Comparative Effects of LY3020371, a Potent and Selective mGlu2/3 Receptor Antagonist, and Ketamine, a Non-Competitive NMDA Receptor Antagonist in Rodents: Evidence Supporting the Use of mGlu2/3 Antagonists for the Treatment of Depression.


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Abstract

The ability of the NMDA receptor antagonist ketamine to alleviate symptoms in patients suffering from treatment resistant depression (TRD) is well documented. In this report, we directly compare in vivo biological responses in rodents elicited by a recently discovered mGlu2/3 receptor antagonist (LY3020371) with those produced by ketamine. Both LY3020371 and ketamine increased the number of spontaneously active DA cells in the ventral tegmental area of anesthetized rats, increased O2 in the anterior cingulate cortex, promoted wakefulness, enhanced the efflux of biogenic amines in the prefrontal cortex, and produced antidepressant-related behavioral effects in rodent models. The ability of LY3020371 to produce antidepressant-like effects in the forced-swim assay in rats was associated with cerebral spinal fluid (CSF) drug levels that matched concentrations required for functional antagonist activity in native rat brain tissue preparations. Metabolomic pathway analyses from analytes recovered from rat CSF and hippocampus demonstrated that both LY3020371 and ketamine activated common pathways involving GRIA2 and ADORA1. A diester analog of LY3020371 (LY3027788) was an effective oral prodrug; when given orally, it recapitulated effects of intravenous doses of LY3020371 in the forced-swim and wake-promotion assays, and augmented the antidepressant-like effects of fluoxetine or citalopram without altering plasma or brain levels of these compounds. The broad overlap of biological responses produced by LY3020371 and ketamine supports the hypothesis that mGlu2/3 receptor blockade might be a novel therapeutic approach for the treatment of TRD patients. LY3020371 and LY3027788 represent molecules that are ready for clinical test of this hypothesis.
Introduction

The naturally occurring amino acid glutamate plays a preeminent role in excitatory neurotransmission within the mammalian central nervous system. Rapid cellular responses to glutamate are mediated by membrane-associated, glutamate-gated ion channels (ionotropic glutamate receptors, iGluRs) which consist of four pharmacologically defined receptor types (AMPA, Kainate, NMDA and delta) each of which are formed by heteromeric assemblies of multiple subunit proteins (AMPA: GluA1-4; Kainate: GluK1-5; NMDA: GluN1, GluN2A-D, GluN3A,B; delta: GluD1,2) (Traynelis et al., 2010). Glutamate also mediates slower, second-messenger-dependent processes through its activation of a family of G-protein coupled, seven transmembrane receptors (metabotropic glutamate receptors, mGluRs). These receptors (mGlu$_1$-mGlu$_8$) have historically been divided into three groups on the basis of amino acid sequence homology, agonist pharmacology, and preferred G-protein coupling partners (Pin and Duvoisin, 1995).

Considerable scientific attention has been given to agents that enhance mGlu$_2$/3 receptor signaling, primarily owing to the observation that ligands that either directly or allosterically enhance the activity of these receptors can produce robust efficacy in preclinical tests that are controlled by elevated synaptic glutamate drive, including those associated with psychiatric disorders (Monn et al., 1997; Helton et al., 1998; Moghaddam et al., 1998; Cartmell et al., 1999; Kłodzińska et al., 1999; Cartmell et al., 2000; Nakazato et al., 2000; Schoepf et al., 2003; Takamori et al., 2003; Swanson et al., 2005; Rorick-Kehn et al., 2007; Jones et al., 2011; Ago et al., 2012), and neurologic conditions (Neugebauer et al., 2000; Simmons et al., 2002; Jones et al., 2005; Du et al., 2008; Kumar et al., 2010; Caraci et al., 2011). Heightened interest came from the demonstration of clinical efficacy in both anxiety (Dunayevich et al., 2008) and schizophrenia patients (Patil et al., 2007), though in the latter case antipsychotic efficacy may be restricted to patient subpopulations (Kinon et al., 2015; Nisenbaum et al., 2016).

As part of a research effort aimed at the identification of potent and selective antagonists for mGlu2/3 receptors, we discovered LY3020371 (Fig. 1; Smith et al., 2012; Chappell, 2016). This molecule was characterized as a highly potent and selective...
orthosteric antagonist of recombinant human mGlu2/3 receptors, endogenously expressed rat cortical and hippocampal mGlu2/3 receptors, and native mGlu2/3 receptors present in synaptosomes prepared from surgically resected human brain tissue (Witkin et al., 2016b). We further demonstrated that when administered by the intravenous route at doses between 0.3-10 mg/kg, exposures of LY3020371 in the cerebrospinal fluid (CSF) fully covered concentrations required to effectively block mGlu2/3 receptor activation in native tissues (Witkin et al., 2016b). Based upon these data, LY3020371 was considered an exemplary tool with which to probe the biological significance of mGlu2/3 receptor antagonism in vivo by parenteral dosing. In this account, we describe the results of a comparative assessment of in vivo electrophysiological, neurochemical, behavioral, and metabolomic responses elicited by LY3020371 and ketamine in rodents, highlighting the broadly overlapping biological effects induced by these agents in vivo. In addition, we describe the attributes of LY3027788 (Fig. 1), a diester form of LY3020371, and its utility in producing biologically-relevant plasma concentrations of active moiety (LY3020371) following oral administration in rodents.
Methods.

All experiments were conducted according to the Guidelines for Care and Use of Laboratory Animals under protocols approved by an institutional animal care and use committee and monitored by animal care and use groups at a local level. We only used male rodents in the present series of experiments for two reasons; first, laboratory precedent and our historical data set in males, and second, ease of animal housing in our vivarium which requires isolated rooms for males and females. Experiments performed in the UK were in accordance with the UK Animals Scientific Procedures Act (1986) and Lilly UK ethical review.

Compounds. LY3020371 and its diester prodrug, LY3027788 (Smith et al., 2012), and LY354740 (Monn et al., 1997) were synthesized at Eli Lilly and Company. Imipramine was purchased from Sigma Chemical Co (St. Louis, MO) and NBQX, Ketamine and (+)-ketamine from Tocris Bioscience (Ellisville, MO). LY3020371, LY354740, and NBQX were dissolved in water and titrated to solution with dilute NaOH. LY3027788.HCl was suspended in 10% acacia and dosed orally, whereas all other compounds were given by i.v. (LY3020371), i.p. (LY3020371, LY354740) or s.c. injection (NBQX). All compounds and vehicle were administered in a volume of 10 ml/kg (mice) or 1 or 2 ml/kg (rats).

Pharmacodynamic effects of LY3020371 in comparison to ketamine.

Dopamine neuron activity in the ventral tegmental area of rats. Male Sprague-Dawley rats (230 to 350 g) from Taconic (Germantown, NY) or Harlan Laboratories (Indianapolis, IN) were anesthetized using chloral hydrate (400 mg/kg, i.p.), followed by supplemental doses as needed. Rats were placed on a heating pad to maintain body temperature at 37°C throughout the procedures. An i.v. catheter, consisting of PE10 tubing (Becton Dickinson, Sparks, MD) connected to a 1 mL tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ) via 30 gauge hypodermic needle (1 inch length; Exel International, Los Angeles, CA), was placed in the jugular vein for administration of test
compounds and supplemental anesthesia. The catheter was secured into the vein using suture thread (3-0 Black Braided Silk; Roboz Surgical, Rockville, MD), tied tightly enough to prevent slippage but not so tight as to constrict blood flow. Following catheter implantation, rats were positioned tightly into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), a hole was drilled in the skull overlying the target VTA coordinates (AP: -5.3 to -5.7 mm, ML: +0.5 to +0.9 mm, DV: -6.0 to -8.5 mm, relative to bregma), and the dura was carefully resected. A single barrel micropipette (Radnoti Starbore Capillary Tube, 1.8 mm O.D.; pulled using a Narishige PE-2 vertical puller; broken back to a tip diameter approximately 2 to 6 μm and filled with 2 M NaCl solution; impedance 3 to 10 MOhms) was mounted into a Burleigh Inchworm 8200 micro-drive for single-unit extracellular recordings.

Electrophysiological signals were amplified by a Dagan 2400 pre-amplifier (low cut 300 Hz, high cut 3000 Hz; gain 1-fold). Data were recorded and analyzed using a Micro1401 data acquisition system with Spike2 software (Cambridge Electronic Design, Ltd; Cambridge, England). Electrophysiological properties of spontaneously active VTA dopamine cells were characterized by lowering recording electrodes slowly (3 μm/sec) through the dorsoventral extent of the VTA along 9 to 12 pre-defined tracks (separated by 200 μm) in a grid pattern throughout the entire nucleus. Dopamine cells were identified and distinguished from non-dopamine cells based on their unique long-duration (2.5 to 4 ms), triphasic waveform, and slow irregular firing rate (2 to 10 spikes/sec). For each track, the number of spontaneously active dopamine cells, the average firing rate and the percentage of spikes occurring in bursts were analyzed. A burst was defined as a period of rapid cell firing, with burst initiation identified by an interspike interval (ISI) <80 msec between two consecutive action potentials, and ISI >160 msec indicating the end of the burst.

