Preclinical Characterization of (*R*)-3-((3*S*,4*S*)-3-fluoro-4-(4-hydroxyphenyl)piperidin-1-yl)-1-(4-methylbenzyl)pyrrolidin-2-one (BMS-986169), a Novel, Intravenous, Glutamate *N*-Methyl-D-Aspartate 2B Receptor Negative Allosteric Modulator with Potential in Major Depressive Disorder^S

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ABSTRACT

(*R*)-3-((3*S*,4*S*)-3-fluoro-4-(4-hydroxyphenyl)piperidin-1-yl)-1-(4-methylbenzyl)pyrrolidin-2-one (BMS-986169) and the phosphate prodrug 4-((3*S*,4*S*)-3-fluoro-1-((*R*)-1-(4-methylbenzyl)-2-oxopyrrolidin-3-yl)piperidin-4-yl)phenyl dihydrogen phosphate (BMS-986163) were identified from a drug discovery effort focused on the development of novel, intravenous glutamate *N*-methyl-D-aspartate 2B receptor (GluN2B) negative allosteric modulators (NAMs) for treatment-resistant depression (TRD). BMS-986169 showed high binding affinity for the GluN2B subunit allosteric modulatory site ($K_i = 4.03\text{--}6.3$ nM) and selectively inhibited GluN2B receptor function in *Xenopus* oocytes expressing human *N*-methyl-D-aspartate receptor subtypes ($IC_{50} = 24.1$ nM). BMS-986169 weakly inhibited human ether-a-go-go-related gene channel activity ($IC_{50} = 28.4$ μ M) and had negligible activity in an assay panel containing 40 additional pharmacological targets. Intravenous administration of BMS-986169 or BMS-986163 dose-dependently increased GluN2B receptor occupancy and inhibited in vivo [3H](+)-5-methyl-10,11-dihydro-

5H-dibenzo[*a,d*]cyclohepten-5,10-imine ([3H]MK-801) binding, confirming target engagement and effective cleavage of the prodrug. BMS-986169 reduced immobility in the mouse forced swim test, an effect similar to intravenous ketamine treatment. Decreased novelty suppressed feeding latency, and increased ex vivo hippocampal long-term potentiation was also seen 24 hours after acute BMS-986163 or BMS-986169 administration. BMS-986169 did not produce ketamine-like hyperlocomotion or abnormal behaviors in mice or cynomolgus monkeys but did produce a transient working memory impairment in monkeys that was closely related to plasma exposure. Finally, BMS-986163 produced robust changes in the quantitative electroencephalogram power band distribution, a translational measure that can be used to assess pharmacodynamic activity in healthy humans. Due to the poor aqueous solubility of BMS-986169, BMS-986163 was selected as the lead GluN2B NAM candidate for further evaluation as a novel intravenous agent for TRD.

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Introduction

Major depressive disorder (MDD) is a prevalent disease and a leading cause of global disability (Ustün et al., 2004). In the United States alone, recent reports show a prevalence of 6.8% with 15.4 million individuals affected and an overall economic burden of ~\$200 billion (Greenberg et al., 2015). Although

therapeutics targeting the monoaminergic systems have been available for many years, many patients fail to show an adequate treatment response. In this regard, results from the STAR*D (Sequenced Treatment Alternatives to Relieve Depression) study showed that only one-third of patients treated with a selective serotonin reuptake inhibitor achieved remission, with 40% of those requiring more than 8 weeks of treatment for resolution of their symptoms (reviewed by Gaynes et al., 2009). An additional 50% of patients showed partial improvement but still exhibited residual symptoms (McClintock et al., 2011), which increases the probability of future relapse and poorer functional and psychosocial outcomes (Paykel et al., 1995; Fava 2006). Finally, almost one third of patients were resistant to treatment and failed to respond to additional antidepressant drug switching and augmentation approaches. Clearly, a high unmet medical need exists for new agents with novel mechanisms that show rapid and improved efficacy in patients with MDD and treatment-resistant depression (TRD).

In the search for novel mechanisms, *N*-methyl-D-aspartate (NMDA) receptor antagonists have been intensely investigated following the first demonstration that the nonselective NMDA receptor channel blocker ketamine, is efficacious in TRD patients (Berman et al., 2000). The clinical response to ketamine was remarkable in that a single, i.v., subanesthetic dose produced rapid symptom relief within hours of dosing that was sustained for up to 7 days. Subsequent studies have confirmed that acute ketamine treatment rapidly achieves a 66%–77% response rate and 31% remission rate in TRD patients and have also extended these findings to patients with bipolar depression (reviewed by Lener et al., 2017). Although this unique efficacy profile is widely accepted, ketamine also produces undesirable effects, including transient dissociative and psychotomimetic effects that emerge during the infusion but resolve within ~2 hours of dosing and may therefore be manageable within the treatment environment. However, additional concerns, including the potential for neuronal injury and abuse, have led to the search for alternative approaches that may retain the efficacy profile of ketamine but with fewer liabilities. In this regard, agents that selectively inhibit the glutamate NMDA 2B (GluN2B) receptor subtype have received attention. This target has been the focus of extensive drug development efforts aimed at the treatment of conditions such as stroke, Parkinson's disease, and chronic pain (Layton et al., 2006). GluN2B receptors possess an amino terminal domain allosteric binding site that can be targeted to develop subtype-selective, negative allosteric modulators (NAMs) (Mony et al., 2009). In a small proof-of-concept study in selective

serotonin reuptake inhibitor-resistant MDD patients, a single i.v. administration of the GluN2B NAM CP-101,606 [(1*S*,2*S*)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol] achieved a 60% response rate and 30% remission rate 5 days after dosing with an infusion regimen that produced minimal dissociative effects (Preskorn et al., 2008).

Preclinical results also show that GluN2B NAMs have a ketamine-like antidepressant profile, and that the effects of ketamine may involve the GluN2B receptor subtype. Acute treatment with the GluN2B NAM Ro 25-6981 (4-[(1*R*,2*S*)-3-(4-benzylpiperidin-1-yl)-1-hydroxy-2-methylpropyl]phenol) activates the mechanistic target of rapamycin (mTOR) signaling pathway and increases synaptic protein expression, effects thought to underlie the rapid antidepressant effect of ketamine (Li et al., 2010). Direct infusion of the mTOR inhibitor rapamycin into the medial prefrontal cortex prevents the antidepressant-like behavioral effects of Ro 25-6981, and treatment with the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzof[*l*]quinoxaline-2,3-dione) inhibits antidepressant activity and mTOR pathway activation after acute ketamine or Ro 25-6981 administration, suggesting common mechanisms (Maeng et al., 2008; Li et al., 2010). On the other hand, genetic deletion of GluN2B subunits in cortical pyramidal neurons mimics and occludes the effects of ketamine on mTOR pathway activation and synaptic protein synthesis, excitatory synaptic transmission, and depression-like behavior (Miller et al., 2014). Although it should be acknowledged that not all groups are able to demonstrate the mTOR pathway activation effect of ketamine (Popp et al., 2016), these results have led to renewed interest in GluN2B NAMs and their potential as rapid-acting antidepressant agents for TRD (Miller et al., 2016).

The present studies describe the pharmacological characterization of BMS-986169 [(*R*)-3-((3*S*,4*S*)-3-fluoro-4-(4-hydroxyphenyl)-piperidin-1-yl)-1-(4-methylbenzyl)pyrrolidin-2-one], a novel GluN2B NAM identified during a drug discovery program focused on the identification of an i.v. therapeutic for TRD. We also present results for BMS-986163 [4-((3*S*,4*S*)-3-fluoro-1-((*R*)-1-(4-methylbenzyl)-2-oxopyrrolidin-3-yl)piperidin-4-yl)phenyl dihydrogen phosphate], the phosphate prodrug of BMS-986169, which was developed to address the poor aqueous solubility of the parent molecule.

Materials and Methods

Animals. Studies were conducted at either Bristol-Myers Squibb (BMS, Wallingford, CT) or at the Biocon Bristol-Myers Squibb Research Center (BBRC, Bangalore, India).

BMS Studies. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility and maintained in accordance with the guidelines of the Animal Care

ABBREVIATIONS: aCSF, artificial cerebrospinal fluid; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ANOVA, analysis of variance; BBRC, Biocon Bristol-Myers Squibb Research Center; BMS, Bristol-Myers Squibb; BMS-986163, 4-((3*S*,4*S*)-3-fluoro-1-((*R*)-1-(4-methylbenzyl)-2-oxopyrrolidin-3-yl)piperidin-4-yl)phenyl dihydrogen phosphate; BMS-986169, (*R*)-3-((3*S*,4*S*)-3-fluoro-4-(4-hydroxyphenyl)-piperidin-1-yl)-1-(4-methylbenzyl)pyrrolidin-2-one; CANTAB, Cambridge Neuropsychological Test Automated Battery; CL, confidence limit; CP-101,606, (1*S*,2*S*)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol; CSF, cerebrospinal fluid; fEPSP, field excitatory postsynaptic potential; FST, forced swim test; GluN, glutamate *N*-methyl-D-aspartate; hERG, human ether-a-go-go-related gene potassium channel; HFS, high-frequency stimulation; list-DMS, list delayed match to sample; LMA, locomotor activity; LTP, long-term potentiation; MDD, major depressive disorder; MK-0657 (CERC-301), 4-methylbenzyl-(3*S*,4*R*)-3-fluoro-4-[(2-pyrimidinylamino)methyl]-1-piperidinecarboxylate; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohept-5,10-imine; mTOR, mechanistic target of rapamycin; NAM, negative allosteric modulator; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzof[*l*]quinoxaline-2,3-dione; NMDA, *N*-methyl-D-aspartate; NSF, novelty suppressed feeding; Occ50, dose producing 50% occupancy; PEI, polyethylenimine; qEEG, quantitative electroencephalogram; RM, repeated measures; Ro-04-5595, 1-[2-(4-chlorophenyl)ethyl]-1,2,3,4-tetrahydro-6-methoxy-2-methyl-7-isoquinolinol; Ro 25-6981, 4-[(1*R*,2*S*)-3-(4-benzylpiperidin-1-yl)-1-hydroxy-2-methylpropyl]phenol; TRD, treatment resistant depression.

and Use Committee of the Bristol-Myers Squibb Company, the "Guide for Care and Use of Laboratory Animals," and the guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Research protocols were approved by the Bristol-Myers Squibb Company Animal Care and Use Committee. In vivo [^3H](+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine ([^3H]MK-801) binding studies were conducted in singly housed, male Sprague Dawley rats (170–250 g; ~6–8 weeks of age) supplied with a previously implanted jugular vein catheter (Hilltop Laboratory Animals Inc., Scottsdale, PA). Ex vivo GluN2B occupancy studies were conducted in male Sprague Dawley rats housed two rats/cage purchased from Charles River Laboratories (Raleigh, NC; 5–7 weeks of age) or Harlan (Indianapolis, IN; 7–9 weeks of age). Ex vivo long-term potentiation (LTP) studies were conducted in male Sprague Dawley rats housed two rats/cage purchased from Harlan (4–6 weeks of age). Cage size for both single- and pair-housed rats was 10.25 $\times$ 14 inches, and animals were housed in temperature- (21 $\pm$ 2°C) and humidity-controlled (50% $\pm$ 10%) rooms maintained under a 12-hour light/dark cycle (lights on at 06:00 hours) with food and water available ad libitum. Cognitive performance, behavioral observational studies, and quantitative electroencephalogram (qEEG) studies were conducted in separate cohorts of male cynomolgus monkeys (*Macaca fascicularis*; 5–7 years of age, 5.0–8.5 kg). Monkeys were typically pair housed [cage size (width/depth/height): 64 $\times$ 31 $\times$ 33 inches] and fed standard monkey chow (Teklad global 20% protein Primate Diet 2050; Harlan). Subjects for cognitive testing were fed sufficient quantities to ensure normal growth while maintaining motivation to perform cognitive tasks (Weed et al., 1999); subjects for behavioral observation were food restricted overnight prior to compound administration. For all subjects, water was continuously available except during studies, and fresh fruit or dietary enrichment was provided twice weekly. Toys and foraging devices were routinely provided, and television programs were available in the colony rooms. Subjects were fitted with plastic or metal neck collars (Primate Products, Immokalee, FL).

