data analysis.

[35S]GTPγS Binding to Membranes from CHO Cells Expressing the Rat mGlu6 Receptor and to Membranes from L929 Sa Cells Expressing the Human mGlu4 Receptor. Membrane preparations were performed as described above (see [35S]GTPγS Binding” under Materials and Methods). Membranes, compound, and glutamate were diluted in assay buffer (10 mM HEPES acid, 10 mM NaCl, 0.3 mM MgCl2, and 10 μM GDP). Assay mixtures included 10 μg of membrane protein and were preincubated with compound alone or together with glutamate for 30 minutes at 37°C. [35S]GTPγS was added at a final concentration of 0.1 nM and incubated for another 30 minutes at 37°C. Reactions were terminated by rapid filtration through Unifilter-96 GF/B filter plates (Packard, Meriden, CT) using a 96-well Packard FilterMate Harvester.

Selectivity of JNJ-40068782 in Additional Radioligand Binding Assays. JNJ-40068782 was tested at a concentration of 10 μM by CEREPS (Celle L’Evescault, France) for its inhibition of radioligand binding to a battery of neurotransmitter and peptide receptors, ion channels, and transporters.

Data Analysis. For both [35S]GTPγS and Ca2+ mobilization assays, data were processed using either the Lexis software interface developed at Janssen Research and Development or GraphPad Prism version 4.02 (GraphPad Software, Inc., La Jolla, CA) and were calculated as percentage of the control agonist challenge. Sigmoid concentration-response curves plotting percentages of effect versus the log concentration of the test molecule were analyzed using nonlinear regression analysis.

To further describe the signaling of glutamate in the presence of a PAM, the fold shift of the glutamate concentration-response was plotted as a function of the concentration of modulator in GraphPad Prism version 4.02 using nonlinear regression; interpolation was then used to allow estimates of concentrations needed for a 5-fold shift.

Inhibition of [3H]JNJ-40068782 binding data were fitted to a one-site binding model (GraphPad Prism version 4.02) and estimates of dissociation constants (Kd) were derived using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Saturation binding experiments were analyzed using nonlinear regression (one-site binding hyperbola; GraphPad Prism version 4.02).

In Vivo Studies

All animal experiments were carried out in strict accordance with the Declaration of Helsinki and the European Communities Council Directive of 24th November 1986 (86/609/EEC) and were approved by the Animal Care and Use Committee of Janssen Research and Development as well as the local ethical committee.

Pharmacokinetic Analysis. Animals were given free access to food and water overnight before dosing and throughout the study. Male Sprague-Dawley rats were given a single oral dose of 10 mg/kg. Blood samples were taken at 0.5, 1, 2, 4, 8, and 24 hours from the tail vein using EDTA as the anticoagulant. Male Swiss mice were dosed with a dose of 10 mg/kg s.c., and blood samples were taken at 0.5, 1, and 1.5 hours after dosing. Plasma was obtained following centrifugation, extracted using protein precipitation, and subsequently analyzed for JNJ-40068782 using a liquid chromatography/tandem mass spectrometry method.
mass spectrometry qualified research method on an API4000 instrument (Applied Biosystems, Foster City, CA).

Sleep-Wake Architecture Study in Rat. Male Sprague-Dawley rats (Charles River Laboratories, Les Oncins, France) weighing 250–300 g at the time of surgery were used. The effect of oral administration of JNJ-40068782 (administered orally 2 hours after light onset using a dose volume of 10 ml/kg) on sleep-wake organization in rats was determined as described in Ahnou et al. (2009). In brief, sleep polysomnographic variables were determined in 32 adult, male Sprague-Dawley rats (n = 8 in each group), which were chronically implanted with electrodes for recording the cortical electroencephalogram (EEG), electrical neck muscle activity, and ocular movements. Polysomnographic data were scored with an automated sleep stages analyzer, scoring each 2-second epoch before averaging stages over 30-minute periods. Sleep-wake state classifications were assigned based upon the combination of dynamics of five EEG frequency domains, integrated electrical neck muscle activity (EMG), ocular movements (EOG), and body activity level as follows: active wake; passive wake; rapid eye movement (REM) sleep; intermediate stage (pre-REM transients); light non-REM sleep; and deep non-REM sleep. Different sleep-wake parameters such as amount of time spent in each state were investigated over 20 postadministration hours.