For each dose level, spontaneously active dopamine cells were characterized throughout 2 to 3 tracks. Doses were administered (i.v., 10 min pretreatment) to n = 6 rats in ascending, cumulative order, such that vehicle was administered 10 min prior to the first track, 0.3 mg/kg LY3020371 was administered prior to track 4, 1 mg/kg LY3020371 was administered 10 min prior to track 7, and 3 mg/kg LY3020371 was administered 10 min prior to track 7.
prior to track 10. In a different cohort, the AMPA antagonist, NBQX (10 mg/kg, i.v.), was tested for its effects on VTA dopamine cells when administered alone, and also its ability to block the increase in spontaneously active dopamine cells produced by LY3020371 (3 mg/kg, i.v.) in n = 8 rats. Finally, the NMDA antagonist ketamine HCl (tested as the racemate, 17 mg/kg i.v., 10 min pretreatment) was tested alone in a separate cohort of n = 6 rats (formulations not corrected for salt weight). LY3020371 and NBQX were dissolved in sterile water with the addition of 1N NaOH (titrated to pH 7-8 using 8.5% lactic acid in water). Ketamine was dissolved in sterile water.

The percentage of spikes occurring in bursts was determined using Spike2 software (version 5.21, CED Ltd, Cambridge, England). The mean number of spontaneously active dopamine cells, the mean firing rate, and the mean percentage of spikes occurring in bursts were analyzed using separate repeated-measures ANOVAs, followed by Dunnett’s post-hoc comparisons vs. respective vehicle (SAS v9.3, SAS Institute, Cary, NC). For comparison purposes, effects of LY3020371, NBQX and ketamine were calculated as percent change from vehicle, by dividing each drug group mean by its respective vehicle group mean, and multiplying by 100, and plotted together on the same graph.

**Effects on brain oxygen.** Male, Wistar rats (n=36, Batch B21046) were implanted with carbon paste electrodes (CPEs) in anterior cingulate cortex (ACC) (AP +2.0 mm, ML ±0.5 mm, DV -2.0 mm from dura,) and the striatum (STR) (AP +1.2 mm, ML ± 3.4 mm, DV -4.2 mm from the dura,). At time of surgery, animals weighed 250-320 g.

At the time of testing, animals weighed 420-700 g. Animals were cabled for 15 to 30 minutes pre-dosing to establish a stable tissue oxygen baseline signal. At Time 0, animals were treated with either vehicle (saline ip), LY3020371 (1, 3, or 10 mg/kg, i.p.) or (S)-(−)-ketamine HCl (10 mg/kg, s.c.) and oxygen signals recorded for 90 minutes. Animals received all treatments, dosed once weekly per animal in a randomized, within subjects design. At the conclusion of the session they were returned to their home cage. The study lasted five weeks.

Four-channel potentiostats (EA164 Quadstat) and 16-channel E-Corder data acquisition systems with Chart software (all eDAQ, Australia) were used for monitoring
and recording oxygen signals. Data were sampled at 1 KHz and downsampled to 1Hz for the purposes of analysis.

Oxygen response time courses from ACC and STR were normalized to a time point immediately before injection time and post-injection AUC calculated for statistical analysis. All statistics were calculated using STATISTICA v.9. A general linear model approach was used in a repeated measures, within subjects design. A main effect of dose was followed with Tukey’s post-hoc comparisons to determine the effect of each treatment group relative to the vehicle group. Following exclusions due to O2 signal quality, AUC statistical outliers and histological verification of CPE placements, 11 animals from the ACC group and 17 animals from the STR group remained in the final analysis.

**Effects on Neurochemical Efflux in the Rat Medial Prefrontal Cortex.**

Microdialysis probes (MAB 4.7.4 Cu 6kDa cut-off; RoYem Scientific Ltd., U.K.) were implanted under isoflurane anesthesia into the medial prefrontal cortex of male Wistar rats (approximately 300-400 g; Charles River, U.K.) using the following coordinates (from bregma and dura surface; probe angled at 12 degrees): AP = +2.8 mm, LM = +1.5 mm, DV = -5.0 mm.

The day after probe implantation, animals were connected to a liquid swivel suspended on a counterbalanced arm, allowing the probes to be perfused with artificial cerebrospinal fluid containing NaCl (141mM), KCl (5mM), MgCl$_2$ (0.8mM), and CaCl$_2$ (1.5 mM) via an infusion pump flowing at 1.5 µL/min. Following a 90-minute pre-sample washout period, dialysate samples were collected at 20-minute intervals and immediately frozen on dry ice prior to analysis by liquid chromatography-mass spectrometry (LC-MS/MS After six baseline samples (120 minutes), animals were injected with drug (LY3020371, 10 mg/kg i.p. dissolved in water buffered to pH 6-7 with 1N NaOH; S-(+)-ketamine hydrochloride 10 mg/kg free base s.c. dissolved in 5% glucose), or corresponding vehicle.

To each thawed dialysis sample (29 µL) was added: 20 µL buffer (1M Bis-Tris, pH10), 20 µL mixed deuterated standard and 260 µl 0.1% w/v dansyl chloride (in acetone). The samples were vortexed and heated at 65°C for 30 min, then dried under N$_2$ and re-suspended in 40 µL 50:50 (v/v) ACN:water (containing 10 mM ammonium
formate and 0.06% formic acid). The samples were then centrifuged at 13000 RPM for 10 min at ambient temperature and 35 µL pipetted into 03-FIVR vials; 10 µL was injected onto the LC-MS/MS using a CTC PAL HTC-xt Autosampler.

Chromatographic separation of dansylated samples (including drug standards) was performed under a 13-minutes gradient (including washout and a re-equilibration step) using Shimadzu LC-20AD XR binary pumps (plus a CBM-20 controller) and a 2.6 µm Phenomenex Kinetex, XB-C18 HPLC column. Mobile Phase A consisted of acetonitrile/water 5:95 (v/v), 2 mM ammonium formate, and 0.06% formic acid, and mobile Phase B, acetonitrile/water 95:5 (v/v), 2 mM ammonium formate and 0.06% formic acid.

An AB Sciex API5500 was operated in two periods: Period 1, positive Turbo Ion Spray (TIS) mode for detection of acetylcholine, and Period 2 with positive and negative TIS modes for all other analytes (negative for glycine, glutamate, and glutamine) using a 20 millisecond dwell time. For most dansylated analytes, the dansyl fragment (m/z 170 or 171) was the abundant product ion and was subsequently used for multiple reaction monitoring (MRM). Additional fragment ions were selected for the MRM of 5-HT (m/z 380), DA (m/z 619), glutamate (m/z 144, negative mode), glutamine (m/z 234, negative mode), glycine (m/z 234, negative mode), DHPG (m/z 386), telemethyl histamine (m/z 188) and “non-dansylated” ACh (m/z 87).

Data were expressed as a percentage of a pre-injection control period, obtained by averaging three samples prior to drug delivery (=100%) and expressing values as a percentage of this value. The amount of analyte in each microdialysate sample was recorded as a peak area or height. Calibration curves were also constructed to allow measurement of LY3020371 or S-(+)-ketamine in the same microdialysate sample. Statistical analyses were undertaken using either ANOVA with Repeated Measures (RM/Fit), to compare response profiles following drug or vehicle administration, or by One-way ANOVA (using JMP, SAS Institute) for comparison of area under the curve (AUC). A probability value of p<.05 was considered to be statistically significant.

Metabolomic Analysis. We used an in-house mass spectrometry based metabolomics platform which allowed routine detection of > 5000 metabolic features. In this study the
resulted data sets yielded statistically significant abundant changes in > 240 metabolites, excluding peptides and intact lipids (Xia et al., 2012; Tolstikov et al., 2013). Statistical analysis was performed with JMP 9.03 and MetaboAnalyst 2.0. Linear regression and curve clustering techniques to the time course expression profile of all the metabolites under different treatment schemes were applied to extract information related to altered metabolism at different time points. Ingenuity Systems (http://www.ingenuity.com/) was used to carry out pathway and network analyses.

Animals were dosed (i.p.) with vehicle, 10 mg/kg LY3020371, or 10 mg/kg ketamine. Doses were based upon comparable effects in the rat forced-swim assay. Tissue from hippocampus and cerebrospinal fluid (CSF) were harvested at 1 hr post dosing. The harvest of these samples was synchronized to minimize the influence of diurnal fluctuations of metabolites on the primary dependent measures of drug and dose. Tissue and bio-fluid samples were processed and analyzed in randomized fashion under validated SOPs. Identification of the potential biomarkers, which were not present in GC/LC-MS libraries, was performed using a HILIC-LC-MS discovery platform and further validated with the authentic standards comparisons. Data from multiple platforms were normalized to fresh weights and/or to bio-fluid volumes taken for metabolite extraction and subjected to rigorous bio-statistical analyses.

We used an ANOVA model to compare the effect of treatments. Multiplicity adjusted P values were calculated for each contrast of interest. Additionally, to identify biomarkers and discern the pattern of strong diurnal effect on many metabolites, we applied curve clustering techniques to the expression profile of all the metabolites under different treatment schemes. The significantly altered metabolites were then used to discern the metabolic pathway changes at different time points by bioinformatics analytic methods.