BBRC Studies. All experimental procedures were conducted in accordance with the guidelines set by the Committee for the Purpose of Control and Supervision on Experiments on Animals, Ministry of Environment and Forests, Government of India. An institutional animal ethics committee approved the experimental protocols, and all animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited animal facility. Female *Xenopus laevis* were obtained from Nasco (Fort Atkinson, WI) and housed two frogs/cage in Tecniplast (Buguggiate, Varese, Italy) Xenopus standalone housing. Water temperature and pH were maintained at 18 $\pm$ 1°C and 6.8–7.4, respectively, with regular monitoring of nitrogenous wastes. Frogs were fed with Zeigler adult *Xenopus* diet (*Xenopus* Express, Brooksville, FL) three times per week, and each cage was provided with enrichment tubes and pads. Male Sprague Dawley rats (7–10 weeks of age; Harlan, Livermore, CA) were used for in vitro GluN2B binding studies and were housed three rats/cage (cage size: 12.6 $\times$ 12 inches). Male CD-1 mice were obtained directly from Harlan (Horst, Limburg, Netherlands) for GluN2B occupancy (5–8 weeks of age) and locomotor activity (LMA) studies (6–7 weeks of age) or from in-house breeding colonies derived from Harlan CD-1 mice for the forced swim test (FST; 6–7 weeks of age). Novelty suppressed feeding (NSF) studies were conducted in male BALB/c mice (13–14 weeks of age; Taconic Vivo-bio, Hyderabad, India). Mice were housed five/cage (cage size: 12.8 $\times$ 6.6 inches), and all rodents were held in colony rooms with controlled temperature (22–24°C) and humidity (48%–54%) under a 12-hour light/dark cycle (lights on at 07:00 hours) with food and water available ad libitum unless specified otherwise.

Reagents. BMS-986169, BMS-986163, CP-101,606 [Traxoprodil; free base (in vitro studies) or methanesulfonic acid salt], Ro 25-6981, MK-0657 (CERC-301; 4-methylbenzyl-(3*S*,4*R*)-3-fluoro-4-[(2-pyrimidinylamino)methyl]-1-piperidinecarboxylate), [^3H]Ro 25-6981, and [^3H]MK-801 were synthesized by the BMS or BBRC chemistry groups or the BMS radiosynthesis group. The chemical structures of BMS-986169 and the phosphate prodrug BMS-986163 are shown in

Fig. 1. Chemical structures of BMS-986169 and the phosphate prodrug BMS-986163.

Fig. 1. Sources of other reagents were as follows: (+)MK-801 hydrogen maleate (Sigma-Aldrich, St. Louis, MO), ketamine HCl [Ketaset (Fort Dodge Animal Health, Fort Dodge, IA) or Aneket (Neon Pharmaceuticals, Mumbai, India)], 1-[2-(4-chlorophenyl)ethyl]-1,2,3,4-tetrahydro-6-methoxy-2-methyl-7-isoquinolinol hydrochloride (Ro-04-5595) (Tocris Bioscience, Bristol, UK).

GluN2B Binding K_i Determinations In Vitro. Studies to determine in vitro binding affinity at rat brain GluN2B receptors were conducted at BBRC. For membrane preparation, rat forebrains were thawed on ice for 20 minutes in homogenization buffer composed of 50 mM KH_2PO_4 (pH adjusted to 7.4 with KOH), 1 mM EDTA, 0.005% Triton X-100, and protease inhibitor cocktail (1:1000; Sigma-Aldrich). Thawed brains were homogenized using a Dounce homogenizer and centrifuged at 48,000g for 20 minutes. The pellet was resuspended in cold buffer and homogenized again using a Dounce homogenizer. Finally, the protein concentration was determined using a BCA kit (Sigma-Aldrich), and the homogenate was aliquoted, flash frozen, and stored at -80°C for 3–4 months. To perform competition binding experiments, thawed membrane was passed through a 24-gauge needle and 98 μl (20 μg) of the membrane protein was added to each well of a 96-well plate. Test compounds (2 μl) were added to achieve the desired concentration in each well of the assay plate, incubated with brain membrane for 15 minutes followed by the addition of [^3H]Ro 25-6981 (50 μl , 4 nM final concentration), and further incubation for 1 hour. Nonspecific binding was determined using MK-0657 (40 μM). Membranes were then harvested onto GF/B filter plates using a Filtermate universal harvester (PerkinElmer, Waltham, MA). Prior to membrane transfer, filter plates were presoaked in 0.5% polyethylenimine (PEI) for 1 hour at room temperature and washed three times using cold assay buffer. After membrane transfer, filter plates were washed eight times with cold assay buffer and dried at 50°C for 20 minutes followed by addition of 50 μl of Microscint20 scintillation cocktail (PerkinElmer). Radioactivity was counted using a TopCount (PerkinElmer) after 10 minutes of Microscint addition. Counts per minute were converted to percentage of inhibition and the concentration-response curves plotted and fitted to calculate K_i values using custom-made software. Each experiment had a plate duplicate and was repeated at least twice to obtain an average binding K_i value.

Studies to determine in vitro binding affinity at human (Analytical Biologic Services Inc., Wilmington, DE) and cynomolgus monkey GluN2B receptors were conducted at BMS. Frontal cortical membranes were prepared from frozen brain sections by homogenizing samples at 4°C in hypotonic lysis buffer consisting of 10 mM Tris (pH 7.4), 5 mM EDTA, and protease inhibitors and centrifugation at 32,000g for 20 minutes. The pellet was washed once in buffer

consisting of 50 mM Tris (pH 7.4), 1 mM EDTA, and protease inhibitors and centrifuged at 32,000g for 20 minutes. This pellet was then resuspended in buffer containing 50 mM KH_2PO_4 (pH 7.4 at 25°C), 1 mM EDTA, 0.005% Triton X-100, and 0.1% (v/v) Sigma Protease Inhibitor Cocktail. Aliquots were then frozen on dry ice/ethanol and kept at -80°C . On the day of the assay, frozen aliquots of membrane homogenate were thawed, homogenized, and resuspended to provide 15 μg /well cynomolgus brain protein or 35 μg /well human brain protein in assay buffer (5 mM Tris-HCl, pH 7.4). In saturation binding experiments, the membrane preparation was incubated at room temperature for 120 minutes in the presence of increasing concentrations of [^3H]Ro 25-6981. Nonspecific binding was defined with 10 μM Ro-04-5595. Competition binding experiments were performed using a single concentration of [^3H]Ro 25-6981 (1.5 nM) in the presence of 10 increasing concentrations (in duplicate) of test compound. The reaction was terminated by the addition of 5 ml of ice-cold assay buffer and rapid vacuum filtration through a Brandel Cell Harvester (Brandel, Gaithersburg, MD) using Whatman GF/B filters (PerkinElmer) presoaked in 0.5% PEI. The filtration and washing was completed in less than 30 seconds. The filter was then punched onto a 96-well microbeta sample plate, 200 μl /well of Packard Ultima Gold XR scintillation fluid (PerkinElmer) was added, and the filters were soaked overnight. The filter pads were then counted in an LKB Trilux liquid scintillation counter (PerkinElmer). K_i values from inhibition curves were determined using the "sigmoidal dose-response (variable slope)" non-linear curve fit by GraphPad Prism (version 5.01; GraphPad Software, La Jolla, CA).

Functional Inhibition of NMDA Receptor Subtypes In Vitro. Studies to demonstrate functional inhibition of GluN2B receptor-mediated currents in *Xenopus* oocytes were conducted at BBRC. Prior to surgery, frogs were anesthetized using 3-aminobenzoic acid ethyl ester (1 g/l, pH 7.0 adjusted using sodium bicarbonate), the ovarian lobes were removed using aseptic surgical techniques, and the animals were then singly housed for a 24-hour recovery period with postoperative monitoring for up to a week postsurgery. Following removal, the ovarian lobes were incubated in cold *Xenopus* oocyte buffer A (Biopredic International, Saint Gregoire, France) for 1 hour. The oocytes were then mechanically isolated in small clusters followed by collagenase treatment (1.5–2 mg/ml) for at least 1 hour on a shaking platform at 18°C to remove the follicular layer. The defolliculated oocytes were treated with *Xenopus* oocyte buffer A, B, and C (Biopredic International) for 15 minutes each in series at 18°C and then maintained in Barth's solution (pH 7.4) composed of 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 10 mM HEPES, 0.82 mM MgSO_4 , 0.33 mM $\text{Ca}(\text{NO}_3)_2$, 0.91 mM CaCl_2 , and supplemented with gentamycin (100 μg /ml), penicillin (10 μg /ml), and streptomycin (10 μg /ml). To determine functional inhibition of GluN2B receptors, oocytes were injected with 8–15 ng each of human GluN1a and GluN2B cRNA within 24 hours of isolation. Two electrode voltage clamp recordings were made 2–7 days postinjection. Oocytes were placed in a plexiglass chamber and impaled with glass microelectrodes filled with 3 M KCl. The oocytes were clamped at -40 mV and perfused with buffer containing 90 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.01 mM EDTA, and 0.5 mM BaCl_2 , pH 7.4. NMDA receptor currents were activated by the application of 50 μM glutamate and 30 μM glycine and recorded using an Axoclamp 900A amplifier and pClamp 10 data acquisition software (Molecular Devices, LLC., Sunnyvale, CA). The concentration response was determined using four to seven concentrations of the test compound, and each concentration was applied for 15–20 minutes on a different oocyte. The baseline leak current at -40 mV was recorded at the beginning and the end of the recording, and the full current trace was linearly corrected for any change in leak current. The level of inhibition was expressed as the percentage of the initial glutamate/glycine response in the absence of compound, and the IC_{50} values were obtained by concentration-response curve fitting using the following equation in GraphPad Prism (version 5.01): $Y = \text{bottom} + (\text{top} - \text{bottom}) / [1 + 10^{(\log \text{IC}_{50} - X) * \text{HillSlope}}]$, where $X = \log$ of concentration,

$Y = \text{percentage of inhibition}$, and the top and bottom were constrained to 100 and 0, respectively. To determine functional inhibition at other NMDA receptor subtypes, isolated oocytes were injected with 0.2–0.5 ng of human GluN1a and 0.5–1 ng of human GluN2A or 45–60 ng of human GluN1a and 35–55 ng of human GluN2C or GluN2D. The voltage clamp recording procedure was as described earlier except that oocytes were clamped at -80 mV to record GluN2C or GluN2D receptor currents, and compound was applied only at 3 μM for 10–15 minutes. The level of inhibition at 3 μM was calculated as a percentage of the initial glutamate/glycine-mediated current.