Time spent in each sleep-wake stage was expressed as a percentage of the recording period. A statistical analysis of the obtained data was carried out by a nonparametric analysis of variance of each 30-minute period, followed by a Wilcoxon–Mann–Whitney rank sum test of comparisons with the control group.

Phencyclidine-Induced Hyperlocomotion in Mice. Test compound or solvent was administered alone (to monitor effects on spontaneous locomotion) or together with phencyclidine (PCP; 5 mg/kg) to male NMRI mice (22 ± 4 g and fasted overnight; Charles River Laboratories) 30 minutes before measurement. Both JNJ-40068782 and PCP were injected subcutaneously, and locomotor activity was measured for a period of 30 minutes. ED_{50} values (the doses inducing 50% responders to criterion) were determined as follows.

All-or-nothing criteria for significant (P < 0.05) effects were defined by analyzing a frequency distribution of a large series of historical control data obtained in solvent-treated animals. Based on the criteria obtained in this way, the ED_{50} and corresponding 95% confidence limits were determined according to the modified Spearman–Kärber estimate, using theoretical probabilities instead of empirical ones. This modification allows determination of the ED_{50} and its confidence interval (CI) as a function of the slope of the log dose-response curve. The criterion for drug-induced decrease of spontaneous locomotion was total distance of <2500 counts (4.1% false positives in controls; n = 410). The criterion for drug-induced inhibition of PCP-induced hyperlocomotion was total distance of <5500 counts (3.9% false positives in controls; n = 154).

Results

JNJ-40068782 Acts as a Selective PAM at the mGlu2 Receptor. Figure 2 shows the concentration-response curves for the glutamate-stimulated increase in [^{35}S]GTPγS binding in absence and presence of JNJ-40068782, BINA, LY487379, or LY2607540. JNJ-40068782, similar to the other PAMs, enhances the glutamate-stimulated increase in [^{35}S]GTPγS. It is noteworthy that all compounds magnified the response produced by glutamate, shifting the glutamate concentration-effect curve both upward and leftward. Whereas a concentration of 429 nM BINA, 568 nM JNJ-40068782, or 305 nM LY2607540 increases the glutamate potency (i.e., lowers the glutamate EC_{50} 5-fold, LY487379 causes only a 2-to 3-fold shift in the glutamate concentration-response curve at the highest tested concentration of 1 mM. Maximal decreases in glutamate EC_{50} of 10- to 12-fold are observed for the other compounds.

By quantifying the increase in response at a fixed glutamate concentration, for example, the glutamate EC_{20}, the potency (EC_{50}) of JNJ-40068782 at the human mGlu2 receptor was estimated to be 143 nM (Fig. 2B; Table 1). This value is similar (overlapping CIs) to the potencies of BINA, LY487379, and LY2607540 (respective EC_{50} values of 89, 256, and 95 nM). The maximal response to glutamate was increased to 309 ± 23% upon addition of JNJ-40068782, which is similar to the potentiation seen with BINA and LY2607540 (221 ± 9 and 215 ± 7%, respectively) (Table 1). However, LY487379 showed a more modest, 147 ± 9% increase in glutamate E_{max}. Although both EC_{50} and E_{max} values slightly increased at the rat compared with the human mGlu2 receptor, we found a good similarity between compound potencies obtained in the rat and human assays (Table 1).

At higher concentrations, JNJ-40068782 also increased [^{35}S]GTPγS binding in the absence of glutamate, suggesting that it acts as an "ago-potentiator." Similar profiles were observed for BINA and LY2607540 but not LY487379. It is noteworthy that JNJ-40068782 along with the reference antagonist rat mGlu2 receptors (i.e., did not have any intrinsic activity in the absence of glutamate) (Table 1).

Further profiling of JNJ-40068782 showed that it also facilitated glutamate-induced Ca^{2+} mobilization at human recombinant mGlu2 receptors. In HEK293 cells coexpressing G_{a16}, JNJ-40068782 potentiated the EC_{20} glutamate response with an EC_{50} value of 15 nM (95% CI, 12–19 nM; n = 30). It is noteworthy that, in this assay, JNJ-40068782 did not increase the maximal response of glutamate (E_{max} = 119 ± 2%; also see Fig. 2C). JNJ-40068782 induced intracellular Ca^{2+} mobilization on its own (allosteric agonism) with an EC_{50} of 618 nM (95% CI 497–768 nM) and E_{max} of 81 ± 3% (n = 25). JNJ-40068782 did not show agonist, antagonist, or PAM activity at the human mGlu3 receptor or any of the group I or group III mGlu receptors up to 10 μM (data not shown). Further profiling (CEREP, Poitiers, France) revealed 59 and 85% inhibition of human 5HT_{2A} (determined with [^{3}H]ketanserin) and human cholecystokinin A (determined with [^{125}I]cholecystokinin-8s) receptor binding at 10 μM. The complete selectivity profile based on CEREP data is shown in Supplemental Table 1.