Sleep/Wake Assessments. The parallel groups study design was carried out across 5 treatment days, each separated by at least one week. Animals were assigned in no particular order to a dose group, and no animal received a given dose more than once. In general, animals were not naïve to drug treatments. At least 7 days “washout” preceded and followed any treatment. Adult, male Wistar rats (approximately 250-300 g at time of
surgery, Charles River Laboratories) were anesthetized (2% isoflurane in 100% oxygen) and surgically prepared with a cranial implant that permitted chronic electroencephalogram (EEG) and electromyogram (EMG) recording. Body temperature and locomotor activity were monitored via a miniature transmitter (Minimitter PDT4000G, Philips Respironics, Bend, OR) surgically placed in the abdomen during the same anesthetic event in which the cranial portion was implanted. The cranial implant consisted of stainless steel screws (2 frontal [+3.5 AP from bregma, ±2.0 ML] and 2 occipital [-6.5 AP, ±5.2 ML]) for EEG recording. Two Teflon-coated stainless steel wires were positioned under the nuchal trapezoid muscles for EMG recording. An analgesic (buprenorphine 0.05 mg/kg sc) was administered pre-operatively, at the end of the surgery day and the morning of the first post-operative day. To provide additional pain relief, Metacam (meloxicam) 0.15 mg/kg po was administered for 6 days post-surgery. An antibiotic (Ceporex (cefalexin) 20 mg/kg, p.o.) was administered 24 hr before and again immediately before surgery, and for 7 days after surgery. At least 3 weeks were allowed for recovery.

Animals were individually housed with a custom engineered flexible tether connected at one end to the commutator and at the other end to the animal’s cranial implant. Animals were maintained on a 24-hour light-dark cycle (LD 12:12) at 21 ± 2°C with ad lib available food and water. Light intensity averaged 35-40 lux at mid-level inside the cage. Relative humidity averaged 50% approximately. Animals were undisturbed for 48 hours before and after each treatment.

Amplified EEG 10,000-fold (band-pass filtered 1-30Hz: Grass Corp., Quincy, MA, USA) with an initial digitization rate of 400 Hz and amplified EMG (band-pass 10-100 Hz, RMS integration) were collected from the fixed electrodes. Concurrently, body temperature, locomotor activity, food and drink-related activity were recorded. SCORE2004™, an automated sleep-wake and physiological monitoring system was used to record and determine vigilance state, which is described and documented elsewhere (Van Gelder et al., 1991; Seidel et al., 1995; Olive et al., 1998; Phillips et al., 2012). Vigilance states were classified on-line as NREM sleep, REM sleep, wake, or theta-dominated wake every 10 seconds using EEG period and amplitude feature extraction and ranked membership algorithms. A fast Fourier transform was used to calculate the
spectral power of EEG in each 10 second epoch in 0.1 Hz bins. Offline, individually taught EEG-vigilance-state templates and EMG criteria differentiated states of arousal.

The post-treatment observation time was divided into 3 post-dosing periods. Time of dosing was defined as hour=0, and each hour includes measurements up to and not including the following hour. LY3020371 was dosed by the i.v. route and LY3027788.HCl was dosed by the oral route at 5 hr after the lights on period. Period 1 was defined as the first 7 hours post-dosing, denoted as hours 0 to 6, and during which the lights were on; period 2 was defined as the subsequent 12 hours, i.e., 7 to 18, during which the lights were off. The measured outcomes were summarized in each period by computing either the mean hourly, maximum hourly or the cumulative value across each period.

All outcomes were analyzed by a mixed-model repeated measures analysis of variance using treatment (drug dose) and treatment date as factors. Adjusted means and standard errors were summarized for each treatment group.

Antidepressant-Like Effects

Forced-Swim Assay. All animals were experimentally- and drug-naïve at the time of testing and were used for only one experiment.

Rats. Male Sprague-Dawley rats (250-275 g, from Harlan Sprague-Dawley) were received 7 days prior to testing. They were housed 4 rats per cage. Animals weighed about 300 g when tested. Animals were brought to the testing room at least 1 hr prior to testing. Rats were placed in clear plastic cylinders (diameter: 18 cm; height: 40 cm) filled with water (22-25°C) to a depth of 16 cm for 15 minutes. On the next day, the same procedure was followed in the presence of vehicle or drug (i.v.); immobility time of each rat was recorded for the first 5 minutes of the experiment and the rats were then removed from the chamber, dried, and warmed. A rat was regarded as immobile when floating motionless or making only those movements necessary to keep its head above the water. The data—immobility (sec)—was copied into a JMP data sheet, and analyzed by One-way ANOVA followed by post-hoc Dunnett’s tests if significant at p<0.05.

Mice. Male, NIH-Swiss mice (20-25g, Harlan Sprague-Dawley, Indianapolis, IN) were used. In other experiments, mice with receptor deletions were studied (mGlu2 -/-, see
below); these mice were bred by heterozygote x heterozogote breeding and used as littermates for -/- and +/+ mouse comparisons (Taconic Farms). Mice were placed in clear, plastic cylinders (diameter: 10 cm; height: 25 cm) filled to 6 cm with 22-25°C water for six minutes. Different groups of mice were pretreated with either vehicle or LY3020371 (i.p., 30 min prior to testing) or LY3027788.HCl (p.o., 60 min prior). The duration of immobility was recorded during the last 4 minutes of a 6-minute trial. A mouse was regarded as immobile when floating motionless or making only those movements necessary to keep its head above the water. Data were analyzed by post-hoc Dunnett’s test with alpha level set at 0.05. The amount of time spent immobile was measured. Means ± SEM were subjected to ANOVA followed by Dunnett’s test with p<.05 set as the error rate for statistical significance.

**mGlu2 -/- mice.** mGlu2 -/- mice were generated by homologous recombination as described in detail (Zhai et al., 2002; Linden et al, 2005) Separate mGlu2 and mGlu3 receptor knockout mouse strains were generated by homologous recombination as previously described for the mGlu8 receptor knockout mice (Zhai et al., 2002). Briefly, DNA fragments containing mouse mGlu2 and mGlu3 receptor coding regions were isolated from a 129SVJ genomic library. In both cases, an expression cassette of the neomycin resistance gene (Neo) was inserted into the third exon. Each targeting construct was injected into the R1 line of mouse embryonic stem cells. The homologous recombinants were confirmed by Southern hybridization analysis and these embryonic stem cells were injected into murine C57Bl/6 blastocysts. The resulting chimeric males were mated with ICR (CD-1) females. Male offspring carrying the null allele were backcrossed for three generations (N3) with ICR(CD-1) females. Heterozygous offspring were then interbred to obtain age-matched wildtype and receptor knockout mice with genotyping as previously described (Linden et al., 2005).

**Enhancement of quinpirole-induced locomotor activity.**

Sensitization within the dopamine pathways can be measured in a number of ways, one of which is to evaluate the increased sensitivity to the locomotor effects of dopaminergic agonists (Willner, 1997, D'Aquila et al., 2000). A mouse model has been disclosed that was used to evaluate the impact of antidepressant treatments on the behavioral effects of the dopamine D₃/2 agonist, quinpirole (Marsteller et al., 2009).
Male balbC mice were used in these studies. Locomotor activity was measured with a 20-station Photobeam Activity System (San Diego Instruments, San Diego, CA, USA) with seven photocells per station. Animals were weighed, injected with compound i.p., for LY3020371 and p.o. for LY3027788.HCl and returned to their home cages for 0.5 hr. They were then injected with quinpirole and placed back in the home cages for an additional 2 hrs after with they were placed in the locomotor activity boxes (40.6x20.3x15.2 cm). Locomotion was assessed for a 2 hr period post quinpirole dosing. Data were collected as total ambulation (where ambulation was defined as the sequential breaking of adjacent photobeams) in 10 min intervals for the entire period. The mean of the total ambulations for the 4th hr of the experimental session was summarized. ANOVA with post-hoc Dunnett’s test were used to evaluate the dose-response functions separately of drug alone or drug plus quinpirole. Student’s t-test was used to compare each drug dose alone vs. drug dose given with quinpirole. Ten mice were used per group and each animal was used only once.

**Drug combination studies.** Doses of fluoxetine HCl, citalopram HCl, and LY3027788.HCl were selected based upon prior dose-effect studies and are as follows: fluoxetine (10 mg/kg, i.p. 30 min); citalopram (0.3 mg/kg, i.p., 30 min); and LY3027788.HCl (10 mg/kg, p.o., 60 min prior). These doses were again shown in this study to be inactive (no statistically-significant difference compared to vehicle controls) when given alone when studied under the forced-swim assay in mice (methods as described above) and were used for drug combination experiments. Doses of each drug alone and the drug combination were evaluated by post-hoc Dunnett’s test with p<0.05 being assigned as statistically significant. For studies evaluating drug synergy, a synergy analysis was conducted using the method of Bliss Independence (Greco et al. 1995; Fitzgerald et al. 2006); using the endpoint of % inhibition, an ANOVA was applied to test the coefficient of the interaction term in a 2x2 full factorial model of the two compounds.