Ex Vivo GluN2B Occupancy in Mice and Rats. Studies to determine ex vivo GluN2B occupancy in rats were conducted at BMS. For dose-response studies, rats ($n = 4$ –5/group) were randomly assigned to receive an i.v. injection via the tail vein of vehicle (0.9% saline, pH 7.4 for BMS-986163 or 30% hydroxypropyl- β -cyclodextrin/70% citrate buffer, pH 4 for BMS-986169; 2 ml/kg), BMS-986169 (0.03, 0.3, 1, or 3 mg/kg), or BMS-986163 (0.1, 0.3, 1, 3, or 10 mg/kg) 15 minutes prior to decapitation, and blood and brains were collected. In a separate study, the time course of GluN2B occupancy was determined from 5 to 120 minutes after i.v. dosing with 3 mg/kg BMS-986169 ($n = 4$ /group). In addition to collection of plasma and brain, a terminal cerebrospinal fluid (CSF) sample was also collected from the cisterna magna for measurement of drug concentrations. Finally, in parallel with [^3H]MK-801 binding studies, satellite groups of rats with jugular vein catheters were randomly assigned to receive i.v. BMS-986163 or vehicle treatment ($n = 3$ /group) to determine GluN2B occupancy and drug exposure. Following collection, the brain was placed on ice, the cerebellum was removed, and the forebrain was dissected along the midline into left and right hemispheres, snap frozen in isopentane on dry ice, and stored at -80°C . Blood samples were centrifuged at 3500 rpm for 5 minutes at 4°C to separate the plasma, and plasma and CSF samples were stored at -80°C . On the day of occupancy determinations, one forebrain hemisphere from each animal was thawed and homogenized in 7 volumes of an assay buffer containing 50 mM KH_2PO_4 , 1 mM EDTA, 0.005% Triton X-100, and 1:1000 dilution of Sigma protease inhibitor P3843 (pH 7.4) using a Polytron homogenizer (Fisher Scientific, Hampton, NH). In a 96-well plate, 100 μg of tissue (0.4 mg/ml; tested in triplicate) was incubated with 5 nM [^3H]Ro 25-6981 in the assay solution at 4°C for 5 minutes. The nonspecific binding was defined by inclusion of 10 μM Ro 25-6981. At the end of the incubation, the reaction was stopped by filtration through FPXLR-196 filters (Fisher Scientific) that had been soaked in 0.5%–1.0% PEI for 1 hour at 4°C . The filters were washed twice with ice-cold assay solution, and the radioactivity was measured using a Wallac Microbeta liquid scintillation counter (PerkinElmer). For each sample, specific binding was calculated by subtracting the value of the nonspecific binding from that of the average total binding and percentage of occupancy calculated as $(1 - \text{specific binding in drug treated} / \text{specific binding in vehicle treated}) \times 100\%$. For estimation of the dose producing 50% GluN2B occupancy (Occ_{50}), a one-site binding model using nonlinear regression was fitted to the mean occupancy achieved at each dose (GraphPad Prism version 7.02). To estimate the Occ_{50} drug concentration in plasma, brain, or CSF, the same curve fit was applied to plots of occupancy/exposure results for individual subjects.

Additional GluN2B occupancy determinations were also conducted at BBRC in mice completing behavioral assessment in the FST ($n = 4$ /group) or in satellite groups dosed in parallel with NSF and LMA subjects ($n = 4$ /group). Mice were rapidly decapitated, and brain and blood samples were collected. Forebrain membranes were prepared as described earlier except for the addition of 3 ml of assay buffer for 160 mg of tissue and homogenization by the Polytron for 10 seconds followed by 30 strokes using a Dounce homogenizer. On the day of the experiment, brain homogenates were thawed on ice and passed three times through a 24-gauge needle. In a 96-well plate, 200 μl (6.4 mg/ml) of tissue homogenate (tested in triplicate) was incubated with 6 nM [^3H]Ro 25-6981 in assay buffer at 4°C for 5 minutes on a shaking platform. The membrane was harvested onto a GF/B filter plate

(treated with 0.5% PEI for 1 hour at 4°C), then the filter plate was dried at 50°C for 20 minutes and 50 μ l of Microscint20 (PerkinElmer) was added. After shaking of the filter plate for 10 minutes, the radioactivity was measured using a TopCount (Perkin Elmer). Non-specific binding was determined by incubating the membrane from vehicle-treated animals with 10 μ M Ro 25-6981.

In Vivo [3 H]MK-801 Binding in Rats. Studies to determine inhibition of in vivo [3 H]MK-801 binding were conducted at BMS using methods previously described (Fernandes et al., 2015). For the dose-response study, rats with previously implanted jugular vein catheters were randomly assigned ($n = 4$ /group) to treatment with either vehicle (0.9% saline, 2 ml/kg, i.v.), BMS-986163 (0.1, 0.3, 1, 3, 10, or 30 mg/kg, i.v.), or MK-801 (5 mg/kg, i.v.) to define the specific binding window. All rats received [3 H]MK-801 (0.2 μ Ci/g body weight) 5 minutes later and were decapitated 10 minutes after [3 H]MK-801 administration (i.e., 15 minutes after BMS-986163 treatment). For the time-course study, rats ($n = 4$ /group) were dosed with BMS-986163 (3 mg/kg, i.v.) 5–120 minutes prior to euthanasia, with all subjects receiving [3 H]MK-801 10 minutes prior to sacrifice. At the appropriate time point, the brain was collected, the cerebellum and brain stem were removed, and one forebrain hemisphere was weighed and homogenized in 30 volumes of ice-cold 5 mM Tris-acetate buffer (pH 7.0). A 600- μ l sample of the homogenate was filtered through GF/B Whatman filters (presoaked in ice-cold Tris-acetate buffer) on a filter box connected to a vacuum source. Filters were washed four times with ice-cold Tris-acetate buffer, and then placed in a scintillation vial with 10 ml of scintillation fluid (Optiphase supermix; PerkinElmer) overnight. Radioactivity in the scintillation vial was counted using a Wallac Microbeta liquid scintillation counter. The total [3 H]MK-801 binding was determined by measuring radioactivity in the forebrain of animals treated with vehicle, whereas nonspecific binding was determined by measuring the radioactivity in animals treated with MK-801. For each sample, specific binding was calculated by subtracting the value of the nonspecific binding from that of the total binding, and percentage of inhibition was calculated as $(1 - \text{specific binding in drug treated} / \text{specific binding in vehicle treated}) \times 100\%$. For estimation of the dose producing 50% inhibition of MK-801 binding, data were fitted to a one-site binding model using nonlinear regression as described previously.

Mouse FST. Studies to evaluate GluN2B NAM treatment effects on immobility in the mouse FST were conducted at BBRC. All studies were conducted between 09:00 and 13:30 hours and were performed under dim light and low noise conditions. Behavior was recorded and quantified using the automated CleverSys forced swim test apparatus (CleverSys, Reston, VA), and investigators were unblinded to dosing solutions. Animals ($n = 8$ –11/group) were randomly assigned to treatment with either vehicle (30% hydroxypropyl- β -cyclodextrin/70% citrate buffer, pH 4; 5 ml/kg, i.v.), CP-101,606 (3 mg/kg, i.v.), or BMS-986169 (0.3, 0.56, 1, or 3 mg/kg, i.v.) and 15 minutes later were placed in plexiglass swim tanks (20-cm diameter, 40-cm height) filled with water up to a height of 20 cm at a temperature of $25 \pm 1^\circ\text{C}$. In a separate study, mice were randomly assigned to treatment ($n = 9$ –10/group) with either vehicle (0.9% saline; 10 ml/kg, i.v.) or ketamine (1, 3, or 10 mg/kg, i.v.) and tested 30 minutes after dosing. Swim tanks were positioned inside a box made of plastic and separated from each other by opaque plastic sheets to the height of cylinders. Individual animals were placed in one of the three swim tanks, and behavior was recorded in a 6-minute testing session. The total immobility duration, defined as the time spent passively floating with only small movements necessary to keep the nose/head above water and remain afloat during the 6-minute test, was measured using the automated CleverSys software. Results were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test (GraphPad Prism version 7.02). Upon completion of testing (~25 minutes after drug treatment), a subset of animals ($n = 4$ /group) were euthanized by rapid decapitation, and blood and brain samples were collected for GluN2B occupancy and drug exposure measurements as previously described.

NSF in Mice. Studies to examine the effect of GluN2B NAM treatment on NSF were conducted at BBRC. Prior to treatment, all mice were briefly (~1 minute) placed in a restrainer to acclimatize them to the i.v. dosing conditions for 2 consecutive days. Animals were then randomly assigned to treatment ($n = 12$ –15/group) with CP-101,606 (10 mg/kg, i.v.), BMS-986163 (1, 3, or 10 mg/kg, i.v.), or their respective vehicles (25% hydroxypropyl- β -cyclodextrin or phosphate buffer, pH 7.4, respectively; 5 ml/kg, i.v.) and then food deprived for 21 hours with water available ad libitum. On the following day (i.e., 24 hours after treatment), individual mice were placed in a novel Perspex arena (40 $\times$ 40 $\times$ 40 cm; Coulbourn Instruments, Allentown, PA) covered on three sides with white paper, with the fourth side left transparent to enable behavioral evaluation. A light source was positioned on the top to produce a light intensity of ~1000 lux in the center of the arena. One food pellet (standard chow feed) was placed on the top of a 60-mm Petri plate placed in the center of the arena. The session began with the animal placed in one corner of the arena facing away from the food pellet. The latency to begin eating the pellet (i.e., latency to feed) was recorded by an observer blinded to drug treatment. Feeding was defined as the mouse holding the food pellet with its forepaws and biting it, and testing was terminated either when the mouse started to feed or after 6 minutes had elapsed. After evaluation in the novel arena, the mouse was transferred to its home cage and food consumption was monitored for 30 minutes (home cage feeding). Results were analyzed by either two-tailed, unpaired t test (CP-101,606 vs. vehicle) or one-way ANOVA followed by Dunnett's post-hoc test (GraphPad Prism version 7.02). Satellite groups of mice ($n = 4$ /treatment) were dosed in parallel with the NSF subjects, and plasma and brain samples were collected 15 minutes later for exposure and GluN2B occupancy determinations.