Modulation of [^{35}S]GTPγS Binding by JNJ-40068782 at Native Rat mGlu2 Receptor. Despite extensive washing of the rat brain sections, in the absence of exogenous glutamate, there remained a basal level of [^{35}S]GTPγS binding that could be reduced by mGlu2/3 receptor antagonists (data not shown), suggesting that there was sufficient residual endogenous glutamate to produce a degree of mGlu2 and/or mGlu3 receptor activation. As a result, glutamate produced much smaller responses in rat brain sections than observed in the recombinant human mGlu2 cell line, with 10 μM glutamate producing a maximal response of approximately 25% over basal, for example. The [^{35}S]GTPγS binding to rat brain sections nevertheless does give a clear illustration that JNJ-40068782 can modulate the effects of glutamate at native rat brain mGlu2 receptors. Hence, JNJ-40068782 was able to increase the [^{35}S]GTPγS binding.
binding to rat brain slices in the absence of exogenous glutamate (Fig. 3). Although this may imply that JNJ-40068782 has intrinsic direct agonist effects at native rat mGlu2 receptors (i.e., can activate mGlu2 receptors in the absence of glutamate), the possibility that this may be a modulation of endogenous glutamate that was not removed during washing of the slices cannot be excluded. In the presence of 10 μM glutamate, JNJ-40068782 produced a robust increase in [35S]GTPγS binding, particularly in the cortex, striatum, and hippocampus (Fig. 3). Potentiation of glutamate by JNJ-40068782 was evident at the lowest concentration of the allosteric modulator, and quantification of these data showed that although 10 μM glutamate or 1 μM JNJ-40068782 alone increased [35S]GTPγS binding in the cortex by ~10 and ~20%, respectively, the combination led to a stimulation of ~80%. In combination with glutamate, a maximal stimulation of ~175% was detected after the addition of 10 or 100 μM JNJ-40068782; in the absence of glutamate, 100 μM JNJ-40068782 increased the response to 125%. Hence, although the potency of JNJ-40068782 on [35S]GTPγS binding at native rat receptors is reduced relative to recombinant systems, presumably as a result of the complication of endogenous glutamate, the anatomic distribution of the responses (i.e., preferential stimulation of the binding in the cortex, striatum, and hippocampus) is consistent with an mGlu2 receptor-mediated response.

**JNJ-40068782 Does Not Affect [3H]LY341495 Binding but Increases [3H]DCG-IV Binding.** To ascertain its allosteric character, JNJ-40068782 was tested for displacement of the prototypic orthosteric (i.e., glutamate-site) mGlu2 receptor antagonist [3H]LY341495 binding to human mGlu2 receptor–expressing membranes. [3H]LY341495 binding to the human mGlu2 receptor was not inhibited upon the addition of 10 μM JNJ-40068782 (Fig. 4A). In contrast to the lack of effect of JNJ-40068782 on the binding of the antagonist radioligand, binding of the radiolabeled orthosteric agonist [3H]DCG-IV was increased up to 2.5-fold upon the addition of JNJ-40068782 at 3 μM (Fig. 4B).
To assess whether JNJ-40068782 alters the apparent affinity of an agonist for the orthosteric binding site, saturation binding of the agonist [3H]DCG-IV was performed in the absence or presence of an EC20 concentration of glutamate. Figure 5A clearly demonstrates that there was a leftward shift in the [3H]DCG-IV binding curve in the presence of JNJ-40068782. Quantification of these data shows that in the absence of JNJ-40068782, the affinity of [3H]DCG-IV for the orthosteric binding site was 240 ± 39 nM (average ± S.E.M. of six experiments), whereas with the PAM present, KD decreased approximately 8-fold, reflecting increased affinity (Table 2). Similar results were seen with other mGlu2 receptor PAMs. The number of binding sites, represented by the Bmax value, was unchanged. The linearity of the Scatchard analysis of the saturation binding data illustrates that both in the absence and presence of JNJ-40068782, the binding of [3H]DCG-IV is best described by a single binding site. To see whether the same effects were seen for the natural agonist glutamate, glutamate-mediated inhibition of [3H]DCG-IV binding was assessed in the absence and presence of 3 μM JNJ-40068782. In line with previous data, JNJ-40068782 not only increased the maximal binding but also enhanced the affinity of glutamate, i.e., lowered the IC50 of glutamate by ∼5-fold (4.5- and 6.7-fold found in two independent experiments) (Fig. 5B).