**Plasma and brain analysis under drug combination.** Three mice from each group were sacrificed immediately post experiment and plasma and brain were collected on ice and dry ice, respectively. Plasma and brain levels of each drug alone were
analyzed. Brain samples were weighed and a 3-fold volume of water/methanol (4:1, v/v) was added prior to homogenization with an ultrasonic tissue disrupter. Control (naïve) brain tissue was also homogenized to generate control homogenate for preparation of calibration standards. Stock solutions containing 1 mg/mL of each of the analytes were diluted to produce working solutions which were then used to fortify control plasma or control brain homogenate to produce calibration standards with concentrations ranging from 1 to 5000 ng/mL. Aliquots of each study sample, calibration standard, and control samples were then transferred to 96-well plates, mixed with acetonitrile and internal standard to precipitate sample proteins, centrifuged to pellet insoluble material, and the supernatant evaporated. Extracts were reconstituted in 0.5% heptafluorobutyric acid in water. Study samples were analyzed by LC-MS/MS using a Sciex API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS; Foster City, CA) equipped with a TurboIonSpray interface, and operated in positive ion mode. Citalopram and fluoxetine were chromatographically separated using a Betasil C18 Dash, 5µm 20x2.1mm (Thermo, Cat# 70105-022150). LY3020371 was chromatographically separated using a 2.1 mm x 50 mm, Atlantis T3 column (Waters, Milford, MA). The pumps were Shimadzu LC-10AD units with a SCL-10A controller (Kyoto, Japan), and a Gilson 215 liquid handler (Middleton, WI) or a Leap CTC liquid handler (LEAP Technologies, Carrboro, NC) was used as the autosampler. Water/1M NH₄HCO₃ (2000:10, v/v) (Mobile Phase A), and MeOH/1M NH₄HCO₃ (2000:10, v/v) (Mobile Phase B) was used for citalopram and fluoxetine and 0.2% formic acid in water (Mobile Phase A), and MeOH/Acetic Acid (3:1, v/v) was used for LY3020371. The selected reaction monitoring (SRM) (M+H)+ transition m/z were 360.1 > 159.1 (LY3020371), 325.2 > 262.2 (citalopram) and 310.1 > 148.1 (fluoxetine).

Prodrug Pharmacokinetics. Male CD-1 mice, male NIH Swiss mice, and male Sprague Dawley rats were employed (all from Harlan Industries, Indianapolis, IN). Animals had free access to food and water at all times, except overnight prior to dosing and 4 hours after dosing. LY3027788.HCl (Smith et al., 2012) was administered orally (p.o.) as a suspension in hydroxyl-ethyl cellulose (1%), Tween 80 (0.25%), and Dow antifoam (0.05%). LY3020371.HCl dissolved in saline was employed for studies involving
intravenous (i.v.) administration. For studies requiring assessment of pharmacokinetics over an extended period of time, blood was collected at 0.08, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h post i.v. administration and at 0, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h post oral administration, while in the mouse brain/plasma experiment, a single time point (1h) was used. Samples were centrifuged and the plasma was stored at -70 °C until analyzed. Cerebrospinal fluid was collected from the cisterna magna by syringe with up to 4 samples per animal and stored at -70 °C until analyzed.

Aliquots of thawed plasma were mixed with acetonitrile and an analog internal standard solution. Samples were centrifuged at 3200 rpm for 10 min and the supernatant evaporated. Extracts were reconstituted in 0.5 % heptafluorobutyric acid and analyzed by LCMS/MS using two Shimadzu LC-20AD pumps (Kyoto, Japan), a Leap PAL autosampler (Carrboro, NC), and a Sciex API 4000 (monkey and mouse samples) or API 5000 (rat samples) triple quadrupole mass spectrometer (Applied Biosystems/MDS; Foster City, CA) equipped with a TurboIonSpray interface, and operated in positive ion mode. Chromatographic separation was accomplished on a 2.1 mm x 50 mm, Atlantis T3 column (Waters, Milford, MA) using a binary mobile phase. The initial mobile phase system was composed of 0.2% formic acid in water (mobile phase A) and MeOH/Acetic Acid (3:1, v/v; mobile phase B). The selected reaction monitoring (M+H) transition was m/z 360.1 > 159.2 (API 4000) or 360.1 > 159.0 (API 5000). Pharmacokinetic parameters were calculated by non-compartmental analysis using Watson 7.4 (Thermo Fischer Scientific).
Results.

Pharmacodynamic Effects of LY3020371 in Comparison to Ketamine. Several studies were conducted in rats to compare effects of LY3020371 and ketamine in different brain areas.

Dopamine neuron activity in the ventral tegmental area (VTA) of rats. LY3020371 was characterized for its ability to modulate dopamine cell activity in the VTA and compared to effects of ketamine. Acute administration of LY3020371 (0.3 to 3 mg/kg, i.v.) significantly increased the number of spontaneously active dopamine cells in the VTA (Fig. 2A), an effect similar to ketamine (Fig. 2A; Witkin et al., 2016a). LY3020371 did not significantly affect the firing rate (Fig. 2B) or the percentage of spikes occurring in bursts (Fig. 2C). The AMPA receptor antagonist, NBQX (10 mg/kg, i.v.), prevented the LY3020371-induced increase in the number of spontaneously active VTA dopamine cells (p<0.05), without producing any effects on its own (p>.05) (Fig. 2A).

Effects on Brain Oxygen. These experiments examined the changes in brain oxygen in two brain areas induced by LY3020371 or ketamine. LY3020371 (i.p.) produced dose-dependent increases in tissue oxygen in the anterior cingulate cortex (ACC) (Fig. 3) but not in the striatum (STR) (Fig. 3). The dose of ketamine tested also robustly increased tissue oxygen levels in both regions.

Effects on neurochemical efflux in the rat medial prefrontal cortex. In vivo microdialysis studies were undertaken in rats to investigate the sensitivity of neurotransmitter systems to modulation by ketamine and to LY3020371 in order to explore neurochemical commonalities in their actions. (+)-Ketamine (10 mg/kg, s.c.) significantly increased the efflux of multiple neurochemicals with time courses and overall response profile (Area Under the Curve, AUC) for the 3 hr post dosing as shown in Fig. 4.

The time course for effects and the overall response profile (AUC) for the 3 hr post-dosing period for LY3020371 on efflux of brain neurochemicals are shown in figure 5. LY3020371 (10 mg/kg, i.p.) produced a significant increase in the mPFCx compared to vehicle controls for the neurochemicals shown. Other analytes, including glycine and
glutamine, failed to change significantly after drug administration (not shown). Measurement of LY3020371 in the same microdialysate samples revealed an average concentration of approximately 35 nM at one hr after administration (Fig. 5). Recovery across the probe was not calculated, so the true extracellular concentration can only be approximated. Based on a 10-20% recovery, which may be expected for this particular flow-rate and size of molecule, this value would be 10-20 times the dialysate concentration (i.e. 350-700 nM).

Wake-promotion. LY3020371 was characterized using the SCORE-2000™ bioassay system that can detect wake-promoting effects via EEG electrodes. Effects were compared to those observed with ketamine. Of n = 115 animal-dosings, n = 113 yielded usable data. The average age at the time of dosing was 148 days and the average weight was 517 g. Ketamine (10 mg/kg, s.c.) produced increases in wake-promotion of rats followed by a rebound hypersomnolence starting at about hr 16 (Fig. 6). LY3020371 increased cumulative wake time of rats in a dose- and time-dependent manner without rebound hypersomnolence with significant increases being observed at the lowest dose tested of 1 mg/kg, i.v. (Fig. 6).

Antidepressant-Like Effects in SSRI-Sensitive and Insensitive Models: Effects Comparable to Those of Ketamine

Forced-swim assay. Both ketamine and LY3020371 produced an antidepressant-like behavioral signature in rats, significantly decreasing immobility time, with a minimaleffective dose of 17 mg/kg for each compound when dosed i.p. (28.6 % decrease for ketamine; 19.2% decrease for LY3020371). Imipramine, given as a positive control, was as efficacious at 15 mg/kg, i.p. (21.7% decrease).

When dosed intravenously, the potency of LY3020371 but not ketamine increased with doses as low as 0.3 mg/kg being active in the forced-swim assay in rats (Fig. 7). The plasma concentration of ketamine at 10 mg/kg = 200 ng/mL, which is equal to plasma exposures associated with clinical efficacy in depressed patients (Zarate et al., 2012).
The dependence of the effects of i.v. ketamine or LY3020371 on mGlu2/3 receptors was evaluated in the presence of the mGlu2/3 receptor agonist LY354740 (3 mg/kg, i.p.). Under these conditions, the mGlu2/3 receptor agonist fully prevented the antidepressant-like effects of ketamine and of (Fig. 7). LY354740 (3 mg/kg, i.p.) did not significantly affect immobility in rats when given alone (272 ± 16 sec, p>0.05 compared to vehicle control). In order to further evaluate the role of mGlu2/3 receptors in the antidepressant-like effects of LY3020371, effects of LY3020371.HCl were tested in mGlu2 receptor -/- mice. In this study, the antidepressant phenotype was produced in mGlu +/+ mice but not in mGlu2 -/- mice (Fig. 7, bottom panel).

Effects of ketamine in the forced-swim assay have previously been shown to be dependent upon AMPA receptors (c.f., Maeng at al., 2008; Witkin et al., 2016a). In the present study, we now document a comparable dependence upon AMPA receptors of the antidepressant-like effects of LY3020371. Under these conditions, NBQX (10 mg/kg) was able to completely prevent the antidepressant-like effects of LY3020371 without significant effects on its own (Fig. 7). Given alone, NBQX produced immobility times of 275 ±16 sec (p>0.05 compared to vehicle control).