During the initial pharmacological characterization of the NSF assay, studies were conducted to examine the effect of ketamine treatment following i.p. administration, a dosing route commonly used to examine the antidepressant-like effect of this agent. Subjects ($n = 12$ –15/group) were treated with vehicle (Milli-Q water, 10 ml/kg) or ketamine (3, 10, or 30 mg/kg) and tested in the NSF assay 24 hours later as described earlier.

Ex Vivo Hippocampal LTP in Rats. Studies to examine the effects of GluN2B NAM treatment on hippocampal LTP were conducted by BMS. Rats were handled and acclimatized to the i.v. dosing restrainer for up to 5 minutes each day for 3 days prior to dosing. On the morning of drug treatment, rats were restrained and dosed via the tail vein by an investigator unblinded to treatment with either BMS-986169 (1 or 3 mg/kg) or vehicle (30% hydroxypropyl- β -cyclodextrin/70% citrate buffer; 2 ml/kg). Typically, either two rats (one treated, one control) or four rats (two treated, two control) were randomly assigned to treatment each day for subsequent LTP measurements 24 or 72 hours later. Each dose of BMS-986169 or time point was examined as a separate study with independent vehicle-treated controls ($n = 5$ –8/group). At the appropriate time point, rats were rapidly decapitated, and brain tissue was removed and quickly submerged into ice-cold cutting solution containing 100 mM sucrose, 60 mM NaCl, 3 mM KCl, 7 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.25 mM NaH_2PO_4 , 0.5 mM CaCl_2 , 5 mM D-glucose, 0.6 mM ascorbate, and 28 mM NaHCO_3 . The cerebellum and frontal cortex were removed, and the brain was cut down the midline. Transverse slices (350 μ m) were cut from the middle of the hippocampal formation with a vibratome (Leica VT1000S; Leica Microsystems, Wetzlar, Germany) in ice-cold cutting solution bubbled with 95% O_2 /5% CO_2 . Brain slices were transferred to a 50:50 mixture of cutting solution and artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 3 mM KCl, 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.25 mM NaH_2PO_4 , 2 mM CaCl_2 , 10 mM D-glucose, and 36 mM NaHCO_3 and oxygenated with 95% O_2 /5% CO_2 at room temperature (26°C) for 30 minutes. Slices were then switched to 100% aCSF and continued to be oxygenated with 95% O_2 /5% CO_2 at 30°C for at least 30 minutes of recovery. Up to eight slices were recorded simultaneously using a modified multislice recording configuration (Graef et al., 2013). In brief, two separate tissue slice

chambers, each with a capacity for four isolated tissue slices, were perfused with warm (32–34°C), oxygenated aCSF using a gravity perfusion system. A flow rate of 2 ml/min for each four-slice recording chamber was regulated by maintaining a constant fluid level in a secondary solution chamber through the use of a solenoid valve (Harvard Apparatus, Holliston, MA), a float, and an IR beam detector (NV-253SD; Med Associates, Fairfax, VT). Four vehicle slices were placed in one of the four-slice recording chambers, and four slices from a rat treated with test agent were placed in the other four-slice recording chamber, with the chambers used for each condition being alternated on subsequent experimental days. Once all slices were oriented in the recording chambers, custom-made recording and stimulating electrodes (0.2- μ m diameter platinum/iridium wire) were positioned in the stratum radiatum layer of the Cornu Ammonis area 1 (CA1) region of the hippocampus. Field responses were elicited with a single monophasic stimulation from two twisted wires (0.1 ms) every 20 seconds (3 Hz) using individual IsoFlex stimulus isolator units driven by a Master 8 stimulus generator (A.M.P.I.; Jerusalem). The strength of each stimulus was adjusted to elicit 30%–50% of the maximum response. Baseline signals were recorded for 20 minutes, then LTP was induced by the following high-frequency stimulation (HFS) protocol: two trains of 80 stimulations at 100 Hz at the same stimulus intensity with a 60-second interval between trains. This HFS protocol was repeated two more times at 20 and 40 minutes following the first bout of HFS. Following this conditioning stimulus, a 1-hour test period was recorded where responses were again elicited by a single stimulation every 20 seconds (3 Hz) at the same stimulus intensity. Signals were amplified with a differential amplifier and were digitized and sampled at 10 kHz with a DigiData 1440 (Molecular Devices, Sunnyvale, CA), recorded using Clampex 10.0 acquisition software, and analyzed with the Clampfit 10.0 software package (Molecular Devices). Data from all four slices from each animal (control and treated) on each experimental day were averaged to obtain mean LTP values for each subject. Only data from slices where the baseline responses were greater than 0.1 mV and did not vary by more than 10%, and demonstrated at least a 10% potentiation of the baseline response were used for analysis. These inclusion criteria were prespecified prior to the LTP recordings and resulted in average values from two to four slices per subject. The total numbers of slices excluded from analysis across all studies were as follows: LTP amplitude: vehicle = 9/78 (11.5%), BMS-986169 = 9/79 (11.4%); LTP slope: vehicle = 8/78 (10.3%), BMS-986169 = 9/79 (11.4%). The numbers of slices excluded from analysis for individual studies were as follows: study 1: vehicle (24 hours) = 1/27 slices, 3 mg/kg BMS-986169 (24 hours) = 3/27 slices (slope) or 4/27 slices (amplitude); study 2: vehicle (72 hours) = 4/31 slices, 3 mg/kg BMS-986169 (72 hours) = 4/32 slices; study 3: vehicle (24 hours) = 3/20 slices (slope) or 4/20 slices (amplitude), 1 mg/kg BMS-986169 (24 hours) = 2/20 slices (slope) or 1/20 slices (amplitude). One-tailed paired *t* tests comparing corresponding test periods between control and treated animals were used to test for statistical significance.

Locomotor Activity in Mice. Studies to examine the effect of GluN2B NAM treatment on mouse locomotor activity were conducted at BBRC and performed using an automated LMA apparatus. All testing was conducted between 09:00 and 13:30 hours in a dark room with low noise conditions, and investigators were unblinded to dosing solutions. Mice were habituated to individual chambers (40 × 40 × 40 cm; Coulbourn Instruments) for 2 hours and then randomly assigned to treatment. In an initial study, the dose response to ketamine was examined in mice (*n* = 8–9/group) treated with vehicle (Milli-Q water, 5 ml/kg, i.v.) or ketamine (3, 10, 30 mg/kg, i.v.). In the subsequent study, mice (*n* = 7–8/group) were treated with either vehicle (25% hydroxy- β -cyclodextrin/75% water; 5 ml/kg, i.v.), ketamine (30 mg/kg, i.v.), or BMS-986169 (3, 10, or 30 mg/kg, i.v.). Animals were returned to the chamber, and activity data were acquired every 250 ms from the floor panel sensor and expressed as ambulatory distance (centimeters) recorded in 10-minute time bins using TruScan 2.0 software (Coulbourn Instruments). Results were analyzed by two-way repeated-measures

(RM) ANOVA followed by Dunnett's post-hoc analysis (GraphPad Prism version 7.02). Separate satellite groups (*n* = 4/treatment) were dosed in parallel, and plasma and brain tissue was collected 15 minutes later to determine drug concentrations and GluN2B occupancy.

Abnormal Behaviors in Cynomolgus Monkeys. Studies to examine the effect of GluN2B NAM treatment on spontaneous behaviors in monkeys were conducted by BMS. Prior to treatment, baseline behavioral profiles were established by observing home cage behavior for 15 minutes/wk for 3 weeks. Behaviors were evaluated using a 12-item behavioral checklist previously used to demonstrate psychotomimetic-like behaviors following treatment with amphetamine or ketamine in macaques (Castner and Goldman-Rakic 1999; Roberts et al., 2010; Supplemental Table 1). When a given behavior was observed, a mark was made in the appropriate time bin. Each study was organized using a Latin-square design, and the observer was blinded to treatment. A 5-minute predose observation was made 30 minutes before treatment with vehicle (30% hydroxypropyl- β -cyclodextrin/70% water, 0.4 ml/kg, i.m.), ($\pm$)ketamine (1 mg/kg, i.m.), or BMS-986169 (1, 3, or 5.6 mg/kg, i.m.), and behavior was evaluated 0–5, 5–10, 10–15, 30–35, and 60–65 minutes post-treatment (*n* = 8). To examine a higher dose of 10 mg/kg BMS-986169, a second blinded study was conducted using a Latin-square design and a two-injection protocol whereby 2 × 0.4-ml/kg injections were given to achieve a total dose of 10 mg/kg BMS-986169, 1 mg/kg ketamine, or two injections of vehicle (*n* = 8). The dose of ketamine used for comparison was selected from a separate study showing a dose-dependent increase in behavior after ketamine administration (*n* = 12). Behaviors were summed over the entire session and expressed in terms of change from preinjection baseline. Composite measures of “all abnormal behaviors” (behaviors 2+4+6+7+10+11; Supplemental Table 1) and “hallucinatory/dissociative” (behaviors 7+11; Supplemental Table 1) were calculated and analyzed by one-way RM ANOVA followed by Dunnett's post-hoc test (GraphPad Prism version 7.02). On completion of the evaluation, subjects were placed in a primate restraint chair, and a plasma sample (~75 minutes postdose) was collected from the saphenous vein for measurement of drug concentrations.

