

**Pharmacological and Pharmacokinetic Properties of a Structurally-Novel, Potent, Selective  
mGlu2/3 Receptor Agonist: *In Vitro* Characterization of LY404039**

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**Abbreviations:** 2*R*,4*R*-APDC = 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylate; 4CPG = (*S*)-4-carboxyphenylglycine; AMPA =  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; cAMP = cyclic adenosine monophosphate; DCG-IV = (2*S*,1'*R*,2'*R*,3'*R*)-2-(2,3-dicarboxycyclopropyl)glycine; DHPG = 3,5-dihydroxyphenylglycine; L-AP4 = L-2-amino-4-phosphonobutyrate; LY354740 = (1*S*,2*S*,5*R*,6*S*)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate; LY379268 = (-)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate; LY341495 = 2*S*-2-amino-2-(1*S*,2*S*-2-carboxycycloprop-1-yl)-3-(xant-9-yl) propanoic acid; LY404039 = (-)-(1*R*,4*S*,5*S*,6*S*)-4-amino-2-sulfonylbicyclo[3.1.0]hexane-4,6-dicarboxylic acid; MCPG = (+)- $\alpha$ -methyl-4-carboxyphenylglycine; mGlu = metabotropic glutamate receptor; MSOP = (*RS*)- $\alpha$ -methylserine-*O*-phosphate; NMDA = *N*-methyl-*D*-aspartate; PCP = phencyclidine

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## **Abstract**

Group II metabotropic glutamate (mGlu) receptor agonists, including LY354740 and LY379268, have demonstrated efficacy in animal models of anxiety and schizophrenia, and LY354740 decreased anxiety in human subjects. Herein, we report the *in vitro* pharmacological profile and pharmacokinetic properties of another potent, selective, structurally-novel mGlu2/3 receptor agonist (-)-(1*R*,4*S*,5*S*,6*S*)-4-amino-2-sulfonylbicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY404039) and provide comparisons with LY354740. Similar to LY354740, LY404039 is a nanomolar potent agonist at recombinant human mGlu2 and mGlu3 receptors ( $K_i = 149$  and  $92$ , respectively) and in rat neurons expressing native mGlu2/3 receptors ( $K_i = 88$ ). LY404039 is highly selective for mGlu2/3 receptors, showing more than 100-fold selectivity for these receptors, versus ionotropic glutamate receptors, glutamate transporters, and other receptors targeted by known anxiolytic and antipsychotic medications. Functionally, LY404039 potently inhibited forskolin-stimulated cAMP formation in cells expressing human mGlu2 and mGlu3 receptors. Electrophysiological studies indicated that LY404039 suppressed electrically-evoked excitatory activity in the striatum, and serotonin-induced  $L$ -glutamate release in the prefrontal cortex; effects reversed by LY341495. These characteristics suggest LY404039 modulates glutamatergic activity in limbic and forebrain areas relevant to psychiatric disorders; and that, similar to LY354740, it works through a mechanism which may be devoid of negative side effects associated with current antipsychotics and anxiolytics. Interestingly, despite the slightly lower potency (~2-5-fold) of LY404039 versus LY354740 in binding, functional and electrophysiological assays, LY404039 demonstrated higher plasma exposure and better oral bioavailability in pharmacokinetic experiments. Collectively, the current data indicate that LY404039 may be valuable in the treatment of neuropsychiatric disorders, including anxiety and psychosis.

## **Introduction**

Glutamate receptors are comprised of two receptor families: ionotropic glutamate (iGlu) and metabotropic glutamate (mGlu) receptors. iGlu receptors are ligand-gated ion channels that mediate fast synaptic transmission and include NMDA, AMPA, and kainate receptor subtypes. Metabotropic glutamate receptors are G-protein linked receptors that mediate multiple second messenger systems (Cartmell and Schoepp, 2000; Pin and Duvoisin, 1995). mGlu receptors have been further divided into three groups based on receptor sequence homology, second messenger systems, and pharmacological properties. Group I mGlu receptors (mGlu1, mGlu5) are positively coupled to the activation of phosphoinositide hydrolysis and calcium mobilization (Cartmell and Schoepp, 2000). At these receptors, 3,5-dihydroxyphenylglycine (DHPG) is a selective agonist, while (*S*)-4-carboxyphenylglycine (4CPG) is an antagonist (Schoepp et al., 1999a). Group III mGlu receptors (mGlu4, 6, 7, 8) are negatively coupled to adenylyl cyclase and cyclic AMP (cAMP) formation (Cartmell and Schoepp, 2000). At group III mGlu receptors, L-2-amino-4-phosphonobutyrate (L-AP4) is a potent agonist and (*RS*)- $\alpha$ -methylserine-*O*-phosphate (MSOP) is a selective antagonist (Schoepp et al., 1999a; Swanson et al., 2005). Group II mGlu receptors (mGlu2, mGlu3) are also negatively coupled to adenylyl cyclase and the formation of cAMP (Cartmell and Schoepp, 2000). (*2S,1'R,2'R,3'R*)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV) and *2R,4R*-4-aminopyrrolidine-2,4-dicarboxylate (*2R,4R*-APDC) are selective agonists at group II receptors, while (+)- $\alpha$ -methyl-4-carboxyphenylglycine (MCPG) demonstrates antagonist properties (Pin and Duvoisin, 1995; Schoepp et al. 1995, 1996, 1999a). Each mGlu receptor subtype is differentially distributed in diverse brain regions, allowing for the possibility of selectively altering neurotransmission in specific synapses (Cartmell and Schoepp, 2000).

The group II mGlu receptors function primarily as autoreceptors modulating GABAergic and monoaminergic neurotransmitter release directly (Cartmell and Schoepp, 2000) and via various modulatory actions on astrocytic mGlu3 receptors (Bruno et al., 1998; Moldrich et al., 2002). They also play a major role in modulating the release of  $L$ -glutamate, and are highly expressed in the hippocampus,

amygdala, and prefrontal cortex (Ohishi et al., 1993a,b; Schoepp et al., 2003). Their dense localization in limbic and forebrain areas associated with such neuropsychiatric disorders as drug abuse, anxiety, and schizophrenia suggest that they may be useful pharmacological targets for the development of novel medications for these disorders (Swanson et al., 2005). In support of this, potent, selective and systemically-active mGlu2/3 receptor agonists, (1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate monohydrate (LY354740) and (-)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268) were discovered (Monn et al., 1997, 1999) and have been widely characterized in animal models. Preclinical experiments with LY354740 and LY379268 have indicated efficacy in animal models of anxiety (Helton et al., 1998; Linden et al., 2005; Shekhar and Keim, 2000), drug withdrawal (Helton et al., 1997; Vandergriff and Rasmussen, 1999), epilepsy (Klodzinska et al., 2000; Monn et al., 1997), and schizophrenia (Cartmell et al., 1999; Moghaddam and Adams, 1998; Schoepp et al., 1999b). Importantly, this compound does not produce unwanted side effects typically observed with benzodiazepines and neuroleptics, such as sedation, abuse liability, dependence, and motor side effects (Gudex, 1991; Sachdev, 2005). This lack of undesirable side effects is likely related to the ability of LY354740 to modulate excessive L-glutamate release that occurs with the aforementioned disorders, rather than directly inhibiting fast synaptic transmission (Swanson et al., 2005).