### Binding of [3H]JNJ-40068782 to Recombinant Human mGlu2 Receptors

JNJ-40068782 was tritiated and used to characterize the binding to human recombinant mGlu2 receptors (Fig. 6A). Binding was saturable and was best-fit to a single-site model defined by a KD of 12 ± 4 nM with a Bmax of 4.5 ± 1.9 pmol/mg protein (n = 12). At a concentration of 10 nM, the level of specific binding was in the region of 70%. It is noteworthy that, in the presence of agonist (either glutamate, DCG-IV, or LY354740), the apparent affinity of [3H]JNJ-40068782 was increased (average KD in the presence of 100 μM glutamate was 4.0 ± 1.2 nM; n = 4), with the saturation curve being leftward-shifted (Fig. 6B). In each of the four experiments where binding was measured in the absence or presence of glutamate, the Bmax value of [3H]JNJ-40068782 was increased upon addition of the agonist, with an average increase of 2-fold.

The binding of [3H]JNJ-40068782 to human recombinant mGlu2 receptors was inhibited by JNJ-40068782, with a mean IC50 of 38 nM (95% CI 27–49 nM; n = 9) (Fig. 7A). In addition, BINA, LY483739, and LY2607540 inhibited [3H]JNJ-40068782 with respective IC50 values of 385 nM (95% CI 277–847 nM; n = 9) (Fig. 7B).

### Table 1: Functional activity of JNJ-40068782, BINA, LY487379, and LY2607540 at human and rat recombinant mGlu2 receptors, in the absence or presence of an EC20 concentration of glutamate

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity in Absence of Glutamate</th>
<th>Activity in Presence of EC20 Glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Emax (%)</td>
<td>EC50 (nM)</td>
</tr>
<tr>
<td>Human mGlu2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JNJ-40068782</td>
<td>69 ± 9</td>
<td>925 (573–1493)</td>
</tr>
<tr>
<td>BINA</td>
<td>60 ± 3</td>
<td>572 (141–2324)</td>
</tr>
<tr>
<td>LY487379</td>
<td>20 ± 3</td>
<td>&gt;50.000</td>
</tr>
<tr>
<td>LY2607540</td>
<td>28 ± 2</td>
<td>720 (631–821)</td>
</tr>
<tr>
<td>Rat mGlu2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JNJ-40068782</td>
<td>31 ± 3.7</td>
<td>&gt;30.000</td>
</tr>
<tr>
<td>BINA</td>
<td>35 ± 4.9</td>
<td>&gt;30.000</td>
</tr>
<tr>
<td>LY487379</td>
<td>7.4 ± 3.9</td>
<td>&gt;30.000</td>
</tr>
<tr>
<td>LY2607540</td>
<td>13 ± 1.5</td>
<td>&gt;30.000</td>
</tr>
</tbody>
</table>

Fig. 3. Binding of [35S]GTPγS to rat brain sections revealed by quantitative autoradiography. (A) Sagittal rat brain sections were incubated with various concentration of JNJ-40068782 in the absence or presence of 10 μM glutamate. The top left brain section represents, in the absence of glutamate and JNJ-40068782, the basal [35S]GTPγS binding. (B) Quantification of the data represented in the images. Bar graphs represent the average (n = 3 rats) percent stimulation by glutamate and/or PAM over basal binding.
whether the interaction between these molecules and JNJ-40068782 is completely competitive, Hill slopes of the inhibition curves are close to 1 and binding of [3H]JNJ-40068782 was fully displaced upon addition of compound (i.e., the same level of nonspecific binding was observed for all structurally unrelated modulators), suggesting a competitive interaction. Hence, IC50 values were also converted to Ki values according to the Cheng-Prusoff equation (Ki was 20 nM for JNJ-40068782, 215 nM for BINA, 65 nM for LY483739, and 55 nM for LY2607540). Additional studies showed that the negative allosteric modulator 4-[3-(2,6-dimethylpyridin-4-yl)phenyl]-7-methyl-8-trifluoromethyl-1,3-dihydrobenzo[b][1,4]diazepin-2-one (RO4491533) also fully displaced [3H]JNJ-40068782 [IC50 of 16 nM (95% CI 7–39 nM; n = 4); Ki of 9 nM; data not shown].