Cognitive Impairment in Cynomolgus Monkeys. Studies to examine the effect of GluN2B NAM treatment on cognitive performance were conducted at BMS using a list delayed match to sample (list-DMS) procedure described previously (Weed et al., 2016). Subjects (*n* = 9) received prior treatment with other pharmacological agents and were given at least a 2-month drug-free period prior to the start of these studies. During the procedure, animals were seated in restraint chairs and placed within a sound-attenuated chamber containing a touch-sensitive computer monitor controlled by the Monkey CANTAB (Cambridge Neuropsychological Testing Automated Battery) software (Lafayette Instruments, Lafayette, IN). Following correct responses, a dispenser delivered 190-mg banana flavored pellets (Bioserv, Frenchtown, PA). Animals were first trained to proficiency on the standard CANTAB DMS task in which an abstract stimulus is presented in the “sample” phase, and after a memory delay, the sample stimulus plus three distracter stimuli are presented in the “choice” phase. Animals then progressed to a list-DMS procedure in which three different sample stimuli are presented first followed by their choice phases which occur sequentially based on their respective memory delays. Thus, the three trials are nested within the longest memory delay, thereby reducing the overall session length. The memory delays used in this study were 2, 20, and 45 seconds. Completion of the list was followed by a 10-second screen blank, after which a new list was presented for a total of 20 lists in each test session. During both the sample and choice phases, the subject had 5 seconds to respond to the stimulus, otherwise the trial was considered an omission. If the omission was in the sample phase, the choice phase for that sample stimulus was not presented, but the timing of the sample and choice phases for other stimuli remained unchanged. All subjects were habituated to receiving injections prior to testing. Evaluation of performance after vehicle treatment (25% hydroxypropyl- β -cyclodextrin/75% water, 0.4 ml/kg, i.m.)

occurred prior to, during, and at the end of the dose-response function (total of four vehicle determinations). The effect of BMS-986169 treatment was tested twice weekly (Monday and Thursday) with baseline performance sessions on Tuesday and Friday. On test days, the cohort was divided roughly in half, and one half received a given dose of BMS-986169 and the other half received a different dose. Drug doses were thus administered in a mixed order across test days and across subjects by investigators unblinded to treatment. In the first study, the effect of BMS-986169 (0.1, 0.3, 1, or 3 mg/kg, i.m.) administered 30 minutes prior to testing was examined to determine the dose-effect relationship. In a follow-up study, vehicle or 1 mg/kg BMS-986169 was administered 3 or 5 hours prior to testing to examine the time course of the effect. List-DMS test sessions were ~30 minutes long, and on completion, blood samples were collected from the saphenous vein for analysis of plasma drug concentrations. Sample collection times, therefore, correspond to 1 hour postdose for the dose-response study and 3.5 or 5.5 hours for the time-course study. Plasma and CSF drug concentrations were also investigated after dosing with BMS-986169 (0.3–5.6 mg/kg, i.m.) in a separate cohort of cynomolgus monkeys previously implanted with vascular and CSF lumbar access ports ($n = 2/\text{group}$). Blood samples were collected into K₂EDTA tubes on ice, plasma was separated by centrifugation at 1330g for 10 minutes, and plasma and CSF samples were stored at -80°C until analyzed.

The primary measure for these studies was performance accuracy expressed as the percentage of correct trials (% accuracy = $[\text{correct trials}] / ([\text{correct trials} + \text{errors}]) \times 100$). Secondary measures included 1) latency to respond in the choice phase (milliseconds between onset of stimulus presentation and touching of monitor) and 2) percentage of task completed (trials with a response in both sample and choice phase/trials presented). Omissions were not included in the percentage correct measure, but contributed to percentage of task completed. Percentage correct accuracy was analyzed by two-way RM ANOVA followed by Dunnett's post-hoc test. For the time-course study, performance after vehicle treatment was similar regardless of the pretreatment time (0.5, 3, or 5 hours), and the average was therefore used in the analysis. Latencies to respond in the choice phase (averaged across delays) and percentage of task completed for the entire session were analyzed by one-way RM ANOVA followed by Dunnett's post-hoc test. In addition, for the long delay condition, the difference between performance after vehicle and drug treatment was calculated for each subject as follows: difference = % correct at long delay(drug treatment) – % correct at long delay(vehicle treatment). All statistical analyses were conducted using GraphPad Prism (version 7.02).

qEEG Studies in Cynomolgus Monkeys. Studies to examine the effect of GluN2B NAM treatment on the qEEG response were conducted by BMS using methods described previously (Keavy et al., 2016). Six cynomolgus monkeys served as subjects for the qEEG studies, and each had been used previously in pharmacological studies, with a drug-free period of at least 1 month prior to testing. Subjects were surgically implanted under isoflurane anesthesia with radiotelemetry transmitters (Kongsberg Instruments, Inc., Monrovia, CA) attached to two sets of EEG leads placed over the dura: one set with leads over the frontal cortex (with a reference over parietal cortex; roughly F6-P6 in the 10–20 system), and the second set with leads over the auditory cortex (roughly C6-CP6 in the 10–20 system). Buprenorphine (0.01–0.03 mg/kg, i.m.) was administered after surgery and for a further 2–3 days twice daily with extended treatment as needed if subjects showed signs of discomfort. After full recovery (approximately 3–4 weeks), the implanted radiotelemetry device was tested to ensure a good EEG signal, and suitable subjects were enrolled in the study. On each test day, prior to the start of qEEG recordings, subjects were seated in a primate restraint chair and an i.v. catheter was placed in a saphenous vein for drug administration. Animals were allowed to acclimate to a quiet testing chamber outfitted with an antenna to receive telemetry signals and a camera to monitor the animal during the session. After a 20-minute baseline recording, animals were treated with either vehicle (0.9% saline; 0.4 ml/kg, i.v.) or BMS-986163 (0.12, 0.36, or 1.2 mg/kg, i.v.) by investigators

unblinded to treatment, and the qEEG was measured for a further 90 minutes. The i.v. catheter was removed at the end of the session, and animals were tested once weekly with treatments assigned using a Latin square design. Analog signals from the telemeters were digitized at 1000 Hz by PowerLab DAQ systems (ADInstruments, Colorado Springs, CO). Raw EEG waveforms were Fourier transformed using a Hanning (cosine bell) window with 50% overlap between data blocks and an FFT block size of 1024. Overall total power (0.5–55 Hz) as well as power within the different frequency bands was recorded for delta (0.5–4 Hz), theta (4–9 Hz), alpha (9–13 Hz), beta 1 (13–19 Hz), beta 2 (20–30 Hz) and gamma (30–55 Hz) bandwidths. The relative power (power in a band/overall total power) was determined as the average value from 1-minute bins across the 90-minute session and analyzed by two-way RM ANOVA followed by Holm-Sidak post-hoc test (GraphPad Prism version 7.02). Area under the curve over the entire 90-minute period for each power band was calculated from the time-course data and analyzed by RM ANOVA followed by Dunnett's post-hoc test (GraphPad Prism version 7.02). Finally, using the area under the curve results, Cohen's d estimate of effect size was calculated using unpaired statistics (<http://www.uccs.edu/~lbecker/>).

Plasma and CSF drug concentrations were also investigated after dosing with BMS-986163 (1.2 mg/kg, i.v.) in cynomolgus monkeys previously implanted with vascular and CSF lumbar access ports ($n = 2/\text{time point}$). Sample collection, processing, and storage were as previously described.

Quantification of BMS-986169 and BMS-986163 in Samples from In Vivo Studies. For exposure analysis of BMS-986169 and BMS-986163 in plasma, brain, and CSF, rats and monkeys were dosed and samples were collected as described. BMS-986163 was stabilized ex vivo by collecting blood samples into tubes containing 4% K₂EDTA dissolved in 1 M phosphate buffer, pH 7.4 (5% of the blood volume) and maintained at 4°C . Plasma was gathered within 15 minutes of blood collection and stored at -80°C until analysis. For brain samples, tissue was homogenized and diluted with blank plasma. CSF samples were prepared in the same manner as plasma. An aliquot of 50 μl of calibration standard or study sample (plasma/tissue/CSF) was added to 200 μl of acetonitrile containing internal standard (Ro 25-6981 at 100 nM) and transferred to a Strata protein precipitation filter plate (Phenomenex, Torrance, CA). Plates were vortexed on a plate shaker for 5 minutes at 300 rpm, centrifuged at 4000 rpm for 5 minutes, and the eluent was collected into a 96-well collection plate (Waters Corporation, Milford, MA). A volume of 4 μl was injected for liquid chromatography–mass spectrometry/mass spectrometry analysis. Liquid chromatography–mass spectrometry/mass spectrometry analysis was performed on a Waters Acquity UPLC (Waters Corporation) interfaced to an API4000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) equipped with a Turboionspray source. The source temperature was set at 550°C and the ionspray voltage was set to 4.5 kV. Samples were maintained at 10°C for the duration of the analysis. Separations were performed on a Waters Acquity BEH C18 (2.1 $\times$ 50 mm, 1.7 μm) column maintained at 60°C . The mobile phase, which consisted of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), was delivered at a flow rate of 700 $\mu\text{l}/\text{min}$. Separation was achieved using a gradient elution starting at 95% aqueous mobile phase and going to 95% organic mobile phase. Nitrogen was used as nebulizer and auxiliary gas with a pressure and flow rate of 80 psi and 7 l/min, respectively. Data acquisition used selected reaction monitoring operating mode. Standard curves used for quantification were fitted with linear regression weighted by reciprocal concentration, $1/x^2$. Data and chromatographic peaks were quantified using Analyst version 1.4.2 software (Sciex).

Results

BMS-986169 Potently Inhibits [³H]Ro 25-6981 Binding In Vitro. Saturation binding experiments showed that [³H]Ro 25-6981 labeled a single population of receptors in

membranes prepared from rat forebrain, cynomolgus monkey frontal cortex or human frontal cortex with Kd values of 2.23 (n = 3), 0.93 (n = 1) and 1.99 nM (n = 2), respectively. BMS-986169, CP-101,606 and MK-0657 potently and completely displaced [³H]Ro 25-6981 specific binding in all species (Supplemental Figs. 1 and 2; Table 1).

BMS-986169 Selectively Inhibits GluN2B Receptor Function. To demonstrate that BMS-986169 inhibits GluN2B receptor function, electrophysiological recordings were made in *Xenopus* oocytes expressing human GluN1a/GluN2B receptors. Application of BMS-986169 produced a concentration-dependent inhibition of glutamate/glycine activated currents; the IC₅₀ value was 24.1 nM [95% confidence limits (CL) 20.1–28.9] determined from a 7-point concentration-response curve (n = 3–5/concentration) (Fig. 2). In comparison, the IC₅₀ values determined following 15-minute incubation with CP-101,606 or Ro 25-6981 were 10.6 (95% CL 7.7–14.6) and 9.3 nM (95% CL 8.4–10.4), respectively (Fig. 2). BMS-986169 showed minimal inhibition of glutamate/glycine-activated currents at other NMDA receptor subtypes; the mean ± S.D. inhibition at 3 μM was –2.4% ± 2.6%, 8.6% ± 6.4%, and 7% ± 3.8% at human GluN1a/GluN2A, GluN1a/GluN2C, or GluN1a/GluN2D receptors, respectively (n = 2–3). BMS-986169 was also examined in a broad panel of in vitro assays at 40 additional pharmacological targets with no additional activity of relevance identified (Supplemental Table 2). Finally, BMS-986169 showed weak functional inhibition at human ether-a-go-go-related gene potassium channel (hERG) channels; the IC₅₀ determined by patch-clamp electrophysiology in HEK293 cells expressing hERG was 28.4 μM.