Clinical studies with LY354740 demonstrated initial efficacy in human models, using such paradigms as fear-potentiated startle and ketamine-induced working memory deficits in healthy volunteers, and CO<sub>2</sub>-induced panic in panic-prone patients (Grillon et al., 2003; Krystal et al., 2005; Schoepp et al., 2003). Importantly, LY354740 was well-tolerated in those studies. However, the clinical development of LY354740 has been hampered by low oral bioavailability, due to minimal absorption in the gastrointestinal tract and inadequate penetration through the blood-brain barrier (Johnson et al., 2002; Bueno et al., 2005). Although recent attempts to improve the oral bioavailability of this compound have met with good success (Rorick-Kehn et al., 2006), further research has been devoted to discovering additional potent and selective mGlu2/3 receptor agonists. Herein, we report the *in vitro* pharmacological profile and pharmacokinetic properties of the structurally-novel mGlu2/3 receptor agonist (-)-

(1*R*,4*S*,5*S*,6*S*)-4-amino-2-sulfonylbicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY404039, Fig 1; Monn et al., in press) and provide comparisons with LY354740. We characterize the efficacy, potency, and selectivity of LY404039 and LY354740 at human and rat mGlu and iGlu receptors, as well as binding at glutamate transporters and receptors implicated in the efficacy of current anxiolytic and antipsychotic medications, including adrenergic, monoaminergic, and GABAergic receptors. We also characterize the ability of LY404039 to modulate  $L$ -glutamate release in two different paradigms, electrically-evoked EPSPs in striatal slices and serotonin-induced EPSCs in prefrontal cortical slices, and present a pharmacokinetic comparison between LY404039 and LY354740 following oral administration.

## **Methods**

### **Receptor Binding Assays**

Cell lines expressing human mGlu2, mGlu3, mGlu1a, mGlu5a, mGlu4a, mGlu6, mGlu7a, and mGlu8 receptors were derived as previously described (Schoepp et al., 1997) and cultured in Dulbecco's modified Eagles medium (DMEM) with 5% dialyzed fetal bovine serum, 1 mM glutamine, 1 mM sodium pyruvate, 50 mg/ml Geneticin G418, and 0.2 mg/ml hygromycin B. Confluent cultures were passaged weekly. These cells are referred to as Rat Glutamate Transporter (RGT) cells. RGT cells are AV12-664 cells (American Type Culture Collection, accession number CRL 9595) which have been stably transfected with a glutamate transporter (GLAST) to prevent the accumulation of  $L$ -glutamate into the cell media (Schoepp et al., 1997). Membranes from mGlu receptor-expressing RGT cells were prepared by harvesting adherent cells from confluent T-150 flasks with a cell scraper. Cells and media were then placed in 50 ml conical tubes and centrifuged at 4°C at 1000 x  $g$  for 5 min. The supernatant was removed and the pellet was frozen at -10°C until use. Preliminary binding experiments with fresh, or frozen and thawed, cellular membrane pellets showed no differences in specific or non-specific binding. Therefore, frozen pellets, thawed at room temperature for about 10 minutes were routinely used. Washed cell membranes were prepared by adding 20 ml of 10 mM potassium phosphate buffer pH 7.6 at 5°C 100 mM potassium bromide and homogenizing with an Ultra-Turrax tissue mixer for 15 s at 90% output. The homogenate was centrifuged in a Sorvall RC-5B SS34 rotor at 48,000 x  $g$  for 10 min at 5°C. This last step was repeated two times, and the final pellet was resuspended in 10 ml of the same buffer and reserved on ice until initiating the binding assay.

#### *[<sup>3</sup>H]-LY341495 Binding: Group II mGlu Receptors*

[<sup>3</sup>H]-LY341495 binding was assayed in a reaction mixture containing 10 mM potassium phosphate (pH 7.6), 100 mM potassium bromide and 1 nM [<sup>3</sup>H]-LY341495, (final vol. 500 ml). The incubation was initiated by the addition of the membrane suspension, (~15 mg membrane protein), and allowed to continue on ice for 30 min. Incubation was terminated by rapid filtration with a Brandel cell harvester through glass fiber Whatman GF:B filters pre-wet with the same potassium phosphate buffer

assay buffer (at 4 °C). The filters were washed five times with 1 ml buffer. Filter sections were transferred to minivials and 5 ml of Fisher Scientific ScintiSafe liquid scintillation cocktail was added to each vial. Vials were allowed to set for several hours prior to counting on a Beckman LS6000 liquid scintillation counter. Protein concentrations were quantified by a modified Bradford Pierce Comassie microassay. Nonspecific binding was determined in the presence of 1 mM L-glutamate.

#### *[<sup>3</sup>H]-LY341495 Binding: Group III mGlu Receptors*

Binding procedures were similar to those described previously (Johnson et al. 1999). Membranes from cells expressing recombinant human group III mGlu receptors were prepared the same as described for group II mGlu receptor membranes. To start the reaction, washed tissue (0.05–0.20 mg protein) was added to [<sup>3</sup>H]-LY341495 (10 nM) and appropriate concentrations of test compounds in assay buffer. Final assay volume was 0.5 ml. Nonspecific binding was defined with 1 mM L-glutamate or 1 mM L-serine-O-phosphate (for mGlu7a). Assay plates were incubated on ice for 45 min and the reaction was terminated by rapid filtration (Whatman GF/B filters), and washed twice with 1 ml ice-cold assay buffer. Filters were placed in minivials and scintillation cocktail added. Protein concentration was determined using the Pierce Coomassie micro assay.

#### *[<sup>3</sup>H]-LY341495 Binding to Rat Brain Membranes*

Brain tissue was obtained by decapitating adult male Sprague-Dawley rats (150–250 g; Harlan, Indianapolis, IN) in accordance with the Eli Lilly and Company animal care and use policies and prepared as described previously (Wright et al., 1994). The forebrain (cortex, striatum, and hippocampus) was used; the brain tissue was homogenized in 30 mM Tris-HCl + 2.5 mM CaCl<sub>2</sub> buffer (pH 7.6 at 5° C) and washed three times by centrifugation, incubated for 30 min at 37° C followed by three more washes, and then resuspended in 10 volumes of buffer and frozen at -20° C. Frozen pellets of rat brain homogenate were thawed on the day of assay and washed three times with ice-cold assay buffer (10 mM potassium phosphate containing 100 mM potassium bromide, pH 7.6). To start the reaction, tissue (0.02–0.06 mg of protein) was added to deep-well polypropylene microtiter plates, which contained [<sup>3</sup>H]-LY341495 (1 nM) and appropriate concentrations of test compounds in assay buffer. Final assay volume was 0.5 ml.



Nonspecific binding was defined with 1 mM L-glutamate. Assay plates were incubated on ice for 30 min, and bound and free radioligands were separated by rapid filtration with 5 x 1 ml of ice-cold assay buffer using Whatman GF-B filters (Brandel Inc., Gaithersburg, MD). Protein concentration was determined using the Pierce Coomassie microassay.