The risk of tolerance development to the antidepressant-like effects of LY3020371 was evaluated by dosing LY30200371 or vehicle for 4 consecutive days, and then testing on day 5 with LY3020371 (i.v.). In this study, 4 days of prior treatment with LY3020371 did not significantly alter the anti-immobility effects of LY3020371 when tested on day 5. The percent decrease in immobility at 1 mg/kg was 84.8% (4-day vehicle-treated) vs 87.9% (4-day LY3020371-treated) and 78.3% (vs 4-day vehicle-treated) vs 81.2% (4-day LY3020371-treated).

The potential impact of a priori exposure to other antidepressant medications like the selective serotonin uptake inhibitors (SSRIs) on the antidepressant-like effects of LY3020371 was tested in rats. In this study, rats were dosed for 14 consecutive days with either drug vehicle or citalopram (10 mg/kg) and then tested with LY3020371 two days later. Both 1 (84.8 vs. 83.9% decrease for vehicle-treated vs. citalopram-treated rats, respectively) and 3 mg/kg (78.3 vs. 81.4% decrease for vehicle-treated vs. citalopram-treated rats, respectively) were active in the rat forced-swim assay after 14
days of prior exposure to citalopram, the same as was observed after 14 d dosing with vehicle.

Time-course experiments were conducted in rats in the forced-swim assay from 0.5 to 24 hrs post i.v. dosing at 1, 3, and 10 mg/kg. Both the maximal effect of LY3020371 and the duration of action were increased as a function of dose (Fig. 8).

**Relationship of LY3020371 CSF drug levels to antidepressant-like effects.** CSF concentrations of LY3020371 were collected in a group of rats exposed to 1, 3, or 10 mg/kg, i.v. at times from 0.5 to 24 h post dosing (Witkin et al., 2016a). These were previously shown to account for 3-6% of plasma drug concentrations. CSF concentrations of drug were a function of both dose and time (Fig. 9, bottom left). With all doses tested, LY3020371 (i.v.) produced CSF concentrations at some time points that were above the IC$_{50}$ for functional antagonism in rat hippocampal slices (Witkin et al., 2016a). Antidepressant-like effects (reduction in immobility time in the forced-swim assay) of LY3020371, i.v., were linearly and positively associated with CSF drug levels (Fig. 9, bottom right).

**Metabolomic analysis.** Based upon the dose-effect functions defining potency and efficacy of ketamine and LY3020371 (i.p.), we selected doses of 10 mg/kg as producing effects most comparable to one another. Under these conditions, ketamine significantly decreased immobility time whereas LY3020371 just missed statistical significance. We then used these doses to compare the metabolomic profile of ketamine and LY3020371. We detected more than 240 polar metabolites, excluding peptides and intact lipids. Of these, several analytes displayed significant differentiation from those detected in vehicle-treated animals in both CSF and in hippocampus (Table 1). In hippocampal tissue, both ketamine and LY3020371 significantly altered levels of pyruvic acid and of hydroxylamine relative to vehicle-treated rats; methylnicotinamide was positively altered in both hippocampus and the CSF of rats treated with LY3020371 (Table 1).

Utilizing pathway analysis of the analytes detected, predicted metabolic changes in hippocampus were uncovered for the ketamine-(Fig. 10) and LY3020371-(Fig. 11) treated rats that both overlapped and were distinct from one another. Specifically, predicted activation or upregulation of the following analytes were detected for both ketamine and LY3020371: ADORA1, GRIA2, MAP2, prostaglandin D2, and
phosphocreatine. Pathway analysis predicted inhibition or downregulation of the following analytes for both ketamine and for LY3020371: kynurenic acid, glycine, NPPA, SLC1A1, and EPO.

**Plasma pharmacokinetics following oral administration of prodrug LY3027788.HCl.** Oral administration of diester prodrug LY3027788.HCl (Fig. 1) led to the rapid and dose-proportionate appearance of the pharmacologically active species LY3020371 in plasma of both mouse (Fig. 12A, Table 2) and rat (Fig. 12B, Table 3). No detectable plasma levels of either diester LY3027788 or individual monoesters were observed. In a separate study (Fig. 13C), oral bioavailability of LY3020371 in rats was determined by comparing plasma exposures of LY3020371 following administration of prodrug LY3027788.HCl (8.74 mg/kg, p.o., a dose equivalent to 5 mg/kg of LY3020371) with those achieved via i.v. administration of LY3020371.HCl (1.1 mg/kg, a dose equivalent to 1 mg/kg LY3020371) (Table 4). From this study, the bioavailability of LY3027788 was calculated to be 42.9 ± 12%. Plasma and CSF concentrations of LY3020371 following single oral doses of LY3027788.HCl are shown in figure 13D.

**CSF levels of LY3020371 following oral administration of prodrug LY3027788.HCl in rat.** Mean plasma and cerebrospinal fluid (CSF) levels of LY3020371 were determined following oral administration of LY3027788.HCl (10 and 60 mg/kg). CSF levels of LY3020371 were apparent at the first assessment (1 h), peaked at 2 h and persisted through the full (24 h) course of the experiment (Fig. 12D, Table 5). Mean concentrations of LY3020371 in CSF significantly lower than those observed in plasma at each assessed time point (CSF/ plasma ratios based on mean AUC were 5.4% (10 mg/kg) and 3.7% (60 mg/kg) while CSF/ plasma ratios based on C_{max} were 1.4% (10 mg/kg) and 1.8% (60 mg/kg). The CSF/ plasma ratio appeared to increase over time (from 0.7% to 230% at 10 mg/kg dose, 1.3% to 13% at 60 mg/kg dose), suggesting that clearance of LY3020371 from the central compartment is slow compared to that from plasma. These results are similar to previously disclosed findings for rat CSF/ plasma pharmacokinetics following i.v. dosing of LY3020371 (Witkin et al., 2016) as well as to those associated with mGlu2/3 receptor agonists possessing similar physiochemical characteristics (Monn et al. 2015a,b).
Brain levels of LY3020371 following oral administration of prodrug LY3027788.HCl in mouse. Mean brain and plasma levels of LY3020371 were determined in the mouse 1 h following oral dosing of LY3027788.HCl (5.6, 17.6 and 29.9 mg/kg, Table 6). Similar to CSF/plasma ratios found in the rat, the brain/plasma ratios in the mouse were determined to be 3.7% and 2.3% following oral doses of 17.6 and 29.9 mg/kg, respectively. Brain levels of LY3020371 in the mouse following an oral dose of 5.6 mg/kg of LY3027788.HCl were below levels of detection (14 nM).

Behavioral effects of the oral prodrug, LY3027788.HCl. In the mouse forced-swim assay, LY3027788.HCl was potent and efficacious with a minimal effective dose of 16 mg/kg, p.o. (equivalent to 10 mg/kg active moiety LY3020371). (Fig. 13A). The ED_{60} in this assay was 8.2 mg/kg (equal to 4.7 mg/kg of active moiety LY3020371). In the locomotor activity assay in mice, a single dose of LY3027788.HCl (16 mg/kg, p.o.) enhanced the locomotor stimulant effects of quinpirole (Fig. 13B). Further, as with LY3020371, oral administration of the prodrug form, LY3027788.HCl, dose-dependently increased the wake time of rats without engendering rebound hypersomnolence (Fig. 13C). The amount of time spent awake compared to vehicle control that was induced by single oral doses of LY3027788.HCl (10 mg/kg, 20 mg/kg, 30 mg/kg) was 40 ± 16 min, 44 ± 13 min, and 106 ± 8 min, respectively.

Drug combination studies. Individual doses of fluoxetine, citalopram, and LY3027788.HCl were selected based on their lack of effect when given alone in the forced-swim assay in mice. Fluoxetine (10 mg/kg, i.p. 30 min prior), citalopram (0.3 mg/kg, i.p., 30 min prior) and LY3027788.HCl (10 mg/kg, p.o., 60 min prior) were without effect when given alone also in the present study (Fig. 14, top panels). However, when given in combination with LY3027788.HCl, citalopram produced antidepressant-like effects in this assay that were greater than those produced by either drug alone (Fig. 14). In contrast, the effects of the fluoxetine/LY3027788 combination was additive, having just missed statistical significance for synergy. Under these conditions, giving both compounds in combination did not alter the plasma or brain levels...
of either drug alone (p<0.05). Concentrations of the active/parent moiety LY3020371 after oral dosing of LY3027788.HCl with or without fluoxetine were 2992.8 ± 347.2 (alone) and 3963.7 ± 1036.6 nM (drug combination) for plasma and 63.6 ± 6.4 (alone) and 70.4 ± 7.0 (drug combination) for brain. Concentrations of the active/parent moiety LY3020371 after oral dosing of LY3027788.HCl with or without citalopram were 3294.2 ± 418.6 (alone) and 3043.4 ± 558.6 nM (drug combination) for plasma and 173.1 ± 96.9 (alone) and 188.1 ± 45.4 nM (drug combination) for brain. The concentrations of fluoxetine were 2675.9 ± 220.8 (alone) and 2394.5 ± 1170.6 nM (drug combination) for plasma and 37659 ± 5579 (alone) and 33718 ± 14203 nM (drug combination) for brain. The concentrations of citalopram were 22.6 ± 10.1 (alone) and 29.3 ± 3.8 nM (drug combination) for plasma and 499.3 ± 236.6 (alone) and 704.5 ± 132.0 nM (drug combination) for brain.
Discussion