Intravenous Administration of BMS-986169 or BMS-986163 Achieves High Levels of GluN2B Receptor Occupancy in Rat Brain. Intravenous administration of BMS-986169 produced a dose-dependent increase in ex vivo GluN2B occupancy determined in rat brain homogenates 15 minutes after dosing; the dose achieving 50% occupancy was 0.22 mg/kg (95% CL 0.13–0.37) with 97% occupancy achieved at the highest dose tested (3 mg/kg; Fig. 3A). Treatment with the phosphate prodrug BMS-986163 also produced a dose-dependent increase in GluN2B occupancy determined 15 minutes after i.v. dosing in rats (Fig. 3A). Again, maximal occupancy was achieved at 3 mg/kg BMS-986163 (101%), and the dose achieving 50% occupancy was 0.42 mg/kg (95% CL 0.28–0.61). As expected, GluN2B occupancy was closely correlated with the total plasma concentration of BMS-986169 following dosing of either BMS-986169 or BMS-986163 (Fig. 3B). These results confirm that the prodrug is rapidly converted to the parent drug after i.v. administration and are consistent with other pharmacokinetic studies showing little to no detectable levels of prodrug in the systemic circulation. Brain tissue concentrations of BMS-986169 also showed a strong relationship with GluN2B occupancy (Supplemental Fig. 3A; Supplemental Table 3); the

Fig. 2. BMS-986169 inhibits glutamate/glycine currents in xenopus oocytes expressing human GluN1a/GluN2B receptors. (A) Representative recording showing inhibition of 50 μM glutamate + 30 μM glycine currents following application of 100 nM BMS-986169. (B) Concentration-response curve for inhibition of glutamate/glycine-activated currents. Each point shows the mean ± S.E.M. percentage of inhibition following 15-minute incubations with BMS-986169 (n = 3–5/concentration), CP-101,606 (n = 1–4/concentration), or Ro 25-6981 (n = 1–2/concentration).

average brain tissue/total plasma concentration ratio across all GluN2B occupancy studies was 3. Investigation of the occupancy time course after dosing with 3 mg/kg i.v. BMS-986169 showed that peak occupancy (~93%) was maintained for up to 30 minutes and then declined rapidly to a level of 57% 2 hours after treatment (Fig. 3C). Analysis of BMS-986169 concentrations in plasma (total and free drug concentration), brain tissue, and CSF collected from these subjects showed a strong relationship between drug exposure and occupancy in all compartments (Fig. 3D; Supplemental Table 3). BMS-986169 was detected in CSF at levels similar to the free plasma drug concentration at all time points examined (Supplemental Fig. 3B). The CSF concentration to achieve 50% GluN2B occupancy was 3.5 nM

TABLE 1
Displacement of [³H]Ro 25-6981 binding to native GluN2B receptors in membranes prepared from rat forebrain, cynomolgus monkey frontal cortex, or human frontal cortex

Compound	Rat (Mean Ki ± S.D.)	Monkey (Mean Ki ± S.D.)	Human (Mean Ki ± S.D.)
	nM	nM	nM
BMS-986169	4.03 ± 1.39 (n = 13)	4.2 ± 0.8 (n = 3)	6.3 ± 1.5 (n = 3)
CP-101,606	6.96 ± 0.56 (n = 3)	2.2 ± 0.5 (n = 4)	3.1 ± 0.9 (n = 4)
MK-0657	2.52 ± 0.42 (n = 3)	3.2 ± 0.5 (n = 3)	4.4 ± 1.4 (n = 3)

Fig. 3. Ex vivo GluN2B occupancy in rat brain following tail vein administration of BMS-986169 or the phosphate prodrug BMS-986163. Occupancy dose response (A) and total plasma concentration/occupancy relationship (B) determined 15 minutes after BMS-986169 or BMS-986163 treatment. Occupancy time course (C) and relationship between occupancy and total plasma, free plasma, brain tissue, and CSF BMS-986169 concentration (D) in rats treated with 3 mg/kg BMS-986169. Results are presented as the mean $\pm$ S.E.M. percentage of GluN2B occupancy ($n = 4$ –5/dose) or exposure/occupancy results for individual subjects.

and was consistent with the in vitro GluN2B binding affinity. In addition, GluN2B occupancy, predicted from CSF concentrations and the rat in vitro GluN2B binding K_i , was similar to the measured occupancy in these subjects (Supplemental Fig. 3C). It should be noted that BMS-986169 shows poor oral bioavailability in rats; the oral bioavailability determined after oral dosing with 5 mg/kg BMS-986169 or the prodrug BMS-986163 was 2.5% and 1.7%, respectively.

BMS-986163 Functionally Inhibits NMDA Receptors after i.v. Dosing in Rats. Intravenous administration of the phosphate prodrug BMS-986163 dose-dependently inhibited in vivo [3 H]MK-801 binding with a maximal 41% inhibition achieved at 10 mg/kg (Fig. 4A). Satellite groups of rats receiving jugular vein injections of the same BMS-986163 dosing solutions showed 97% GluN2B occupancy at 10 mg/kg and an Occ50 dose of 0.55 mg/kg (95% CL 0.2–1.47) (Fig. 4A). Further examination of the [3 H]MK-801 binding response confirmed that inhibition saturates at ~40% after treatment with BMS-986163 (mean $\pm$ S.E.M. percentage of inhibition 15 minutes after dosing: 10 mg/kg = 38.4 ± 1.2 ; 30 mg/kg = 43.6 ± 2.7). Inhibition of [3 H]MK-801 binding was also time-dependent; the maximal inhibition achieved after dosing with 3 mg/kg BMS-986163 was 37% and occurred at 15 minutes post-treatment (Fig. 4B). Thereafter, inhibition of [3 H]MK-801 binding declined rapidly over time and was negligible (6% inhibition) by 2 hours post-treatment (Fig. 4B). Again,

GluN2B occupancy determined in satellite groups was high ($\geq 89\%$) for the first 30 minutes after dosing and then declined rapidly to a level of 28% 2 hours after treatment (Fig. 4B). The relationship between total plasma and brain BMS-986169 concentrations and GluN2B occupancy from these combined studies was similar to previous results (Supplemental Fig. 4; Supplemental Table 3).

BMS-986169 Reduces Immobility in the Mouse FST. Treatment with BMS-986169 produced a significant, dose-dependent decrease in immobility [ANOVA: $F(5,53) = 3.547$, $P = 0.0077$] (Fig. 5A) in mice tested in the FST assay. The minimum effective dose was 1 mg/kg, which achieved an average total plasma BMS-986169 concentration and GluN2B occupancy of 268 nM and 73.3%, respectively, determined immediately after completion of the test (Supplemental Table 4). Decreased immobility was also seen in mice treated with CP-101,606 (3 mg/kg, i.v.), which achieved a mean ($\pm$ S.E.M.) GluN2B occupancy of $85 \pm 0.45\%$. As expected, BMS-986169 produced a dose- and exposure-dependent increase in ex vivo GluN2B receptor occupancy; the dose achieving 50% occupancy was 0.4 mg/kg (95% CL 0.16–0.82), and the total plasma and brain drug concentrations achieving 50% occupancy were 102 and 404 nM, respectively (Supplemental Fig. 5; Supplemental Table 3). The average BMS-986169 brain tissue/total plasma concentration ratio in mice was 4. A dose-dependent decrease in immobility

Fig. 4. Inhibition of in vivo [^3H]MK-801 binding and relationship to ex vivo GluN2B occupancy in rats treated with the phosphate prodrug BMS-986163. (A) Dose response determined 15 minutes after i.v. dosing. (B) Time course determined in rats treated with 3 mg/kg (i.v.). Results are presented as the mean $\pm$ S.E.M. percentage of inhibition of [^3H]MK-801 binding ($n = 4/\text{group}$) or percentage of GluN2B occupancy ($n = 3/\text{group}$).

was also seen after i.v. ketamine administration [$F(3,34) = 5.212$, $P = 0.0045$], which was significant at doses of 3 and 10 mg/kg and similar in magnitude to the effects seen with BMS-986169 (Fig. 5B).

BMS-986163 Reduces NSF 24 Hours after i.v. Dosing in Mice. Treatment with BMS-986163 produced a dose-dependent decrease in the latency to feed in a novel environment determined 24 hours after dosing in mice [ANOVA: $F(3,52) = 4.635$, $P = 0.006$] (Fig. 5C). A similar reduction in latency was also observed in CP-101,606-treated animals (unpaired t test: $P = 0.0255$) (Fig. 5C). Home cage food consumption, measured on completion of testing in the NSF assay, was not significantly different from vehicle-treated subjects in mice treated with BMS-986163 [ANOVA: $F(3,52) = 1.976$, $P = 0.1291$] or CP-101,606 (unpaired t test: $P = 0.9731$) (Fig. 5D). Measurement of GluN2B occupancy 15 minutes after dosing in satellite groups of mice showed that 101%

occupancy was achieved at both 3 and 10 mg/kg BMS-986163 (Supplemental Table 4). The mean $\pm$ S.E.M. GluN2B occupancy determined 15 minutes after CP-101,606 (10 mg/kg, i.v.) administration was $92\% \pm 1\%$.

The effect of intravenous ketamine administration was not tested; however, i.p. dosing was examined during our initial characterization of the NSF assay. Ketamine significantly reduced the latency to feed in the novel environment [ANOVA: $F(3,51) = 3.788$, $P = 0.0158$] 24 hours after administration of 10 and 30 mg/kg i.p. with no effect observed on home cage food consumption [ANOVA: $F(3,51) = 1.749$, $P = 0.1687$] (Supplemental Fig. 6).

BMS-986169 Enhances Ex Vivo Hippocampal LTP 24 Hours after Dosing in Rats. Recordings from hippocampal slices taken from vehicle-treated rats showed that application of HFS increased both the amplitude and the slope of the field excitatory postsynaptic potential (fEPSP) response compared with baseline recordings, consistent with the induction of LTP. A single administration of BMS-986169 (3 mg/kg, i.v.) produced a robust enhancement of ex vivo hippocampal LTP measured 24 hours after dosing (Fig. 6). This elevation was apparent for both the amplitude (Fig. 6, A and B) and the slope (Fig. 6, C and D) of the fEPSP response with a significant enhancement over baseline observed following application of all three HFS stimuli. The elevation of LTP was dose-related, with a small but significant elevation of the fEPSP slope observed 24 hours after treatment with BMS-986169 (1 mg/kg, i.v.) (Supplemental Table 5). Finally, the enhancement of LTP was time-dependent and was no longer observed 72 hours after dosing with 3 mg/kg BMS-986169 (Supplemental Table 5).