Binding of [3H]JNJ-40068782 to Rodent Brain Tissue. [3H]JNJ-40068782 bound with a similar affinity to receptors expressed in rat cortex; KD and Bmax were 1064 nM (c.f. 1264 nM at human recombinant mGlu2 receptors) and 14.765.7 pmol/mg protein (n = 6), respectively. IC50 values for displacing cortical mGlu2 receptors were 52 nM (95% CI 34–78 nM; n = 7), 617 nM (95% CI 242–1571 nM; n = 5), 102 nM (95% CI 55–190 nM; n = 5), and 79 nM (95% CI 43–143 nM; n = 3) for JNJ-40068782, BINA, LY483739, and LY2607540, respectively (assuming a competitive interaction, Ki values are 26, 305, 51, and 39 nM, respectively), confirming that these PAMs also bind to native receptors (Fig. 7B).

By use of radioligand autoradiography, we examined [3H]JNJ-40068782 binding distributions in rat brain sections in further detail (Fig. 8A). High specific binding was observed in the hippocampal formation, granular layer of the cerebellum, striatum, and cortex, corresponding very well with the expression pattern demonstrated using the mGlu2/3 receptor antagonist [3H]LY341495. [3H]JNJ-40068782 binding site distribution was similar in wild-type mouse brain. It is important to note that binding was lost in mGlu2 knockout mice, underlining the mGlu2 specificity of the radioligand (Fig. 8B).

Effects of JNJ-40068782 on Sleep-Wake Organization in the Rat. The preceding data collectively demonstrate that JNJ-40068782 not only binds to native mGlu2 receptors but...
also selectively potentiates the functional response at rat brain mGlu2 receptors. As JNJ-40068782 was shown to be brain-penetrant (with $C_{\text{max}}$ of $\sim$1400 ng/ml and brain-to-plasma ratio of $\sim$0.4 after oral dosing of 10 mg/kg in rats), we set out to evaluate some of the in vivo functional effects of this novel mGlu2 receptor PAM.

We have previously shown that selective activation or positive allosteric modulation of the mGlu2 receptor in vivo affects the sleep-wake architecture of rats (Ahnaou et al., 2009). To assess whether JNJ-40068782 has in vivo central activity, we evaluated sleep-wake organization of rats after oral dosing of JNJ-40068782.

The time course of vigilance states over 20 hours following administration of vehicle and JNJ-40068782 is illustrated in Fig. 9. Acute administration of JNJ-40068782 (3, 10, and 30 mg/kg PO) significantly suppressed the time spent in REM sleep and intermediate stage, whereas no major effects on the other sleep-wake stages were observed. Quantification of effect over a 4-hour time period following compound administration revealed a dose-related reduction in time spent in REM sleep with a lowest active dose of 3 mg/kg PO,

### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_D$ (nM)</th>
<th>$B_{\text{max}}$ (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>240 ± 39</td>
<td>14.5 ± 2.8</td>
</tr>
<tr>
<td>JNJ-40068782 (3 μM)</td>
<td>22/39</td>
<td>16.6/7.6</td>
</tr>
<tr>
<td>BINA (3 μM)</td>
<td>47/81</td>
<td>15.5/9.0</td>
</tr>
<tr>
<td>LY487379 (3 μM)</td>
<td>35/53</td>
<td>17.25/7.0</td>
</tr>
</tbody>
</table>

Table 2: Characteristics of $[\text{3H}]$DCG-IV binding to human recombinant mGlu2 receptors in the absence and presence of JNJ-40068782, BINA, and LY487379.

The $K_D$ and $B_{\text{max}}$ of $[\text{3H}]$DCG-IV in the absence of compound is an average ± S.E.M. of six experiments. The same parameters measured in the presence of compound come from two independent experiments (values from both experiments are given).
corresponding to a plasma drug concentration of ~420 ng/ml. REM sleep reduction was accompanied by consistent lengthening in REM sleep onset latency (Fig. 9, upper small bar graph panel in REM sleep stage).