### **Forskolin-Stimulated cAMP Formation and Phosphoinositide Hydrolysis**

Cloned human mGlu receptors mGlu1a, mGlu2, mGlu3, mGlu4a, mGlu5a and mGlu7 were each expressed in "RGT" cells, as described above. RGT cells expressing human mGlu receptors were seeded into 96-well culture plates in DMEM supplemented with 1.2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 5% dialyzed fetal calf serum, 250 µg/ml hygromycin (for selection of mGlu receptor clone expression) and 500 µg/ml G418 (for selection of GLAST protein expression). Cells were maintained at 37 °C in a humidified atmosphere of 6.8% CO<sub>2</sub> in air for 18–24 hr prior to use in second messenger assays.

For phosphoinositide assays, cells expressing mGlu1 and mGlu5 receptors were seeded into 24 well culture plates at 2.5 x 10<sup>5</sup> cells per well in medium containing no added glutamine and cultured at 37° C in a humidified atmosphere of 5% CO<sub>2</sub> in air. After 24 h, the cells were labelled with [<sup>3</sup>H]-myo-inositol (Amersham 4 µCi/ml) for a further 20 h. Cells were washed in assay medium containing HEPES (10 mM), inositol (10 mM) and lithium chloride (10 mM). To test agonist effects, compound or vehicle was added to the cell cultures and incubated for 60 min at 37° C . When tested in antagonist mode, compound or vehicle was added 20 min prior to the addition of agonist quisqualate (0.3 µM) and then further incubated for 60 min. The reaction was terminated by replacing the medium with acetone: methanol (1:1) and the cultures incubated on ice for 20 min. Separation of the [<sup>3</sup>H]-inositol phosphates was carried out by Sep -Pak Accell Plus QMA ion exchange chromatography (Waters, Millipore Ltd., UK). The [<sup>3</sup>H]-inositol monophosphate fraction was eluted with 0.1 M triethyl ammonium bicarbonate buffer and radioactivity was measured by liquid scintillation counting.

For cAMP assays, cells expressing human mGlu2, mGlu3, mGlu4, mGlu6, mGlu7 or mGlu8 were washed (2x with 200 µl/well) with assay medium (Dulbecco's phosphate buffered saline (PBS) plus

3 mM glucose and 500  $\mu$ M isobutylmethylxanthine (IBMX)). The media was replaced with 0.2 ml/well of the same solution and cells preincubated for 30 min at 37° C under 95% O<sub>2</sub>, 5% CO<sub>2</sub>. Each well was then washed two successive times with 200  $\mu$ l of medium. Compounds of interest or water vehicle were added, along with forskolin solution (15  $\mu$ M final concentration group II mGlu receptors, 1  $\mu$ M final concentration group III mGlu receptors, total incubation volume 0.1 ml) and cells were further incubated at 37° C under 95% O<sub>2</sub>, 5% CO<sub>2</sub> for 20 min. The incubation was terminated by adding 0.1 ml of 0.2% triton X-100 lysing solution. Levels of cAMP were determined by an Amersham cAMP-[<sup>125</sup>I] Scintillation Proximity Assay (SPA) Screening Biotrak Assay kit.

### **Receptor Binding Selectivity Profile**

Radioligands and reagents used were obtained from the following commercial suppliers: Aldrich Chemical Company, Inc (Milwaukee, WI), Sigma Chemical Company (St. Louis, MO), Mallinckrodt Chemical Company (Paris, KY), Fisher Scientific (Fair Lawn, NJ), New England Nuclear (Boston, MA), Amersham Corporation (Arlington Heights, IL), and Research Biochemicals Inc (Natick, MA). Receptor binding assays were performed according to methods previously reported (Rasmussen et al., 2000) with minor modifications listed in Table 1.

### **Suppression of Electrically-Stimulated Excitatory Post-Synaptic Potentials**

Coronal slices of striatum were prepared from young (14-22 days old) male Sprague-Dawley rats. Animals were deeply anesthetized with methoxyflurane and decapitated. Their brains were removed rapidly from the skull and immersed in a cold (~ 2° C) NaHCO<sub>3</sub>-buffered saline solution (concentrations in mM): NaCl 126.0, KCl 3.0, MgCl<sub>2</sub> 1.5, Na<sub>2</sub>PO<sub>4</sub> 1.25, CaCl<sub>2</sub> 2.0, NaHCO<sub>3</sub> 26.0, glucose 10.0; pH = 7.4, osmolarity = 300  $\pm$  5 mOsm/liter. The brains were blocked and 300-400  $\mu$ m thick coronal sections were cut through the rostrocaudal extent of the striatum using a Vibroslice (Campden Instruments, London, England). Slices were placed into the continuously oxygenated NaHCO<sub>3</sub>-buffered saline solution warmed to 32° C for 30 min and then maintained at room temperature. After at least one hour of incubation, individual slices were transferred to a recording chamber mounted on an upright microscope and continuously superfused (2-3 ml/min) with oxygenated extracellular solution maintained at 30  $\pm$  0.2° C.

Differential interference videomicroscopy was used to visualize striatal neurons. Whole-cell current-clamp recordings were conducted using patch pipettes fabricated from thin-walled borosilicate glass (Corning 7052, WPI Inc., Sarasota, FL) having resistances of 1 to 4 M $\Omega$  when lowered into the extracellular solution. The pipette solution contained (in mM): K<sup>+</sup>-gluconate 130.0, KCl 10.0, MgCl<sub>2</sub> 2.0, EGTA 1.0, HEPES 10.0, Na<sub>2</sub>ATP 2.0, Na<sub>2</sub>GTP 0.3; pH adjusted to 7.3 with 1 M NaOH, osmolarity of 290-300 mOsm. The extracellular solution contained (in mM): NaCl 125.0, KCl 3.0, CaCl<sub>2</sub> 2.4, MgCl<sub>2</sub> 1.3, NaHCO<sub>3</sub> 26.0, glucose 10.0; pH adjusted to 7.4 with 1 M NaOH, osmolarity of 300  $\pm$  5 mOsm. L-glutamate-dependent excitatory postsynaptic potentials (EPSPs) were isolated by blocking  $\gamma$ -aminobutyric acid<sub>A</sub> and/or B receptors with bicuculline methiodide (1-2  $\mu$ M) and SCH59011 (2  $\mu$ M), respectively. Voltage signals were amplified by an Axoclamp 200B amplifier, low-pass filtered at 5 kHz, and stored on the computer hard-disk for off-line analysis (Clampfit 8.0, Molecular Devices, Sunnyvale, CA). Series resistance (10-30 M $\Omega$ ) compensation was monitored and recordings displaying >30% change in resistance were not included in subsequent analyses. Voltage errors due to the liquid junction potential were subtracted during analysis. Postsynaptic potentials were evoked by constant current single stimulation pulses (100  $\mu$ s, 50-500  $\mu$ A) delivered with a 20 s interstimulus interval using bipolar stimulating electrodes positioned proximal to the recorded neuron. Stock solutions of 10 mM LY404039 and LY354740 in DMSO were aliquoted and stored at -20<sup>o</sup> C until the day of recording. Drugs were added directly to the extracellular superfusion solution at the desired concentration.