A new orthosteric antagonist of mGlu2/3 receptors, LY3020371, was recently reported (Smith et al., 2012) and characterized as a potent and selective orthosteric antagonist in vitro (Witkin et al., 2016b). The pharmacology of LY3020371 from human clonal systems to native tissue functional preparations along with its pharmacokinetic properties suggested that LY3020371 was a well-behaved orthosteric mGlu2/3 receptor antagonist that would block mGlu2/3 receptors in vivo upon systemic dosing (Witkin et al., 2016b). In the present report we document the in vivo effects of LY3020371 and its diester prodrug, LY3027788 (Smith et al., 2012) that predict efficacy in treatment-resistant depression (TRD). The basis for this therapeutic prediction comes from the convergent biological effects of LY3020371 and that of ketamine, an NMDA receptor antagonist that produces rapid and persistent relief of depression symptoms in TRD patients (Zarate et al., 2006; Abdullah et al., 2015). Prior studies have also suggested the efficacy of mGlu2/3 receptor antagonists in depression (Chaki et al., 2004; Witkin and Eiler, 2006; Witkin et al., 2016), and two mGlu2/3 antagonists have entered clinical development: BCI-838 (Yasuhara, 2006; Nakamura, 2006), an oral prodrug of orthosteric antagonist BCI-632 (aka: MGS0039; Nakazato, 2004; Yoshimizu, 2006) and RO4995819 (aka: RG1538, Decoglurant), an mGlu2/3 negative allosteric modulator (Gatti, 2006; clinicaltrials.gov, study NCT01457677).

In the present report, we first documented the common pharmacodynamic effects of ketamine and LY3020371. Both molecules increased the probability of dopamine cell firing in the VTA (see also Witkin et al., 2016a), a brain area known to regulate hedonic valuation and regulate a neurotransmitter system well characterized for its control of mood (Price and Drevets, 2010). This enhancement of dopamine neurotransmission was also observed at the neurochemical level where extracellular levels of dopamine were markedly enhanced in the medial prefrontal cortex (mPFC) of rats with both LY3020371 and ketamine. In behavioral studies, LY3027788.HCl provided immediate enhancement of the functional effects of dopamine as measured in the augmentation of the locomotor stimulant effects of quinpirole, an effect that also occurs with ketamine but not citalopram (Witkin et al., 2016a). LY3020371 also increased oxygen levels in the anterior cingulate cortex (ACC), an effect also observed with ketamine. The ACC is a
brain area controlling mood and is known to be modulated by ketamine in patients (Lally et al., 2015). In addition to dopamine, multiple and overlapping neurotransmitters were enhanced by both LY3020371 and ketamine in the mPFC of behaving rats including the wake- and cognition-regulating monoamines, histamine, and acetylcholine.

LY3020371 was also wake-promoting in rats, an effect reported earlier for the mGlu2/3 receptor antagonist LY341495 (Feinberg et al., 2005). Although ketamine also engenders wake promotion, the striking feature of the effects of blockade of mGlu2/3 receptors is that there is no rebound NREM hypersomnolence.

In rodent models that detect conventional antidepressant drugs such as the forced-swim assay (Cryan et al., 2002), we demonstrated potent and efficacious antidepressant-like effects of LY3020371 like that of ketamine in both rats and in mice. These behavioral effects of LY3020371 were prevented by an mGlu2/3 receptor agonist and were absent in mGlu2-/- mice, attesting to the on-target actions of this molecule. We demonstrated too that the antidepressant-related signature of LY3020371 was positively associated with drug levels in the CSF of rats and that these drug levels were above the IC\textsubscript{50} value for inhibition reported in rat brain tissue preparations (~40 nM) (Witkin et al., 2016a).

One of the compelling features of ketamine as an antidepressant is its sustained effects (Zarate et al., 2006). The question arises in the current context as to whether mGlu2/3 receptor antagonists also produce effects that outlast their kinetics. Our laboratory has not been able to achieve effects even 24 hr post dosing with either ketamine or the mGlu2/3 receptor antagonist LY341495. Other groups have observed longer lasting effects of ketamine and mGlu2/3 receptor antagonists (see Pilc et al., 2013; Dong et al., 2016). However, effects that last beyond a day are not universally observed (Popik et al., 2008; Pilc et al., 2013). We did not evaluate the effects of LY3020371 for this effect. Recently, a metabolite of ketamine was reported to be active and to engender sustained activity (Zanos et al., 2016).

As with ketamine (Karasawa et al., 2005; Maeng et al., 2008; Fukumoto et al., 2016), we showed that the antidepressant-like efficacy of LY3020371 was prevented by blockade of AMPA receptors. AMPA receptor dependence has been reported for other mGlu2/3 receptor antagonists (Gleason et al., 2013; Fukumoto et al., 2016; Witkin et al.,
2016) and has been suggested to be (Alt et al., 2006) and remains (Abdullah et al., 2015) a leading hypothesized transducer of antidepressant efficacy. We independently confirmed this idea with the metabolomics profiling of ketamine and LY3020371. Doses of these molecules (10 mg/kg, i.p.) that were close to or at the dose level producing antidepressant-like effects in rats produced a series of hippocampal analyte changes that predicted enhanced AMPA receptor signaling. These are the first data to compare ketamine and mGlu2/3 receptor antagonism at the metabolomic level and provide additional evidence supporting a critical role for AMPA receptor signaling in the antidepressant responses produced by these agents in rodents as well as additional reason to believe that mGlu2/3 antagonists might produce ketamine-like antidepressant efficacy in patients.

Another protein predicted to be amplified by both ketamine and by LY3020371 from pathway analysis of the metabolomics data was ADORA1 (Adenosine A₁). Adenosine A₁ receptors have previously been shown to have a likely role in driving antidepressant efficacy (see Ortiz et al., 2015; Serchov et al., 2015). The present findings therefore suggest that A₁ receptor activation might be another important downstream pathway in the actions of rapidly-acting antidepressants. Of interest in this regard is a recent report that the potent non-competitive NMDA receptor antagonist (+)-MK-801 might induce antidepressant-like effects in zebra fish through A₁ receptors (da Silva et al., 2015). It is noteworthy that the metabolomics analysis did not uncover mTOR as a potential target for either ketamine or LY3020371 despite reports of mTOR involvement (Li et al., 2010; Dwyer et al., 2012) and the blockade of effects of ketamine and LY3020371 reported here (figure 7). A recent paper has outlined difficulties in recapitulating the data on mTOR expression by ketamine (Popp et al., 2016).

LY3020371 is a highly polar amino diacid with restricted intestinal absorption resulting in poor oral bioavailability in rats (F < 10%, data not shown). This limitation has been overcome with LY3027788.HCl, a diester form of LY3020371 (Smith et al., 2012) that demonstrates rapid absorption and bioconversion to LY3020371 following oral dosing in rodents. Oral doses at or above 10 mg/kg LY3027788.HCl in rats generated CSF levels of LY3020371 that either reached or exceeded the measured antagonist potency for this compound in the rat hippocampal slice (IC₅₀ = 46 nM, Witkin
et al, 2016) and doses at or above 10 mg/kg enhanced wakefulness in rats. Similarly, in mice, functional mGlu2/3 antagonist-associated brain levels of LY3020371 (i.e. concentrations at or above 46 nM) measured 1 h after single oral doses of LY3027788.HCl were attained following oral doses at or above 17.6 mg/kg while statistically significant responses in both the mouse forced swim and quinpirole locomotor sensitization assays were achieved with a oral doses of LY3027788.HCl at or above 16 mg/kg. These results relating central drug concentrations with behavioral responses are consistent with those previously noted for LY3020371 when administered by the i.v. route.

In summary, we have shown here that LY3020371 and oral prodrug LY3027788.HCl engender a host of antidepressant-relevant biological effects that are in common with the known efficacious drug for TRD patients, ketamine, and that these effects can be predicted by drug concentrations measured in the central compartment. Multiple additional commonalities in biological activities have been found between ketamine and other mGlu2/3 receptor antagonists (e.g., Dwyer et al., 2012, 2013; Dong et al., 2016; Fukumoto et al., 2016 Witkin et al., 2016) providing an overall consistent profile across laboratories, biological readouts, and molecules. Finally, while antidepressant efficacy for LY3020371 and its oral prodrug have been demonstrated, evidence that mGlu2/3 receptor antagonism might overcome the side effects that characterize ketamine has not been addressed. A direct comparison of potentially limiting side effects engendered by these two mechanisms will be the subject of a future disclosure to be communicated in due course.
Author Contributions

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Wrote or contributed to the writing of the manuscript: All authors
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**Figure Captions**

**Figure 1.** Structures of LY3020371 and an orally-biovailable prodrug LY3027788.

**Figure 2.** LY3020371 and ketamine (17 mg/kg, i.v.) increased the number of actively firing dopamine neurons in the ventral tegmental area of anesthetized rats (Panel A) [F(3,15) = 6.26, p=0.005]. The effect of LY3020371 is significantly attenuated by the AMPA receptor antagonist NBQX (10 mg/kg, i.p.) (Panel A). Neither LY3020371 or ketamine affected the firing rate (Panel B) or the percentage of spikes occurring in bursts (Panel C). Each bar represents the mean (+SEM) of n = 6 rats.