**Effects of JNJ-40068782 on PCP-Induced Hyperactivity in Mice.** The effects of JNJ-40068782 on spontaneous locomotor activity was measured at doses ranging from 2.5 to 10 mg/kg s.c. (Fig. 10A). The compound produced a modest reduction in spontaneous activity, with the total distance traveled dropping by 20% from 2974 cm in vehicle-treated mice to 2365 cm in animals receiving 20 mg/kg JNJ-40068782.

We subsequently evaluated whether JNJ-40068782 was able to mimic some of the effects of mGlu2/3 agonists in an animal model that is used to predict potential antipsychotic compounds that selectively potentiate the effects of glutamate at mGlu2 receptors (mGlu2 PAMs) can reproduce some of the effects seen with the nonelective mGlu2/3 receptor agonists. Such compounds include N-(4’-cyano-[1,1’-biphenyl]-3-yl-N-(3-pyridinylmethyl) ethanesulfonamide (Johnson et al., 2005), BINA (Galici et al., 2005, 2006), and LY487379 (Johnson et al., 2003; Schaffhauser et al., 2003; Galici et al., 2005). Our studies have provided new pharmacological tools to study the mGlu2 receptor PAM concept, including JNJ-40068782 and [3H]JNJ-40068782. Although the in vitro potencies for the reference mGlu2 receptor PAMs vary from laboratory to laboratory, presumably as a result of differences in receptor expression systems and/or assay methodologies, it nevertheless seems that LY487379 is consistently less potent (with in vitro potency for the human mGlu2 receptor ranging from ~250 nM to ~1.5 µM) than BINA, LY2607540, and JNJ-40068782, all of which may be considered equipotent (with potencies ranging from ~20 nM to 1 µM depending on the assay).

By use of a [35S]GTPγS assay for mGlu2 receptors expressed in CHO cells, there does not seem to be any marked rat versus human species differences for the four above-mentioned PAMs, although EC50 values were generally 2-fold lower, and Emax was 1.5-fold higher between rat and human mGlu2 data. Screening of >100 additional compounds furthermore confirmed this pattern, with no examples of compounds being active in one species but not the other (data not shown; correlation plots can be found in Supplemental Fig. 1). JNJ-40068782 induced a receptor response even in the absence of agonist in some but not all in vitro assays, indicating that the functional effects of this molecule can be different depending on the functional readout as well as the level of receptor expression and hence seem context-dependent. This is in line with the thinking that ligand “behaviors” rather than inherent “properties” can be described and that detection of agonist “behavior” depends on the ligand and sensitivity of the assay used (Langmead, 2012). It also cannot be ruled out that the response can, at least in part, be attributed to the presence of endogenous glutamate.

It is not yet clear why JNJ-40068782 seems to be more potent in the Ca2+ assay compared with the [35S]GTPγS binding assay. Moreover, the maximal response to glutamate was not increased when measuring changes in intracellular Ca2+ concentrations, in contrast to the dramatic increase in response when measuring [35S]GTPγS accumulation. Several reasons may underlie these differences, including the use of different cellular backgrounds, potential differences in receptor and/or G protein expression levels, the transient (Ca2+ versus endpoint ([35S]GTPγS) nature of the assay, potential fluorescent dye saturation, differences in equilibrium binding conditions, or compound solubility during the assay procedure. It can furthermore not be excluded that forced coupling of the mGlu2 receptor to the Ca2+ pathway affects ligand potency compared with using an assay where the
receptor signals via a natural endogenous protein. JNJ-40068782 also enhanced native mGlu2 receptor signaling as shown using GTPγS autoradiography on rat brain slices. Allosteric modulators may enhance the efficacy of the endogenous agonist not only by “magnifying” the intracellular responses produced by the agonist but also by altering the apparent affinity of the agonist, or a mixture of the two (Conn et al., 2009). In the present study, JNJ-40068782, along with BINA and LY487379, was able to increase the affinity of agonists, with the KD for [3H]DCG-IV binding to recombinant human mGlu2 receptors decreasing by ~5- to 10-fold in the presence of mGlu2 PAMs. Moreover, and as expected, although JNJ-40068782 increased the affinity of agonists at the orthosteric binding site, it did not alter the binding of the orthosteric antagonist [3H]LY341495.