### **Suppression of Serotonin-Evoked Post-Synaptic Currents**

Serotonin (5-HT), via activation of 5-HT<sub>2A</sub> receptors, induces L-glutamate release onto the apical dendrites of layer V pyramidal cells in the medial prefrontal cortex (Aghajanian and Marek, 1997). The L-glutamate release induced by 5-HT<sub>2A</sub> receptor activation in the cortex appears to arise from thalamocortical terminals, and previous work has demonstrated that mGlu2/3 receptors function as autoreceptors to modulate L-glutamate release from these terminals (Marek et al., 2000). Brain slices were prepared from male Sprague-Dawley rats (120-200 g) as described previously (Marek et al., 2000).

Briefly, rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and decapitated. Coronal slices (500  $\mu$ M) were cut with an oscillating-blade tissue slicer at a level corresponding to approximately 2.5 mm anterior to bregma. A slice containing the medial prefrontal cortex was then transferred to the stage of a fluid-gas interface chamber, which had a constant flow of humidified 95% O<sub>2</sub>, 5% CO<sub>2</sub>. The slices were perfused in a chamber heated to 34° C with normal ACSF, which consisted of (in mM) NaCl 126, KCl 3, CaCl<sub>2</sub> 2, MgSO<sub>4</sub> 2, NaHCO<sub>3</sub> 26, NaH<sub>2</sub>PO<sub>4</sub> 1.25, and D-glucose 10.

Intracellular recording and single-electrode voltage clamping were conducted in layer V pyramidal cells by using an Axoclamp-2A (Molecular Devices, Sunnyvale, CA) as previously described (Aghajanian and Marek, 1997). Stubby electrodes (~8 mm, shank to tip) with relatively low capacitance and resistance (30-60 MOhms) were filled with 1 M potassium acetate. The cells were voltage clamped at -70 mV. The excitatory post-synaptic currents (EPSCs) recorded under these conditions do not appear to be contaminated by reversed inhibitory postsynaptic currents as previously discussed (Aghajanian and Marek, 1997). The voltage-clamp signals were low-pass filtered (1000 Hz) and data were acquired with a pCLAMP/Digidata 1200 system (Molecular Devices, Sunnyvale, CA). EPSC frequencies were obtained from 10 consecutive episodes (1 s duration) during the baseline and drug treatment periods. Evoked potentials were obtained while holding cells at -80 mV and stimulating the forceps minor in the white matter deep in the cortex.

### **Pharmacokinetic Analysis**

Plasma exposure of LY404039 was measured following a single 1 or 3 mg/kg oral dose to male Sprague Dawley rats or a single 10 mg/kg oral dose to Male Fischer 344 rats of LY404039 in water (adjusted to pH 7.0 with 5N NaOH). Rats were fasted overnight, dosed by gavage, and blood samples were collected using heparin as the anticoagulant from the orbital sinus or by cardiac puncture (final sample). Samples were drawn at 0.5, 1, 2, 3, 5, and 8 hours post dose for the 1 and 3 mg/kg dose, and at 0.25, 0.5, 1, 2, 3, 4, 8, 12, and 24 hours post the 10 mg/kg dose. Plasma was obtained by centrifugation, plasma extracted using solid phase ion exchange, and subsequently analyzed for LY404039 using an LC/MS/MS method on a Sciex API300 instrument.

Plasma exposure of LY354740 was measured following a single oral dose of 10 mg/kg in 5% emulphor to male Fischer 344 rats. Rats were fasted overnight, dosed by gavage, and blood samples were collected using heparin as the anticoagulant from the orbital sinus or by cardiac puncture at 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 24 hours post dose. Plasma was obtained by centrifugation, plasma extracted using solid phase ion exchange, and subsequently analyzed for LY354740 using an LC/MS/MS method on a Micromass instrument.

### Data Analysis

For all binding experiments, affinity constants for the displacers were calculated using nonlinear regression in GraphPad Prism (GraphPad Software, Inc., San Diego, CA) by the one-site competition equation:  $Y = \text{Bottom} + ((\text{Top} - \text{Bottom}) / (1 + 10^{\text{X} \cdot \text{LogEC}_{50}}))$ , followed by the equation:  $K_i = \text{EC}_{50} / (1 + [L] / K_d)$ , where [L] is the concentration of [<sup>3</sup>H]-LY341495.  $K_d$  values for the ligand were determined by saturation curves and Scatchard analysis. Nonspecific binding was defined with 1 mM L-serine-O-phosphate (for mGlu7) or 1 mM L-glutamate (for all other receptors). Bound and free ligands were separated by centrifugation.

The formation of cAMP was expressed as the mean production (pmol/ml). The receptor binding and cAMP experiments were performed on three separate occasions. The phosphoinositide assays were performed in duplicate and each experiment was repeated on two separate occasions. For these experiments, the mean, standard error, and  $\text{EC}_{50}$  or  $\text{IC}_{50}$  values were calculated in GraphPad Prism (San Diego, CA) using a nonlinear regression curve fit with sigmoidal dose response (variable slope).

The frequency and amplitude of serotonin-evoked EPSCs were determined with Mini Analysis Program ([www.synaptosoft.com](http://www.synaptosoft.com); Synaptosoft, Inc., Leonia, NJ) using thresholds of 10 pA and an area of  $\sim 150 \text{ fCs}^{-1}$  for synaptic currents. The determination of  $\text{EC}_{50}$  values for the suppression of 5-HT-induced increases in EPSC frequency or of evoked EPSCs were calculated by nonlinear curve fitting (DeltaGraph 4.0; DeltaPoint, Monterey, CA). The amplitudes of stimulation-evoked EPSPs recorded in striatal neurons were measured using Clampfit 8.0 software (Molecular Devices, Sunnyvale, CA). Comparisons between

groups were made using a one-way ANOVA followed by a Dunnett's post-hoc test (GraphPad Prism 4.0, San Diego, CA).

## Results

### Receptor Binding Assays

Group II mGlu receptor binding affinities for LY354740 and LY404039 were determined by displacement of specific [<sup>3</sup>H]-LY341495 binding in RGT cells expressing recombinant human mGlu2 and mGlu3 receptor subtypes and in cortical tissue prepared from rat forebrain under conditions selectively labeling group II mGlu receptors. As shown in Table 2 and Fig 2, both LY354740 and LY404039 displaced [<sup>3</sup>H]-LY341495 binding with nanomolar potencies: (LY354740: mGlu2,  $K_i = 99 \pm 7$  nM; mGlu3,  $K_i = 94 \pm 10$  nM; rat cortical tissue,  $K_i = 106 \pm 5$  nM; LY404039: mGlu2,  $K_i = 149 \pm 11$  nM; mGlu3,  $K_i = 92 \pm 14$  nM; rat cortical tissue,  $K_i = 88 \pm 15$  nM). Thus, LY404039 is equipotent to LY354740 at human mGlu3 receptors and in rat brain, but slightly less potent at human mGlu2 receptors (Table 2). Overall, both LY404039 and LY354740 are highly selective for group II mGlu receptors. In contrast, neither LY354740 nor LY404039 displaced [<sup>3</sup>H]-LY341495 binding to group III mGlu receptors (mGlu6, mGlu7, or mGlu8;  $K_i$  values > 5000 nM; Table 2, Fig 2).