BMS-986169 Has Marginal Effects on LMA in Mice. Treatment with ketamine produced a significant increase in LMA that lasted 50 minutes after dosing with 10 or 30 mg/kg [treatment effect: $F(3,31) = 19.81$, $P < 0.0001$; time effect: $F(11,341) = 99.19$, $P < 0.0001$; interaction: $F(33,341) = 8.232$, $P < 0.0001$] (Fig. 7A). In comparison, treatment with BMS-986169 had marginal effects on LMA [treatment effect: $F(4,31) = 9.97$, $P < 0.0001$; time effect: $F(11,341) = 62.62$, $P < 0.0001$; interaction: $F(44,341) = 6.76$, $P < 0.0001$] (Fig. 7B). Treatment with 3 mg/kg BMS-986169 had no effect on LMA at any time point. At higher doses, a small but significant increase was observed in the first 10 minutes after dosing, which was substantially lower than the ketamine response (Fig. 7B). A small increase was also observed at the 50–60-minute time point in mice treated with 30 mg/kg. All doses of BMS-986169 were associated with high levels of GluN2B occupancy (range = 96.4%–99.7%) determined 15 minutes after i.v. dosing in satellite groups of mice (Supplemental Table 4).

BMS-986169 Does Not Produce Ketamine-Like Dissociative/Hallucinogenic-Like Effects in Cynomolgus Monkeys. In a previous study, i.m. ketamine administration produced a dose-dependent increase in the incidence of abnormal and dissociative/hallucinogenic-like behaviors; a dose of 1 mg/kg was chosen as the comparator for further investigation (Supplemental Fig. 7). Two independent studies were conducted to examine the effects of intramuscular administration of BMS-986169 on spontaneous behaviors in cynomolgus monkeys; the first examined BMS-986169 at doses of 1, 3, or 5.6 mg/kg, and the second examined BMS-986169 at a dose of 10 mg/kg. Results from repeated-measures ANOVA showed a significant effect of treatment on

Fig. 5. Effect of i.v. administration of BMS-986169 (A) or ketamine (B) on immobility in the mouse FST assay. Results are presented as the mean $\pm$ S.E.M. immobility duration determined 15 minutes after treatment with BMS-986169 or CP-101,606 (CP; 3 mg/kg, i.v.; $n = 8-11$ /group) (A) or 30 minutes after treatment with ketamine ($n = 9-10$ /group) (B). Results were analyzed by ANOVA followed by Dunnett's post-hoc test; ** $P < 0.01$; * $P < 0.05$ compared with vehicle (Veh). Effect of i.v. administration of BMS-986163 or CP-101,606 (10 mg/kg) on novelty-suppressed feeding (C) and home cage feeding (D) determined 24 hours after dosing in mice ($n = 12-15$ /group). Results are presented as the mean $\pm$ S.E.M. latency to feed in the novel environment or quantity of food consumed in the home cage during a 30-minute period beginning immediately after testing in the novel environment. Treatment effects were compared with their respective vehicle by either unpaired t test (CP-101,606 vs. Veh-1) or ANOVA followed by Dunnett's post-hoc test (BMS-986163 vs. Veh-2); * $P < 0.05$; ** $P < 0.01$.

the incidence of abnormal behaviors [study 1: $F(4,28) = 12.06$, $P < 0.0001$; study 2: $F(2,14) = 14.43$, $P = 0.0004$] and dissociative/hallucinogenic behaviors [study 1: $F(4,28) = 6.533$, $P = 0.0008$; study 2: $F(2,14) = 11.08$, $P = 0.0013$]. Dunnett's post-hoc analysis showed that the incidence of these behaviors was significantly increased following ketamine treatment (1 mg/kg, i.m.), whereas BMS-986169 had no effect at any dose tested (Fig. 8). Analysis of total plasma BMS-986169 concentrations on completion of testing (~75 minutes post-treatment) showed dose-dependent exposure ranging from 620 to 10,260 nM (Supplemental Table 6).

BMS-986169 Transiently Impairs Working Memory in Cynomolgus Monkeys. Vehicle-treated monkeys performing the list-DMS task showed a significant decrease in performance accuracy as the delay between the initial presentation of the stimulus and the choice phase increased (mean $\pm$ S.E.M. percentage of accuracy: short delay = $95.6\% \pm 1.3\%$; medium delay = $74\% \pm 4.1\%$; long delay = $63.7\% \pm 4.6\%$; one-way RM ANOVA [$F(2,16) = 33.3$, $P < 0.0001$] followed by Dunnett's post-hoc test compared with short delay). Intramuscular injection of BMS-986169 produced a significant dose- and delay-dependent decrease in performance accuracy in subjects tested 30 minutes after dosing [treatment: $F(4,32) = 39.21$, $P < 0.0001$; delay: $F(2,16) = 116.2$, $P < 0.0001$; interaction: $F(8,64) = 8.533$, $P < 0.0001$]. Further post-hoc

analysis showed that BMS-986169 had no effect on performance accuracy at short delays at any dose tested (Fig. 9A). In contrast, a significant impairment in accuracy was observed at both the medium and long delays in subjects treated with 1 and 3 mg/kg BMS-986169 (Fig. 9A). The lack of effect at short delays indicates that performance of the task per se is not altered by drug treatment. Consistent with these results, treatment with BMS-986169 did not alter percentage of task completion, indicating no effect on motivation to perform the task at any dose (Supplemental Fig. 8A). Although a significant increase in latency to respond was seen at the highest dose, this increase (~300 ms) is only a minor slowing of response time (Supplemental Fig. 8B). The effect of treatment with 1 mg/kg BMS-986169 on performance accuracy was also time-dependent [treatment: $F(3,24) = 18.7$, $P < 0.0001$; delay: $F(2,16) = 103.1$, $P < 0.0001$; interaction: $F(6,48) = 8.112$, $P < 0.0001$], with maximal impairment observed 30 minutes postdose and full recovery achieved by 5 hours after treatment (Fig. 9C). Again, there was no effect on percentage of task completion or response latency consistent with a selective memory impairment (Supplemental Fig. 8, C and D). Across both studies, the degree of impairment, as represented by the difference in the percentage of accuracy from vehicle treatment at the long delay condition, was related to total plasma BMS-986169 concentrations measured on completion

Fig. 6. BMS-986169 (3 mg/kg, i.v.) enhances ex vivo hippocampal LTP 24 hours after dosing in rats. (A and C) Time course of the normalized fEPSP amplitude (A) or slope (C). (B and D) Percentage increase over baseline of the fEPSP amplitude (B) or slope (D) during the last 5 minutes of recording following each HFS application. Results are presented as the mean $\pm$ S.E.M. ($n = 7$ /group). Results at each HFS (B and D) were analyzed by one-tailed, paired t test; * $P < 0.05$ compared with vehicle.

of testing in experimental subjects (Fig. 9, B and D; Supplemental Table 6), with large impairments seen at plasma concentrations ≥ 483 nM. Investigation of plasma exposures in a separate group of subjects confirmed that total plasma BMS-986169 concentrations are stable across the duration of the list-DMS task (i.e., 30–60 minutes postdose; Supplemental Fig. 9A). CSF concentrations of BMS-986169 increased in a dose-dependent manner, ranging from 18.5 to 79 nM at 30 minutes postdose (Supplemental Fig. 9B). The predicted GluN2B occupancy, calculated using CSF concentrations and the monkey in vitro GluN2B binding K_i value (4.2 nM), ranged from 81.5% to 95% across the dose range examined (Supplemental Fig. 9B).

BMS-986163 Alters qEEG Power Band Distribution in Cynomolgus Monkeys. Intravenous administration of BMS-986163 produced robust and dose-related changes in the qEEG power distribution in the beta 1, alpha, and delta power bands. Analysis of time-course data showed that all doses of BMS-986163 reduced the relative power in the beta 1 power band (Supplemental Table 7). At the highest dose (1.2 mg/kg), a significant increase in relative power in the delta band and decrease in the alpha band were also observed (Supplemental Table 7). Time-course results show that treatment effects emerged rapidly and were sustained throughout the 90-minute recording period (Fig. 10A; Supplemental Figs. 10–12). Analysis of results expressed as the area under the relative power curve confirmed these results except for the low-dose (0.12 mg/kg) effect on beta 1 (Fig. 10B; Supplemental Fig. 13; Supplemental Table 8). Cohen's d analysis showed that the relative power changes produced at the highest dose of BMS-986163 were moderate (>0.5) to large (>0.8) with effect sizes of 0.77 (delta), -0.88 (alpha), and -1.01 (beta 1), respectively (Fig. 10C). Investigation of plasma exposure, in

separate subjects dosed with BMS-986163 (1.2 mg/kg, i.v.), showed that maximal total plasma BMS-986169 concentrations were achieved within 5 minutes of dosing, confirming rapid conversion of the prodrug to parent (Fig. 10D) in monkeys. Consistent with these results, the prodrug concentrations in 5-minute plasma samples were negligible and below the assay limits of quantification (<156 nM). CSF concentrations of BMS-986169 were stable over the 90-minute qEEG recording period (Fig. 10D) and associated with a high level of predicted GluN2B occupancy of 97%–98%.

Discussion

Selective inhibition of the GluN2B receptor subtype has been proposed as a strategy to deliver novel therapeutics with ketamine-like antidepressant efficacy and improved tolerability. Using well established in vitro approaches (Mutel et al., 1998; Malherbe et al., 2003; Ng et al., 2008; Risgaard et al., 2013), BMS-986169 was identified as a novel agent with high affinity for the GluN2B amino terminal domain allosteric site ($K_i = 4$ –6 nM) that potently and selectively inhibits GluN2B receptor function ($IC_{50} = 24.1$ nM). BMS-986169 showed negligible activity in a broad assay panel consisting of 40 additional targets. Importantly, BMS-986169 weakly inhibited hERG channels ($IC_{50} = 28.4$ μ M), an off-target liability that has been challenging to address in chemical scaffolds targeting the GluN2B NAM site (Brown et al., 2011; Layton et al., 2011; Müller et al., 2011).