**Figure 3.** Both ketamine and LY3020371 dose-dependently increase tissue oxygen in the anterior cingulate cortex of rats after i.p. dosing. In contrast ketamine but not LY3020371 enhance tissue oxygen in the dorsal striatum of rats. Each point represents mean (± S.E.M.) data from 16 (cingulate) or 17 rats.(striatum). Analysis of the AUC revealed a significant effect of treatment (ACC: F(4,40)=12.79, p <.001; STR: F(4,64) = 15.02, p <.001), and post-hoc comparisons showed that the 10 mg/kg LY3020371 group was significantly different from vehicle in the ACC (p=0.0007), while the 10 mg/kg ketamine group was significantly different from vehicle in both the ACC (p=0.0001) and STR (p=0.0001). * p<.05 **p<.01, ***p<.001 in comparison to the vehicle treatment group.

**Figure 4.** Increases in monoamine efflux in the medial prefrontal cortex of freely-moving rats by S- (+)-ketamine (10 mg/kg, s.c). Each point represents mean (± S.E.M.) data from 6 rats. Data were analyzed by ANOVA; * p<0.05; ** p<0.01; ***p<0.001.

**Figure 5.** Increases in monoamine efflux in the medial prefrontal cortex of freely-moving rats by LY3020371 as a function of time post injection (10 mg/kg, i.p.). Each point represents mean (± S.E.M.) vehicle n = 6, LY3020371 n = 7. Open symbols: LY3020371; filled symbols: vehicle. For all analyses, (F1,11), increases in extracellular levels of the following neurochemicals were significantly different than vehicle control values: 5-HT (F = 14.5; p<.01), 5-HIAA (F = 42.5; p<.0001), DA (F = 63.5; p<.0001), DOPAC (F = 93.1; p<.0001), HVA (F = 73.3; p<.0001), NA (F = 70.5; p<.0001), MHPG (F = 85.3;
p<.0001), DHPG (F = 20.1; p<.001), ACh (F = 54.1; p<.0001), histamine (F = 17.2; p<.01), TMH (F = 20.8; p<.001), GABA (F = 67.9; p<.0001), and glutamate (F = 13.5; p<.01).

**Figure 6.** Both ketamine and LY3020371 increase the time spent awake in freely-moving rats. Each point represents mean (± S.E.M.) data from 8-12 rats. (a). Increases in accumulated time awake over baseline (min) after S-(+)-ketamine (3, 10, and 30 mg/kg, i.p., designated as green, blue, and red lines, respectively). (b). Increases in accumulated time awake over baseline (min) after LY3020371 (1, 3, 10, and 30 mg/kg, i.v., designated as green, blue, and red lines, respectively). (c). Area under the curve for accumulated wake over baseline for S-(+)-ketamine. (d). Area under the curve for accumulated wake over baseline for LY3020371. (e). Changes in REM and non-REM sleep at 7-19 hr for S-(+)-ketamine. (f). Changes in REM and non-REM sleep at 7-19 hr for LY3020371. *p<0.05; **p<0.01; ***p<0.001.

**Figure 7.** Upper Panels: Intravenous ketamine and LY3020371 decrease the time rats are immobile in the forced-swim test in the rat forced-swim assay. Ketamine: F (3, 28) = 14.82, p<0.0001; LY3020371: F (5, 38) = 12.38, p<0.0001. For the dose-response curve, ketamine and LY3020371 were given i.v., 30 min prior to testing. The mGlu2/3 receptor agonist, LY354740 (3 mg/kg, i.p.). The AMPA receptor antagonist NBQX (10 mg/kg, i.p., 60 min prior) attenuated the effects of LY3020371. For the drug combination studies, ketamine was dosed 60 min prior and LY3020371 was given 120 min prior to testing. Each point represents the mean ± SEM of 6-8 rats. V: vehicle; I: Imipramine (30 mg/kg, ip). Open symbols above dose effect curves are molecule + agonist or antagonist. *p<0.05 compared to vehicle control values by post-hoc Dunnett’s test.

Lower Panels: Attenuation of the antidepressant-like effects of ketamine and LY3020371 by an inhibitor of mTOR, AZD8055 in the mouse forced-swim assay. Left: The mTor inhibitor AZD8055 was dosed at 10 mg/kg, p.o., 60 min prior to testing. Ketamine and LY3020371 were given i.p., 30 min prior to testing. Each bar represents the mean SEM results from 7-8 mice*p<0.05 compared to veh (Dunnett's test); $p<0.05 compared to ketamine alone (t- test) Right: The anidepessant-like effects of LY3020371 (10 mg/kg, i.p.) in wild-type mice (WT) are absent in mGlu2/- mouse (KO). Each bar represents the mean + SEM of 8 mice. *p<0.05 compared to WT-veh (Dunnett's test).

**Figure 8.** Time-course of action of LY3020371 in rats under the forced-swim test after intravenous dosing. 1 mg/kg: F (5, 32) = 4.836, p<0.01; 3 mg/kg: F (4, 27) = 4.334, p<0.01; 10 mg/kg: F (5, 32) =
10.42, p<0.0001. Each point represents the mean ± SEM of 6-8 rats. *p<0.05 compared to vehicle control values. Each bar represents the mean ± SEM of 6 rats. *p<0.05 compared to vehicle control values by post-hoc Dunnett’s test.

**Figure 9.** Cerebrospinal fluid concentrations predict efficacy in rat forced-swim assay (R²=0.85, p<0.05) (lower right panel). The numbers adjacent to data points (lower left panel) are the percent decrease in immobility in the forced swim assay. The hashed line (lower left panel) represents the IC₅₀ value for LY3020371 in rat hippocampal slice preparation (IC₅₀ = 46 nM, Witkin et al., 2016a).

**Figure 10.** Metabolomics analysis of cerebrospinal fluid samples from rats after 10 mg/kg, i.p. ketamine.

**Figure 11.** Metabolomics analysis of cerebrospinal fluid samples from rats after 10 mg/kg, i.p. LY3020371.

**Figure 12.** Pharmacokinetics of LY3020371 following oral dosing of diester prodrug LY3027788.HCl in mouse and rat. **A.** Plasma exposures of LY3020371 in the mouse following single oral doses of LY3027788.HCl (■ 4.8 mg/kg, □ 16 mg/kg, ● 27 mg/kg). **B.** Plasma exposures of LY3020371 in the rat following single oral doses of LY3027788.HCl (■ 3 mg/kg, □ 10 mg/kg, ● 30 mg/kg). **C.** Plasma exposures of LY3020371 following either a single 1.1. mg/kg i.v. dose of LY3020371 (■) or 8.74 mg/kg oral dose of LY3027788.HCl (□). **D.** Plasma and CSF concentrations of LY3020371 following single oral doses of LY3027788.HCl. ▲ 10 mg/kg, Plasma; △ 60 mg/kg, Plasma; ■ 10 mg/kg, CSF, □ 60 mg/kg, CSF). Data points are means ± S.D. of 4-12 animals/time point.

**Figure 13.** Some behavioral effects of an oral prodrug form of LY302071, LY3027788.HCl when given orally to rodents. **A.** Antidepressant-like efficacy in the mouse forced-swim assay. Each bar represents the mean ± SEM of 7-8 mice. F (3, 27) = 13.21, p<0.0001. *p<0.05 compared to vehicle control values by post-hoc Dunnett’s test. **B.** Enhancement of the locomotor stimulant effects of quinpirole in mice after oral administration of LY3027788.HCl. F (2, 54) = 21.31, p<0.0001 (dose); F (1, 54) = 18.24, p<0.0001 (treatment, quinpirole + or -); F (2, 54) = 10.60, p<0.0001 (dose x treatment interaction). Each bar represents the mean ± SEM of 9-10 mice. *p<0.05 compared to vehicle control values, # compared to
quinpirole alone, compared to same dose without quinpirole. C. Wake-promoting effects of ly3027788.HCl in freely-moving rats. Each point represents the means of 8-12 SEM rats.