Although our data thus far enabled us to gain an understanding of how PAMs can affect agonist signaling and binding, we set out to study effects on allosteric interaction by agonists, with the use of selective mGlu2 receptor PAM radioligand binding. Radioligands that selectively recognize an allosteric modulatory site have been described for the mGlu1 ([3H]R214127 [Lavreysen et al., 2003]) and mGlu5 ([3H]M-MPEP [Gasparini et al., 2002]; [3H]mPEPy [Cosford et al., 2003]) receptors. Recently, a novel mGlu2/3 receptor agonist radioligand was reported (Wright et al., 2013). However, [3H]JNJ-40068782 is the first selective mGlu2 radioligand that is described extensively. JNJ-40068782 was selective for mGlu2 receptors as defined by CEREP profiling, where a significant interaction at CCK receptors, which are known to be expressed only in very discrete brain regions such as the interpeduncular nucleus and nucleus tractus solitarius (Hill et al., 1987), was observed at 10 μM JNJ-40068782. At relatively high concentrations, JNJ-40068782 also acted on the 5HT2A receptor (IC50, ~10 μM); it is, however, unlikely that it reaches sufficient amounts in vivo to trigger a functional 5HT2A-related effect. Using [3H]ketanserin,
we did not observe in vivo binding up to 30 mg/kg PO (data not shown); also in addition, JNJ-40068782 did not affect sleep-wake behavior in rats, whereas ample evidence exists for modulation of deep non-REM sleep via the 5HT2A receptor (Al-Shamma et al., 2010). Moreover, in rat brain [35S]GTPγS binding experiments, the preferential stimulation of binding in the cortex and striatum is again consistent with JNJ-40068782 selectively modulating the function of mGlu2 receptors. Finally, the selectivity of the radioligand for mGlu2 versus mGlu3 receptors was confirmed by the fact that binding was lost in mGlu2 but retained in mGlu3 receptor knockout mice brains.

[3H]JNJ-40068782 binding met all of the requirements for a ligand well suited to study binding properties, pharmacology, and distribution of mGlu2 receptors. [3H]JNJ-40068782 showed saturable one-site binding, with its affinity (~10 nM) being similar between cloned human receptors and native mGlu2 receptors in rat cortex. The structurally diverse PAMs BINA, LY487379, and LY2607540 seem to share common determinants in the allosteric binding site because, both in recombinant cells and in brain tissue, they could all displace [3H]JNJ-40068782. It is noteworthy that the mGlu2 receptor negative allosteric modulator RO4491533 was also able to inhibit the binding of JNJ-40068782, indicating that both negative and positive allosteric modulators share common determinants of binding within the allosteric modulatory site.

[3H]JNJ-40068782 binding was used to gain more insight into how orthosteric binding would influence binding to the allosteric seven-transmembrane domain. In line with the effect of JNJ-40068782 on agonist binding, demonstrated with both [3H]DCG-IV and glutamate, there was a reciprocal effect of orthosteric agonists (glutamate, DCG-IV, and LY354740) enhancing binding affinity of [3H]JNJ-40068782. Whereas agonists predominantly label the high-affinity or G protein–coupled receptor state, an antagonist shows equal affinity for coupled and uncoupled receptors, or for both the high- and low-affinity states of the receptor. Our finding that the Bmax for [3H]JNJ-40068782 was only approximately half of that of [3H]DCG-IV is interesting and may suggest that PAMs preferentially bind to certain receptor states that are not necessarily similar to those preferentially recognized by DCG-IV. Alternatively, JNJ-40068782 may bind to only one of the subunits within the mGlu2 homodimer, whereas DCG-IV likely binds to both, which might be consistent with previous reports that binding and closure of both extracellular domains within the mGlu receptor dimer are needed for full activity, whereas only a single heptahelical domain is turned on upon receptor activation (Kniazeff et al., 2004; Hlavackova et al., 2005). Even more intriguing is the finding that, in the presence of agonists (glutamate or LY354740), the Bmax of [3H]JNJ-40068782 was consistently increased, opening the possibility of a changed receptor conformation allowing binding to both receptor subunits in the mGlu2 receptor dimer, upon which a “superactivated” state may be imposed.

Because of its specificity, [3H]JNJ-40068782 proved to be suitable for investigation of mGlu2 receptor distribution in brain sections using radioligand autoradiography. These studies showed that the highest level of mGlu2-specific binding was present in the hippocampus, striatum, cortex, and granular layer of the cerebellum. These results correspond to the binding of [3H]LY341495 and to the binding of [3H]LY354740 reported by Richards et al. (2005), where [3H]LY354740 was used under such conditions that it bound selectively to mGlu2 receptors. The distribution of [3H]JNJ-40068782 binding was also comparable to the anatomic distribution of mGlu2 receptor mRNA and protein (Ohishi et al., 1993, 1998; Gu et al., 2008).