The in vivo characterization of BMS-986169 and the phosphate prodrug BMS-986163 focused on 1) confirmation of occupancy and functional inhibition of GluN2B receptors, 2) testing in assays of antidepressant drug-like effects and side

Fig. 7. (A) Increased LMA after i.v. ketamine treatment in mice ($n = 8$ – 9 /group). (B) Effect of BMS-986169 (i.v.) or ketamine (30 mg/kg, i.v.) on LMA in mice ($n = 7$ – 8 /group). Results are presented as the mean $\pm$ S.E.M. ambulatory distance in 10-minute time bins recorded for 2 hours after dosing. Results were analyzed by two-way RM ANOVA followed by Dunnett's post-hoc test; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared with vehicle.

effects, and 3) testing on translational measures that can be investigated in healthy humans. GluN2B occupancy in rodents was measured by ex vivo [3 H]Ro 25-6981 binding (Fernandes et al., 2015) and showed a dose-dependent increase that fully saturated at doses ≥ 3 mg/kg. Occupancy was tightly correlated with exposure, and occupancy/exposure relationships were similar after i.v. dosing with BMS-986169 or BMS-986163, confirming rapid and effective cleavage of the prodrug. Functional inhibition of GluN2B receptors was demonstrated by inhibition of in vivo [3 H]MK-801 binding (Fernandes et al., 2015). [3 H]MK-801 is a radiotracer that labels all NMDA receptor subtypes present in an open conformational state. The assay can be used to show both direct binding site competition following treatment with other NMDA receptor open-channel blockers and binding inhibition due to the functional effects of agents acting at other modulatory sites (Murray et al., 2000; Fernandes et al., 2015). Specifically, the conformational change produced by

Fig. 8. BMS-986169 does not produce abnormal or dissociative/hallucinogenic-like behaviors in cynomolgus monkeys ($n = 8$). Results are presented as the mean $\pm$ S.E.M. total score for all abnormal behaviors (A) or dissociative/hallucinogenic behaviors (B) observed up to 65 minutes after i.m. dosing with vehicle, ketamine (Ket; 1 mg/kg, i.m.), or BMS-986169 (1–10 mg/kg). Results were analyzed by RM ANOVA followed by Dunnett's post-hoc test; $**P < 0.01$; $***P < 0.001$ compared with vehicle.

binding to the GluN2B allosteric site closes channels containing the GluN2B subunit, prevents [3 H]MK-801 from accessing its binding site in the channel pore, and reduces [3 H]MK-801 binding. Previous studies have shown that GluN2B NAMs maximally inhibit [3 H]MK-801 binding by $\sim 60\%$, suggesting that GluN2B-containing NMDA receptors represent 60% of the total NMDA receptor population labeled in vivo (Fernandes et al., 2015). In a similar manner, treatment with BMS-986163 also inhibited [3 H]MK-801 binding in rats, confirming functional NMDA receptor inhibition in vivo. However, in contrast to other agents, the maximal inhibition achieved at doses fully occupying the GluN2B NAM binding site was $\sim 40\%$, suggesting a subpopulation of GluN2B-containing receptors that are less sensitive to functional inhibition by BMS-986169 in vivo. Lower inhibition could reflect functional differences in discrete brain regions expressing GluN2B receptors; the current homogenate assay does not address this question, and GluN2B functional inhibition is not detectable using ex vivo [3 H]MK-801 autoradiography (Lord et al., 2013). Different receptor subtypes—namely, diheteromeric GluN1/GluN2B and triheteromeric GluN1/GluN2A/GluN2B receptors—may also be involved since differences in functional potency and maximal inhibition at tri- versus

Fig. 9. BMS-986169 produces a transient impairment in working memory after i.m. dosing in cynomolgus monkeys ($n = 9$). Effect on list-DMS accuracy determined 30 minutes after dosing with BMS-986169 (0.1–3 mg/kg, i.m.) (A) or at various time points after dosing with 1 mg/kg (i.m.) BMS-986169 (C). Results are presented as the mean $\pm$ S.E.M. percentage correct responses at short, medium, or long delays and were analyzed by two-way RM ANOVA followed by Dunnett's post-hoc test; *** $P < 0.001$ compared with vehicle. Relationship between total plasma BMS-986169 concentration and impairment at long delays determined 30 minutes after dosing with BMS-986169 (B) and at various time points after dosing with 1 mg/kg BMS-986169 (D). Results are presented as the mean $\pm$ S.E.M. difference from vehicle in percentage correct responses at the long delay condition or the mean $\pm$ S.D. total plasma BMS-986169 concentration ($n = 7$ –9/group).

diheteromeric receptors have been reported for CP-101,606 (Hansen et al., 2014). Additional studies, including in vivo approaches such as pharmacological magnetic resonance imaging, may provide further insight into this interesting result (Chin et al., 2011).

To provide evidence of antidepressant drug-like effects, we first tested BMS-986169 in the mouse FST, a simple behavioral assay sensitive to many antidepressant drug classes and multiple NMDA receptor agents (Porsolt et al., 1977; Trullas and Skolnick, 1990; Pilc et al., 2013). As expected, BMS-986169 reduced immobility 15 minutes after dosing, consistent with results for other GluN2B NAMs (Li et al., 2010; Kiselycznyk et al., 2015; Refsgaard et al., 2017). We next showed that BMS-986169 robustly elevated ex vivo hippocampal LTP measured 24 hours after treatment. As evidence of synaptic strengthening, this finding is consistent with the hypothesis that rapid-acting antidepressants enhance synaptic plasticity (Wohleb et al., 2017) and with results for ketamine and the structurally distinct GluN2B NAM CP-101,606 in this assay (Burgdorf et al., 2013; Graef et al., 2015). The effect is

thought to arise from increased expression of synaptic proteins, such as GluR1 and PSD95, and at least two mechanisms downstream of NMDA receptor inhibition may be involved: 1) disinhibition of pyramidal glutamate neurons leading to activity-dependent brain-derived neurotrophic factor release and activation of extracellular signal-regulated kinase/AKT/mTORC1 signaling, and/or 2) activity-independent blockade of extrasynaptic, postsynaptic NMDA receptors leading to reduced eEF2 kinase activity, decreased eEF2 phosphorylation, and desuppression of brain-derived neurotrophic factor and synaptic protein translation (Miller et al., 2016; Murrough et al., 2017). Finally, we showed that BMS-986169 reduced latency to feed in the NSF assay, a model of stress-induced anxiety that is sensitive to chronic, but not acute, treatment with classic antidepressant agents (Dulawa and Hen, 2005). Importantly, we demonstrated efficacy 24 hours after dosing, a result consistent with the sustained efficacy seen after acute ketamine treatment in this assay (Iijima et al., 2012). With respect to target engagement, LTP and NSF results suggest that high levels of GluN2B

Fig. 10. Robust effects on the qEEG power band distribution after i.v. treatment with BMS-986163 in cynomolgus monkeys ($n = 6$). (A) Time course of the effect of 1.2 mg/kg i.v. BMS-986163 on relative power in the beta 1 power band. Results are presented as the mean $\pm$ S.E.M. relative power in 1-minute time bins and were analyzed by two-way RM ANOVA followed by Holm-Sidak post-hoc test. (B) Effect of BMS-986163 on relative power in the beta 1 power band. Results are presented as the mean $\pm$ S.E.M. area under the beta 1 relative power curve (AUC) and were analyzed by RM ANOVA followed by Dunnett's t test; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared with vehicle. (C) Treatment effect size for changes in the qEEG power band distribution. Results show Cohen's d values, calculated using the relative power AUC, for each power band after treatment with BMS-986169. Statistics correspond to results from the AUC analysis as described in (B). (D) Mean ($\pm$ S.D.) total plasma and CSF concentration of BMS-986169 measured in separate subjects after i.v. administration of 1.2 mg/kg BMS-986163 ($n = 2$ /group).

occupancy, sustained for a critical duration, are necessary to deliver efficacy 24 hours after dosing. Robust LTP enhancement was seen at 3 mg/kg, which achieves $\sim 95\%$ peak occupancy and maintains $\geq 80\%$ occupancy for ~ 1 hour. Efficacy in NSF was seen only at 10 mg/kg, whereas peak occupancy was saturated at doses ≥ 3 mg/kg, suggesting the duration of occupancy is important. This initial characterization suggests that BMS-986169/BMS-986163 has potential as a rapid-acting antidepressant and supports progression to further testing in more complex animal models and investigation of the molecular mechanisms involved (Ramaker and Dulawa, 2017).

While BMS-986169 showed low potential to produce ketamine-like hyperactivity or abnormal behaviors, impaired working memory was observed in monkeys performing the CANTAB list-DMS task. We chose i.m. dosing for these studies to ensure stable plasma levels during the 30-minute testing session and to facilitate demonstration of the effect. Importantly, this impairment was transient, fully resolving by 5 hours postdose, and was closely related to plasma exposures with subsequent pharmacokinetic modeling predicting a more rapid resolution after i.v. administration. This profile is similar to other GluN2B NAMs tested in this model

(Weed et al., 2016) and consistent with electrophysiology studies showing that persistent activation of dorsolateral prefrontal cortical delay neurons is dependent upon GluN2B receptors in monkeys performing working memory tasks (Wang et al., 2013; Wang and Arnsten, 2015). The temporal differences between the working memory and hippocampal LTP effects should be noted; working memory deficits coincide with the primary pharmacological effect, i.e., GluN2B receptor inhibition, whereas enhanced 24-hour LTP is measured at a time when downstream events leading to synaptic strengthening have occurred. Given the overlapping exposures and (predicted) occupancy across these assays, the separation of doses providing antidepressant-like effects from those producing transient cognitive impairment within the first hour(s) of dosing may not be possible for this mechanism.

The present results raise important questions about the optimal GluN2B NAM candidate profile. From this perspective, both intravenous (CP-101,606) and oral agents (CERC-301, also known as MK-0657) have been examined in patients. In the case of CP-101,606, a single i.v. infusion improved depression symptoms in a small cohort of TRD patients with minimal dissociative effects (Preskorn et al., 2008). In contrast, although CERC-301 initially appeared promising

(Ibrahim et al., 2012), a recent study showed that treatment with 8 mg daily was not effective in severely depressed TRD patients (www.Cerecor.com). It is possible that target engagement is inadequate at this dose of CERC-301 since preclinical results suggest that exposures reported in MDD patients receiving a 4–8 mg titration (Ibrahim et al., 2012) only briefly (~2 hours) achieve levels predicted to deliver ~50% occupancy (Addy et al., 2009). Management of cognitive impairment may be difficult for oral agents with a long pharmacokinetic half-life if human results confirm the high target engagement requirement for antidepressant effects suggested by preclinical studies. Thus, an i.v. agent may be preferred since exposure can be tightly controlled to deliver transient, high levels of GluN2B occupancy while enabling monitoring for resolution of any transient cognitive impairment within a clinical setting.

To facilitate the progression of BMS-986163, we examined effects on the qEEG. Previous studies have shown robust qEEG power band changes in monkeys treated with GluN2B NAMs (Keavy et al., 2016), and BMS-986163 produced the same pattern of response. As an approach, changes in qEEG-related measures for NMDA receptor antagonists generally align well across species and, although not tested here, other measures such as auditory evoked potentials also have translational utility for GluN2B NAMs (Nagy et al., 2016). Such approaches may provide valuable information; for example, the sustained qEEG effect was not anticipated after i.v. dosing but is likely explained by the relatively stable CSF levels of BMS-986169 during the recording window. Thus, qEEG-related measures provide a noninvasive way to monitor target engagement in humans and potentially aid the optimization of i.v. infusion regimens.

In conclusion, the present results support the further investigation of BMS-986163, the phosphate prodrug of the novel GluN2B NAM BMS-986169, as an i.v. agent with potential for a rapid antidepressant effect.

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