### Forskolin-Stimulated cAMP Formation and Phosphoinositide Hydrolysis

Functional activity of LY354740 and LY404039 at group II and group III mGlu receptors, as measured by the inhibition of forskolin-stimulated cAMP formation is illustrated in Fig 3 (see also Table 3). Both LY354740 and LY404039 are nM potent full agonists at human mGlu2 and mGlu3 receptors, as indicated by the inhibition of forskolin-stimulated cAMP formation (LY354740: mGlu2,  $EC_{50} = 7.9 \pm 0.3$  nM, mGlu3,  $EC_{50} = 21 \pm 2$  nM; LY404039: mGlu2,  $EC_{50} = 23 \pm 1$  nM, mGlu3,  $EC_{50} = 48 \pm 10$  nM). Although these two compounds are similar, LY404039 appears to be 2-3-fold less potent than LY354740 in this functional assay. As shown in Fig 3, both compounds demonstrated >100 fold selectivity for mGlu2 and mGlu3 over other cAMP-coupled mGlu receptors (mGlu4a, 6, 7a, 8a). Neither compound demonstrated activity at group I mGlu receptors (mGlu1a and mGlu5a), as indicated by the inability to stimulate phosphoinositide hydrolysis ( $EC_{50} > 10,000$  nM; Table 3).

### Receptor Binding Selectivity Profile

The binding affinities of LY354740 and LY404039 at ionotropic glutamate receptors, glutamate transporters, and various other receptors that are antagonized or activated to some degree by most antipsychotics or anxiolytics were determined. Neither LY354740 nor LY404039 had any appreciable affinity for ionotropic NMDA, AMPA, or kainate glutamate receptors at concentrations up to 100  $\mu$ M (Table 4). LY354740 and LY404039 demonstrated no affinity for glutamate transporters including rat EAAT1, EAAT2, and EAAT3 at concentrations up to 5 mM. Likewise, no affinity was detected at adrenergic, monoaminergic, benzodiazepine/GABA-ergic, histaminergic, or muscarinic receptors at concentrations up to 10 or 100  $\mu$ M, for LY404039 and LY354740, respectively (Table 4).

### **Suppression of Electrically-Stimulated Excitatory Post-Synaptic Potentials**

In order to demonstrate the activity of LY404039 at native mGlu2/3 receptors, the ability of these compounds to suppress cortically-evoked EPSPs in rat striatal spiny neurons was tested, and compared to that of LY354740. As shown in Fig 4A and 4C, both LY404039 and LY354740 attenuated EPSPs in a concentration-dependent manner with nanomolar potencies in striatal tissue, LY404039:  $EC_{50} = 141$  nM, LY354740:  $EC_{50} = 20$  nM. In contrast to the binding assays, in which LY404039 was demonstrated to be 2-3-fold less potent than LY354740, the difference in potency was ~5-6-fold in this experiment. The suppression of electrically-evoked EPSPs was mediated by activation of mGlu2/3 receptors, as indicated by the reversal of this effect using the selective mGlu2/3 receptor antagonist, LY341495 (1  $\mu$ M) ( $F_{2,17} = 16.0$ ,  $p < 0.0001$ ) (Fig 4B).

### **Suppression of Serotonin-Evoked Post-Synaptic Currents**

LY354740 has been reported to suppress serotonin (5-HT)-evoked EPSCs in rat prefrontal cortical slices ( $EC_{50}$  value = 89.1 nM; Marek et al., 2000). Therefore, only LY404039 was tested here. Since 100  $\mu$ M of 5-HT produces a near maximal increase in the frequency of EPSCs, the effect of LY404039 was tested against this 5-HT concentration. The frequency of EPSCs induced by 100  $\mu$ M 5-HT was  $29.7 \pm 3.9$ /sec (mean  $\pm$  SEM). LY404039 suppressed the frequency of 5-HT-induced EPSCs with an  $EC_{50} = 82.3 \pm 4.4$  nM and with a near maximal suppression of  $85.6 \pm 3.9$  % at 1  $\mu$ M ( $n = 5$ ; Fig. 5). A higher concentration (3  $\mu$ M) was tested in three of the five cells with essentially no additional



suppression of the 5-HT-induced EPSCs. The suppressant action of LY404039 on the 5-HT-induced EPSC frequency was blocked by 1  $\mu$ M of the mGlu2/3 receptor antagonist LY341495 in the three cells tested (data not shown). A twenty minute exposure of the slice to LY341495 decreased the suppressant action of LY404039 from  $39.7 \pm 2.9\%$  to  $24.0 \pm 1.4\%$ .

LY404039 also selectively suppressed the frequency, rather than the amplitude, of the 5-HT-induced EPSCs, suggesting a presynaptic action. In all three cells in which 5-HT induced a significant increase in the EPSC amplitude ( $p < 0.05$ , Kolmogorov-Smirnov test), LY404039 did not alter the EPSC amplitude ( $p > 0.05$ ) at a concentration of the mGlu agonist (100 nM) which significantly decreased the EPSC frequency by  $\sim 50\%$  ( $p < 0.005$ ).

### **Pharmacokinetic Analysis**

Following oral administration of LY404039 to fasted rats at doses of 1, 3, or 10 mg/kg, exposure increased proportionally with dose (Table 5). Comparison of plasma concentrations of LY354740 and LY404039 following a single oral dose of 10 mg/kg of the respective compound indicate similar exposure over a 24 hour period. However,  $C_{max}$  was slightly earlier for LY404039 (2 vs. 3 hours) and nearly double that of LY354740. As seen in Figure 6, this resulted in the majority of the plasma exposure to LY404039 occurring in the first 4 hours post-dose, while the majority of the exposure to LY354740 occurred over the first 8 hours, and did not reach the same plasma concentrations as seen with LY404039.

## ***Discussion***

The current report details the *in vitro* pharmacological and pharmacokinetic profile of a structurally-novel group II metabotropic glutamate receptor agonist, LY404039. We report here that, similar to LY354740 (Schoepp et al., 1997), LY404039 is a nM potent agonist at recombinant human mGlu2/3 receptors and in rat neurons expressing native mGlu2/3 receptors. Also similar to LY354740, LY404039 is highly selective for mGlu2/3 receptors, showing virtually no affinity for group I or group III mGlu receptors, ionotropic glutamate receptors, or glutamate transporters (>100-fold selectivity over these other receptors). Moreover, LY404039 demonstrated no appreciable affinity for receptors implicated in the mechanism of action of current clinically effective antipsychotic and anxiolytic medications, including dopaminergic, serotonergic, muscarinic, adrenergic, GABAergic, histaminergic and benzodiazepine receptors. Functional assays indicated that LY404039 is a full agonist at mGlu2/3 receptors, as measured by the potent inhibition of forskolin-stimulated cAMP production. Interestingly, although similar to LY354740, LY404039 was ~2-3-fold less potent in this assay. Despite the slightly lower potency of LY404039 versus LY354740 in binding and functional assays, LY404039 demonstrated higher plasma exposure and better oral bioavailability in pharmacokinetic experiments in rats [~63% (see Monn et al., in press) as compared to ~10% for LY354740 (Johnson et al., 2002)]. Electrophysiological studies indicated that LY404039 suppressed electrically-evoked excitatory activity in the striatum, an effect reversed by the mGlu2/3 receptor antagonist LY341495, indicating that the suppression of neural activity was likely mediated via mGlu2/3 receptors. The potency of LY404039 at suppressing excitatory activity in the striatum was ~5-6-fold lower than that of LY354740. Moreover, as demonstrated here, LY404039 potently suppressed serotonin-induced  $L$ -glutamate release in the prefrontal cortex, which was also reversed by LY341495, indicating an mGlu2/3 receptor-mediated effect. That the suppression of  $L$ -glutamate release was mediated via mGlu2/3 receptors is important because it demonstrates that this mechanism of action may be capable of demonstrating better efficacy and/or reduced incidence of undesirable side effects when compared with currently available medications.