JNJ-40068782 proved to have appropriate pharmacological and pharmacokinetic properties to evaluate its effects in vivo. It is well established that mGlu2 receptor modulation affects sleep-wake architecture in rats and mice (Feinberg et al.,
02; Ahnaou et al., 2009; Fell et al., 2011). Consistent with these data, we found that JNJ-40068782 dose-dependently decreased the amount of REM sleep in rats when dosed early in the light phase. The effects on sleep-wake organization in rats may well reflect the modulation of physiologic glutamatergic signaling because it has been shown that glutamate levels are increased during REM sleep in rat orbitofrontal and cerebral cortex (Lopez-Rodriguez et al., 2007; Dash et al., 2009). Moreover, because of the translational nature of the sleep-wake EEG paradigm, polysomnography measurements in man may be used as a reproducible and sensitive biomarker. Nevertheless, it remains to be determined to what extent the changes in REM sleep, although a reliable pharmacodynamic marker, have relevance for potential mGlu2-mediated therapeutic efficacy.

As regards potential therapeutic indications for mGlu2 receptor modulation, JNJ-40068782 decreased PCP-induced hyperlocomotion, which is a model in which dopamine D2-related antipsychotics demonstrate robust efficacy. However, the doses at which JNJ-40068782 is active in this model correspond to plasma drug concentrations somewhat higher than those in the sleep-wake organization paradigm, although this may merely reflect the fact that greater drug concentrations may be required to demonstrate efficacy in a challenge model (PCP-induced hyperactivity) compared with a more physiologic situation (sleep-wake). Although these findings provide more evidence that mGlu2 PAMs can mimic aspects of the pharmacology of mGlu2/3 agonists, and hence have the potential to exhibit antipsychotic behavior, studies are ongoing to provide more insight into the translation of compound activities in preclinical animal models into relevant dose levels in a clinical setting.

Collectively, our data indicate that JNJ-40068782 is a selective PAM of native and recombinant rat and recombinant human mGlu2 receptors. It not only enhances the affinity of agonists but also increases the maximal response. In the absence of exogenous glutamate, JNJ-40068782 has a modest degree of intrinsic agonist efficacy. [3H]JNJ-40068782 represents an excellent novel radiotracer to study mGlu2 allosteric interaction, expression, and distribution. With [3H]JNJ-40068782, we show for the first time that agonists also increase the maximal response. In the selective PAM of native and recombinant rat and recombinant human mGlu2 receptors, and hence have the potential to exhibit antipsychotic behavior, studies are ongoing to provide more insight into the translation of compound activities in preclinical animal models into relevant dose levels in a clinical setting.

Acknowledgments

The authors thank Stefan Pype and Claire Mackie for their continuous support, Michel Mahieu and Heidi Huyssmans for technical assistance, and Addex Therapeutics for its collaboration on the mGlu2 PAM discovery program, with special thanks to Francoise Girard, Emmanuel Le Poul, and Guillaume Duve.

Authorship Contributions

Participated in research design: Lavreyens, Langlois, Ahnaou, Drinkenburg, te Riele, Biezens, Van der Linden, Peeters, Megens, Wintmolders, Cid, Trabanco, André, Macdonald, Lütjens, Dautzenberg, Atack.

Conducted experiments: Lavreyens, te Riele, Biezens, Van der Linden, Peeters, Wintmolders.

Contributed new reagents or analytic tools: Cid, Trabanco, André.

Performed data analysis: Lavreyens, Ahnaou, te Riele, Biezens, Van der Linden, Peeters, Megens, Wintmolders.

Wrote or contributed to the writing of the manuscript: Lavreyens, Langlois, Ahnaou, Drinkenburg, te Riele, Biezens, Van der Linden, Peeters, Megens, Wintmolders, Cid, Trabanco, André, Macdonald, Lütjens, Dautzenberg, Atack.

References


Cheng and Prusoff WH (1973) Relationship between the inhibition constant (K) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108.


Galic R, Echemendia NG, Williams LC, de Paulis A, and Conn PJ (2005) A selective allosteric potentiator of metabotropic glutamate (mGlu)2 receptors has effects similar to an orthosteric mGlu2/3 receptor agonist in mice models predictive of antipsychotic activity. J Pharmacol Exp Ther 314:1181–1187.