Figure 14. Top Panels. LY3027788.HCl enhances the antidepressant-like effects of fluoxetine and of citalopram without altering plasma or brain levels of either drug alone. Each bar represents the mean ± SEM of 6-8 mice. Veh: vehicle; Fluox: Fluoxetine (10 mg/kg, i.p. 30 min); Cital: citalopram (0.3 mg/kg, i.p., 30 min); LY: LY3027788.HCl (10 mg/kg, p.o., 60 min prior). *p<0.05 compared to vehicle control values by post-hoc Dunnett’s test. By synergy analysis, only the drug combination of LY3027788 and citalopram was significant (see Methods for statistical methods).
**Table 1.** Analytes differentiating ketamine from LY3020371. Analytes are from hippocampus or cerebral spinal fluid from rats treated with either ketamine or LY3020371 at 10 mg/kg, i.p.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Ketamine - CSF</th>
<th>LY3020371 - CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolite</td>
<td>FC</td>
<td>p.value</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2.39</td>
<td>0.074858</td>
</tr>
<tr>
<td>nicotinamide</td>
<td>2.13</td>
<td>0.000967</td>
</tr>
<tr>
<td>2-deoxy-Erythro-Pentonic acid</td>
<td>1.72</td>
<td>0.022355</td>
</tr>
<tr>
<td>cyclic-AMP</td>
<td>1.51</td>
<td>0.053485</td>
</tr>
<tr>
<td>choline</td>
<td>1.5</td>
<td>3.97E-07</td>
</tr>
<tr>
<td>Phosphocreatine</td>
<td>1.23</td>
<td>0.018469</td>
</tr>
<tr>
<td>succinate</td>
<td>1.22</td>
<td>0.016922</td>
</tr>
<tr>
<td>Methylcysteine</td>
<td>1.21</td>
<td>0.001067</td>
</tr>
<tr>
<td>thiamine-phosphate</td>
<td>1.24</td>
<td>0.006864</td>
</tr>
<tr>
<td>dimethylglycine</td>
<td>1.24</td>
<td>0.088157</td>
</tr>
<tr>
<td>hydroxyproline</td>
<td>1.24</td>
<td>0.026733</td>
</tr>
<tr>
<td>hydroxyphenylpyruvate</td>
<td>1.28</td>
<td>0.002977</td>
</tr>
<tr>
<td>Hydroxyisocaproic acid</td>
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<td>0.015478</td>
</tr>
<tr>
<td>betaine</td>
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<td>0.059023</td>
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<tr>
<td>Glu-Gln</td>
<td>1.42</td>
<td>0.017724</td>
</tr>
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<td>Aminomethylpyrimidine</td>
<td>1.45</td>
<td>0.052945</td>
</tr>
<tr>
<td>t-MIAA</td>
<td>1.48</td>
<td>0.028341</td>
</tr>
<tr>
<td>Betaine</td>
<td>1.54</td>
<td>0.078793</td>
</tr>
<tr>
<td>Argininc acid</td>
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<td>0.035704</td>
</tr>
<tr>
<td>3,4-dihydroxybutanoic acid</td>
<td>1.68</td>
<td>0.094858</td>
</tr>
<tr>
<td>Proline betaine</td>
<td>1.78</td>
<td>0.056244</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>1.99</td>
<td>0.045313</td>
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</table>
| 3,4,5-Trihydroxypentanoic acid | 2.37 | 0.024139 | Glu-Gln: γ-Glutamylglutamate dipeptide; t-MIAA: tele-methylimidazolateacetic acid; PE (16:0): 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine. Pink highlighting indicates increased metabolite levels. Blue highlighting indicates decreased metabolite levels.
Table 2. Mean plasma pharmacokinetics of LY3020371 following single oral doses of diester prodrug LY3027788.HCl in male CD-1 mice

<table>
<thead>
<tr>
<th>LY3027788.HCl dose</th>
<th>mg/kg, p.o.</th>
<th>4.8</th>
<th>16</th>
<th>27</th>
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</thead>
<tbody>
<tr>
<td>LY3020371-equivalent dose</td>
<td>mg/kg, p.o.</td>
<td>2.7</td>
<td>9.1</td>
<td>15.4</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (nM)</td>
<td>1990</td>
<td>10800</td>
<td>15500</td>
<td></td>
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<tr>
<td>LY3020371 Mean Plasma</td>
<td>Tmax (h)</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Pharmacokinetic Parameters</td>
<td>AUC&lt;sub&gt;0-24&lt;/sub&gt; (nM*h)</td>
<td>2830</td>
<td>10900</td>
<td>17100</td>
</tr>
<tr>
<td></td>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>1.6</td>
<td>1.3</td>
<td>1.8</td>
</tr>
</tbody>
</table>
Table 3. Mean plasma pharmacokinetics of LY3020371 following single oral doses of diester prodrug LY3027788.HCl in male SD rats

<table>
<thead>
<tr>
<th>LY3027788.HCl dose</th>
<th>mg/kg, p.o.</th>
<th>3</th>
<th>10</th>
<th>30</th>
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<tr>
<td>LY3020371-equivalent dose</td>
<td>mg/kg, p.o.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (nM)</td>
<td>1540</td>
<td>5590</td>
<td>9160</td>
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<tr>
<td>Mean Rat Plasma Pharmacokinetic Parameters</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>0.5</td>
<td>0.8</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-24&lt;/sub&gt; (nM*h)</td>
<td>4420</td>
<td>14700</td>
<td>30900</td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>1.5</td>
<td>2.9</td>
<td>5.4</td>
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Table 4. Bioavailability of LY3020371 following oral dosing of prodrug LY3027788.HCl. Mean plasma levels of LY3020371 were determined following a single i.v. dose of LY3020371.HCl and single oral dose of diester prodrug LY3027788.HCl in male SD rats.

<table>
<thead>
<tr>
<th>Compound administered (dose, route)</th>
<th>LY3020371.HCl (1.1 mg/kg, i.v.)</th>
<th>LY3027788.HCl (8.74 mg/kg, p.o.)</th>
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</thead>
<tbody>
<tr>
<td>LY3020371-equivalent dose</td>
<td>1 mg/kg</td>
<td>5 mg/kg</td>
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<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (nM)</td>
<td>6867</td>
<td>4130</td>
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<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>n/a</td>
<td>0.5</td>
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<tr>
<td>AUC&lt;sub&gt;0-24&lt;/sub&gt; (nM*h)</td>
<td>5930</td>
<td>12500</td>
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<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>0.77</td>
<td>1.5</td>
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<tr>
<td>V&lt;sub&gt;dss&lt;/sub&gt; (L/kg)</td>
<td>0.458</td>
<td>n/a</td>
</tr>
<tr>
<td>CL (mL/min/kg)</td>
<td>8.7</td>
<td>n/a</td>
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<tr>
<td>F (%)</td>
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<td>42.9</td>
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Table 5. Mean plasma and cerebrospinal fluid concentrations of LY3020371 following single oral doses of diester prodrug LY302778.HCl in SD rats

<table>
<thead>
<tr>
<th>LY302778.HCl dose (mg/kg, p.o.)</th>
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<th>60</th>
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<tr>
<td>LY3020371-equivalent dose (mg/kg, p.o.)</td>
<td>5.7</td>
<td>34.3</td>
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<table>
<thead>
<tr>
<th>C_{max} (nM)</th>
<th>Plasma</th>
<th>7980</th>
<th>13700</th>
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<td></td>
<td>CSF</td>
<td>109</td>
<td>240</td>
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<table>
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<tr>
<th>T_{max} (h)</th>
<th>Plasma</th>
<th>1.0</th>
<th>1.0</th>
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<tbody>
<tr>
<td></td>
<td>CSF</td>
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<td>2.0</td>
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<table>
<thead>
<tr>
<th>AUC_{0-24} (nM*h)</th>
<th>Plasma</th>
<th>17400</th>
<th>46500</th>
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<tr>
<td></td>
<td>CSF</td>
<td>942</td>
<td>1730</td>
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<table>
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<tr>
<th>T_{1/2} (h)</th>
<th>Plasma</th>
<th>3.0</th>
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<tr>
<td></td>
<td>CSF</td>
<td>5.2</td>
<td>4.8</td>
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Table 6. Plasma and brain concentrations of LY3020371 following single oral doses of diester prodrug LY3027788.HCl in NIH Swiss mice

<table>
<thead>
<tr>
<th>LY3027788.HCl dose (mg/kg, p.o.)</th>
<th>17.6</th>
<th>29.9</th>
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<tbody>
<tr>
<td>LY3020371-equivalent dose (mg/kg, p.o.)</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>Mean LY3020371 concentration at 1h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>1365</td>
<td>3316</td>
</tr>
<tr>
<td>Brain</td>
<td>50.9</td>
<td>76.3</td>
</tr>
</tbody>
</table>
Fig. 2

A

Concentration of Dopamine Cells/Track (% of Vehicle)

<table>
<thead>
<tr>
<th>Dose LY3020371 (mg/kg, i.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEH</td>
</tr>
</tbody>
</table>

B

Firing Rate (Spikes/Sec, % of Veh)

<table>
<thead>
<tr>
<th>Dose LY3020371 (mg/kg, i.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>veh</td>
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</tbody>
</table>

C

% of Spikes in Bursts (% of Veh)

<table>
<thead>
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<th>Dose LY3020371 (mg/kg, i.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>veh</td>
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</tbody>
</table>
Fig. 3

--- anterior cingulate cortex ---

--- striatum ---

LY3020371 (mg/kg, i.p.)
Effect of S-(+)-Ketamine (10 mg/kg, s.c.) on Neurotransmitter Efflux in Rat mPFCx

Extracellular Response to S-(+)-Ketamine (10 mg/kg, s.c.) in Rat mPFCx

Concentration of S-(+)-Ketamine in Rat mPFCx Dialysate Samples Following 10 mg/kg (s.c.)

Extracellular Response to S-(+)-Ketamine (10 mg/kg, s.c.) in Rat mPFCx

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Fig. 5
Fig. 6
Fig. 7

![Graph showing immobility results for different treatments.]

- LY354740 (3 mg/kg, i.p.)
- NBQX (10 mg/kg, i.p.)
- LY3020371 (mg/kg, i.v.)

Genotype-Drug Treatment:

- WT
- KO
- KOLY

Note: This article has not been copyedited and formatted. The final version may differ from this version.
Fig. 8

![Bar graph showing time-course of sensitization with different doses of test substance.](chart.png)

- **1 mg/kg, i.v.**
  - Max. decrease = 22%
- **3 mg/kg, i.v.**
  - Max. decrease = 28%
- **10 mg/kg, i.v.**
  - Max. decrease = 36%

**Y-axis:** Seconds of immobility

**X-axis:** Time (h)
Fig. 9
Fig. 10
Fig. 11
Fig. 12
Fig. 13
Fig. 14