The relatively recent discovery of potent, selective compounds that act at various metabotropic glutamate receptor subtypes and the generation of mGlu receptor knockout mice have advanced our understanding of mGlu receptor pharmacology at a rapid pace. Correspondingly, our understanding of the role of individual mGlu receptors in various neuropsychiatric disorders continues to expand, aided in part by the discovery of the selective mGlu2/3 receptor agonists LY354740 and LY379268 (Monn et al., 1997, 1999). Since its discovery, LY354740 has demonstrated broad anxiolytic efficacy using such animal models as fear-potentiated startle, elevated plus-maze, stress-induced hyperthermia, and lactate-induced panic (Helton et al., 1998; Linden et al., 2005; Shekhar and Keim, 2000; Spooren et al., 2002). Additionally, LY354740 and LY379268 have demonstrated efficacy in animal models of drug reinstatement and withdrawal (Baptista et al., 2004; Helton et al., 1997; Vandergriff and Rasmussen, 1999), epilepsy (Klodzinska et al., 2000; Monn et al., 1997), and schizophrenia (Cartmell et al., 1999; Moghaddam and Adams, 1998; Schoepp et al., 1999b). Moreover, LY354740 has produced promising results in initial human tests of anxiety and psychosis (Grillon et al., 2003; Schoepp et al., 2003; Krystal et al., 2005). However, low oral bioavailability (~3-5% in humans) has hindered the clinical development of LY354740. LY404039 resulted from an effort to discover additional potent, selective, orally-active mGlu2/3 receptor agonists for the treatment of psychiatric disorders.

Observed differences between LY404039 and previous mGlu2/3 agonists, including LY354740 and LY379268, suggest that LY404039 could represent a superior pharmacological tool for studying mGlu2/3 receptors in neuropsychiatric and other pathological states. Pharmacokinetic experiments demonstrated that while AUC values were similar between LY354740 and LY404039, oral administration of LY404039 resulted in higher plasma concentrations and a faster T<sub>max</sub>, indicating potential utility in the treatment of acute forms of anxiety disorders (e.g., panic attacks), in addition to other indications, including pain, neuroprotection, drug withdrawal, and schizophrenia (Schoepp et al., 1999b). Moreover, although similar to LY354740 at mGlu2 and mGlu3 receptors, LY404039 demonstrated lower affinity at other mGlu receptor subtypes, such as mGlu6, which are primarily localized in the retina, suggesting reduced likelihood of affecting visual perception. Group II receptors demonstrate a distinctive

perisynaptic and extrasynaptic expression profile in brain areas associated with neuropsychiatric disorders, including the amygdala, hippocampus, and prefrontal cortex: mGlu2 receptors are localized presynaptically, but also in the periphery of the active zone of glutamate release, while mGlu3 receptors are localized predominantly on astrocytes and glia (Cartmell and Schoepp, 2000). Activation of these receptors by LY404039 provides a mechanism through which subtle alterations in neurotransmission (both directly and indirectly) may be achieved, resulting in the suppression of pathological glutamate release without affecting normal synaptic transmission.

Several lines of evidence suggest that excessive  $L$ -glutamate transmission in limbic and cortical areas is associated with the manifestation of psychiatric disorders (Moghaddam, 2002). Noncompetitive NMDA receptor antagonists and hallucinogenic drugs produce an increase in the release of  $L$ -glutamate in the medial prefrontal cortex, and mimic some of the psychotic symptoms of schizophrenia (Aghajanian and Marek, 1997; Krystal et al., 2005). Stress and anxiety disorders are also associated with a hyperglutamatergic state in limbic and cortical regions (Bergink et al., 2004; Moghaddam, 2002). The mGlu2/3 receptor agonist LY354740 has been demonstrated to suppress serotonin-evoked EPSPs in the prefrontal cortex (Marek et al., 2000), as well as reverse behavioral hyperactivity and stereotypy and the increased prefrontal cortical activity induced by administration of the noncompetitive NMDA receptor antagonists MK-801 and PCP (Cartmell et al., 1999; Homayoun et al., 2005; Moghaddam and Adams, 1998). Together with evidence of anxiolytic efficacy (see above), these data suggest that mGlu2/3 receptor agonists may be beneficial for the treatment of such neuropsychiatric disorders as schizophrenia and anxiety (Marek et al., 2000; Moghaddam, 2002). We report here that LY404039 effectively modulates mGlu2/3 receptors *in situ* with a potency similar to that previously reported for LY354740, suggesting that this compound should also demonstrate efficacy in animal models of psychosis and anxiety. Perhaps most importantly, we demonstrate here that despite the *in vitro* similarities between LY354740 and LY404039, oral administration of LY404039 results in much higher plasma levels and bioavailability (63%; Monn et al., in press) than previously observed following oral administration of LY354740 (~10%, Johnson et al., 2002), and a different pharmacokinetic profile. The mechanism by

which LY404039 crosses the gastrointestinal tract is not clearly understood. It will be interesting to measure how this compares across species. In the context of LY354740, we have addressed this issue by using a prodrug approach (LY544344; Rorick-Kehn et al., 2006). Studies in humans should be informative as to whether this approach may be needed for LY404039.

We have identified a structurally-novel, potent, and selective mGlu2/3 receptor agonist LY404039 without significant activity at other glutamate receptors, glutamate transporters, or monoamine receptors. LY404039 modulates neurotransmitter release in several *in vitro* models and exhibits favorable pharmacokinetic properties. In comparison with LY354740, the results reported here indicate that LY404039 may be a superior compound with respect to its drug-like properties. Electrophysiological characteristics indicate that LY404039 modulates glutamatergic activity in limbic and forebrain areas relevant to such psychiatric disorders as anxiety and schizophrenia; and that, similar to LY354740, it works through a mechanism which specifically targets the neural circuitry associated with the hyperglutamatergic state, and which may be devoid of negative side effects associated with currently available antipsychotics and anxiolytics (Gudex, 1991; Sachdev, 2005). Considered together with *in vivo* efficacy in animal models predictive of anxiolytic and antipsychotic efficacy (Rorick-Kehn et al., under review), the current data indicate that LY404039 may be useful for the treatment of neuropsychiatric disorders, including anxiety and psychosis.

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### ***Legends for Figures***

**Figure 1.** Chemical structure of LY404039. For comparison purposes, the chemical structure of LY354740 is also included.

**Figure 2.** Displacement of [<sup>3</sup>H]-LY341495 binding to mGlu receptors by LY354740 or LY404039 in forebrain tissue from adult rats (200 – 500 µg) or RGT cells expressing the designated receptors. Nonspecific binding was defined with 1 mM L-serine-O-phosphate (for mGlu7) or 1 mM L-glutamate (for all other receptors). Each point represents percent of specific [<sup>3</sup>H]-LY341495 bound in absence of compound tested and represents the mean (± SEM) of three experiments performed on separate occasions.

**Figure 3.** Selective inhibition of forskolin-stimulated cAMP formation by LY354740 or LY404039 in cells expressing group II (mGlu2 and mGlu3), but not group III (mGlu4,6,7,8) receptors. Forskolin (15 µM) and LY354740 or LY404039 were incubated with RGT cells expressing the designated receptor. Bound and free ligands were separated by centrifugation. Each point represents the mean (± SEM) of three experiments performed on separate occasions.

**Figure 4.** Effects of LY404039 on EPSPs evoked in striatal neurons *in vitro*. A.) Concentration-response profile of LY404039 on EPSP amplitude in striatal neurons. LY404039 suppressed EPSPs in a concentration-dependent manner having an EC<sub>50</sub> value of 141 nM. Each point represents the mean (± SEM) response (*n*=6) at each concentration tested normalized to the control response amplitude. The points were fit with a four parameter logistic equation. B.) Representative responses of a striatal neuron to local stimulation of glutamatergic inputs during control conditions and in the presence of increasing concentrations of LY404039 (0.03-1.0 µM). C.) The concentration response profile for LY354740 on EPSP amplitude in striatal neurons. Each point represents the mean (± SEM) response (*n*=6) at each concentration tested normalized to the control response amplitude. The points were fit with a four

parameter logistic equation. D.) Effects of the mGlu2/3 receptor antagonist, LY341495, on the LY404039-induced suppression of striatal neuron responses. Application of LY404039 reduced striatal cell EPSPs ( $n=6$ ) by approximately 40%; an effect that was completely reversed by LY341495.

**Figure 5.** Concentration-response curve for the suppression of 5-HT (100  $\mu$ M)-induced EPSCs in neocortical layer V pyramidal cells in the prefrontal cortex by LY404039. Each point represents the mean ( $\pm$  SEM) of five experiments performed on separate occasions (exception:  $n = 3$  at 3  $\mu$ M concentration). The  $EC_{50}$  for LY404039 was  $82.3 \pm 4.4$  nM with a maximal suppression of 85.8%.

**Figure 6.** Time-course of plasma concentrations of LY354740 or LY404039 following oral administration of the respective compounds (10 mg/kg, p.o.). Each point represents the mean ( $\pm$  SEM) of three samples per time point.

**Tables**

**Table 1.** Methodological citations for receptor binding methods.

Receptor	[ <sup>3</sup> H]Radioligand (nM)	Buffer	Tissue	Nonspecific ( $\mu$ M)	Incubate (hr/ $^{\circ}$ C)
$\alpha$ 1-adrenergic	[ <sup>3</sup> H]Prazosin (0.2)	Tris HCl 50 mM pH 7.7	Whole brain	WB4101 0.1	1 23
$\alpha$ 2-adrenergic	[ <sup>3</sup> H]Rauwolscine (0.4)	Tris+EDTA (1 mM) 50 mM pH 6.9	Whole brain	Yohimbine 1	1 23
$\beta$ -adrenergic	[ <sup>125</sup> I]ICP (0.05)	Tris HCl 50 mM pH 7.7	Whole brain	(-)Propranolol 1	1 23
Dopamine D1	[ <sup>3</sup> H]SCH23390 (0.2)	Tris+*salts 50 mM pH 7.4	corpus striatum	SCH23390 0.03	1 23
Dopamine D2	[ <sup>3</sup> H]Raclopride (0.8)	Tris+*salts 50 mM pH 7.4	corpus striatum	Spiperone 0.03	1 23
Benzodiazepine	[ <sup>3</sup> H]Flunitrazepam (1)	Tris HCl 50 mM pH 7.4	Whole brain	Clonazepam 1	1 4
Histamine H1	[ <sup>3</sup> H]Pyrilamine (2)	Na <sub>2</sub> HPO <sub>4</sub> 50 mM pH 7.5	Whole brain	Promethazine 1	0.5 23
GABA <sub>A</sub>	[ <sup>3</sup> H]Muscimol (2)	Tris HCl 50 mM pH 7.4	cortex	GABA 10	1 4
Glutamate NMDA	[ <sup>3</sup> H]CGP 39653 (2)	Tris HCl 50 mM pH 8.0	cerebellum	L-glutamate 100	1 4
Glutamate AMPA	[ <sup>3</sup> H]AMPA (5)	Tris HCl 30 mM + CaCl <sub>2</sub> 2.5 mM+ KSCN 100mM pH 7.5	cerebellum	L-glutamate 100	1 4
Glutamate Kainate	[ <sup>3</sup> H]Kainate (19)	Tris HCl 50 mM pH 8.0	cerebellum	L-glutamate 1000	1 4
Muscarinic	[ <sup>3</sup> H]QNB (1)	Na <sub>2</sub> HPO <sub>4</sub> 50 mM pH 7.4	cortex	Atropine 1	1 23
5-HT <sub>2</sub>	[ <sup>3</sup> H]Ketanserine (0.4)	Tris HCl 50 mM pH 7.4	cortex	Methysergide 10	1 23

\*salts: NaCl (120 mM), KCl (5 mM), CaCl<sub>2</sub> (2 mM), and MgCl<sub>2</sub> (1 mM).

Abbreviations: ICP: Iodocyanopindolol; QNB: quinuclidinyl benzilate.

**Table 2.** Specific binding of LY354740 and LY404039 to subtypes of human cloned mGlu receptors expressed in RGT cells and to native cells in rat forebrain tissue. Data are presented as the mean ( $\pm$  SEM) of three separate experiments.

<b>Preparation</b>	<b>Ki (nM), Mean <math>\pm</math> S.E.M., n = 3</b>	
	<b>LY354740</b>	<b>LY404039</b>
Human mGlu2	99 $\pm$ 7	149 $\pm$ 11
Human mGlu3	94 $\pm$ 10	92 $\pm$ 14
Rat Forebrain	106 $\pm$ 5	88 $\pm$ 15
Human mGlu6	14,463 $\pm$ 1,820	28,637 $\pm$ 5,741
Human mGlu7	28,810 $\pm$ 7,578	120,617 $\pm$ 14,836
Human mGlu8	11,246 $\pm$ 1,253	47,773 $\pm$ 3,505

**Table 3.** Comparison of the potency of LY354740 and LY404039 on second messenger responses in RGT cells expressing subtypes of human mGlu receptors. Data are presented as the mean ( $\pm$  SEM) response of 3-4 separate experiments.

Human Subtype	EC <sub>50</sub> <sup>a</sup> or IC <sub>50</sub> <sup>b</sup> (nM), Mean $\pm$ S.E.M., n = 3-4	
	LY354740	LY404039
Group I (PI Assay)		
mGlu1a	>10,000 <sup>a,b</sup>	>10,000 <sup>a,b</sup>
mGlu5a	>10,000 <sup>a,b</sup>	>10,000 <sup>a,b</sup>
Group II (cAMP Assay)		
mGlu2	7.9 $\pm$ 0.3 <sup>a</sup>	23 $\pm$ 1 <sup>a</sup>
mGlu3	21 $\pm$ 2 <sup>a</sup>	48 $\pm$ 10 <sup>a</sup>
Group III (cAMP Assay)		
mGlu4a	>10,000 <sup>a,b</sup>	>10,000 <sup>a,b</sup>
mGlu6	2,950 $\pm$ 1,390 <sup>a</sup>	16,800 $\pm$ 2,440 <sup>a</sup>
mGlu7a	>100,000 <sup>a,b</sup>	>100,000 <sup>a,b</sup>
mGlu8a	11,500 $\pm$ 2,100 <sup>a</sup>	>10,000 <sup>a,b</sup>

<sup>a</sup> EC<sub>50</sub> values were determined using a nonlinear regression curve fit (sigmoidal, variable slope).

<sup>b</sup> When tested in antagonist mode in the PI and cAMP assays, the agonist concentrations were selected to be at EC<sub>50</sub>-EC<sub>80</sub> values. These were as follows: 0.3  $\mu$ M quisqualate for mGlu1 and mGlu5; 3  $\mu$ M L-AP4 for mGlu4; 0.5  $\mu$ M L-AP4 for mGlu6; 1000  $\mu$ M L-AP4 for mGlu7 and 0.3  $\mu$ M L-AP4 for mGlu8 (mGlu2 and mGlu3 were not tested in antagonist mode).

**Table 4.** Effects of mGlu2/3 receptor agonists at ionotropic glutamate receptors, glutamate transporter subtypes, monoamine and other receptors. The apparent affinity ( $K_i$ ) was determined for each neurotransmitter receptor or transporter. Data are presented as the mean ( $\pm$  SEM) of 2-3 separate experiments. Where affinities are reported as greater than 1  $\mu$ M concentration, this was the highest concentration tested that inhibited <50% specific bound radiolabel in the assay.

	<b><math>K_i</math> or <math>IC_{50}</math>, Mean <math>\pm</math> S.E.M., n = 2-3</b>	
	<b>LY354740</b>	<b>LY404039</b>
iGluR Ligand ( $K_i$ )		
<sup>3</sup> H-CGP39653 (NMDA)	>100 $\mu$ M	>100 $\mu$ M
<sup>3</sup> H-AMPA	>100 $\mu$ M	>100 $\mu$ M
<sup>3</sup> H-Kainate	>100 $\mu$ M	>100 $\mu$ M
Transporter Subtype ( $IC_{50}$ )		
Rat EAAT1	>5 mM	>5 mM
Rat EAAT2	>5 mM	>5 mM
Rat EAAT3	>5 mM	>5 mM
Monoamine Ligand ( $K_i$ )		
Dopamine – D <sub>1</sub>	>100 $\mu$ M	>10 $\mu$ M
Dopamine – D <sub>2</sub>	>100 $\mu$ M	>10 $\mu$ M
Serotonin – 5-HT <sub>2</sub>	>100 $\mu$ M	>10 $\mu$ M
Adrenergic ( $K_i$ )		
$\alpha$ 1-adrenergic	>100 $\mu$ M	>10 $\mu$ M
$\alpha$ 2-adrenergic	>100 $\mu$ M	>10 $\mu$ M
$\beta$ -adrenergic	>100 $\mu$ M	>10 $\mu$ M
Miscellaneous ( $K_i$ )		
Histamine – H <sub>1</sub>	>100 $\mu$ M	>10 $\mu$ M
Benzodiazepine	>100 $\mu$ M	>10 $\mu$ M
GABA <sub>A</sub>	>100 $\mu$ M	>10 $\mu$ M
Muscarinic	>100 $\mu$ M	>10 $\mu$ M

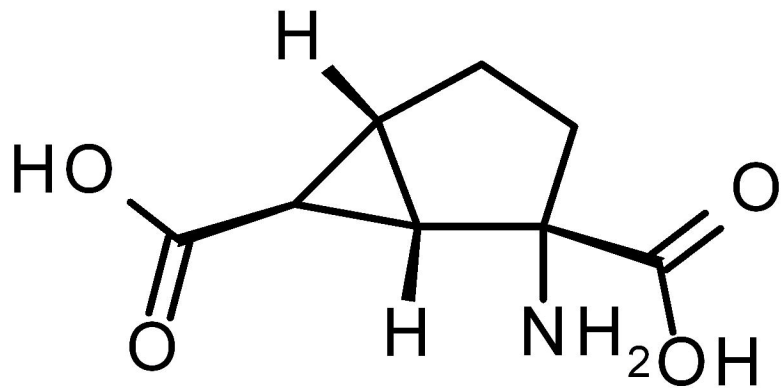


**Table 5.** Pharmacokinetic parameters of LY354740 and LY404039 in fasted rats following 1, 3, or 10 mg/kg oral dose of LY404039 or 10 mg/kg LY354740. Data are presented as the mean ( $\pm$  SEM) of 3 values per time point.  $C_{\max}$  and AUC are rounded to no more than two significant figures.

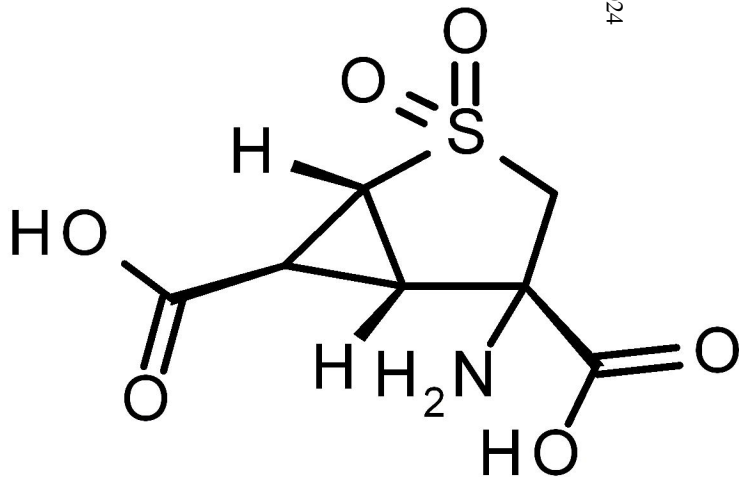
<b>Compound</b>	<b>Dose mg/kg</b>	<b>Route of Administration</b>	<b><math>C_{\max}</math> (ng/ml)</b>	<b><math>T_{\max}</math> (hr)</b>	<b>AUC (<math>\pm</math> SEM) (ng*hr/ml)</b>
LY404039	1	p.o.	212.14	1	581.41 $\pm$ 124.33
LY404039	3	p.o.	552.27	2	1350.79 $\pm$ 269.08
LY404039	10	p.o.	1528.5	2	4516.48 $\pm$ 482.7
LY354740	10	p.o.	848.37	3	4437.19 $\pm$ 515.25

$C_{\max}$  = maximal concentration;  $T_{\max}$  = time to  $C_{\max}$ ; AUC = area under the concentration versus time curve from 0 to the last quantifiable time point.

Figure 1



LY354740



LY404039

Figure 2

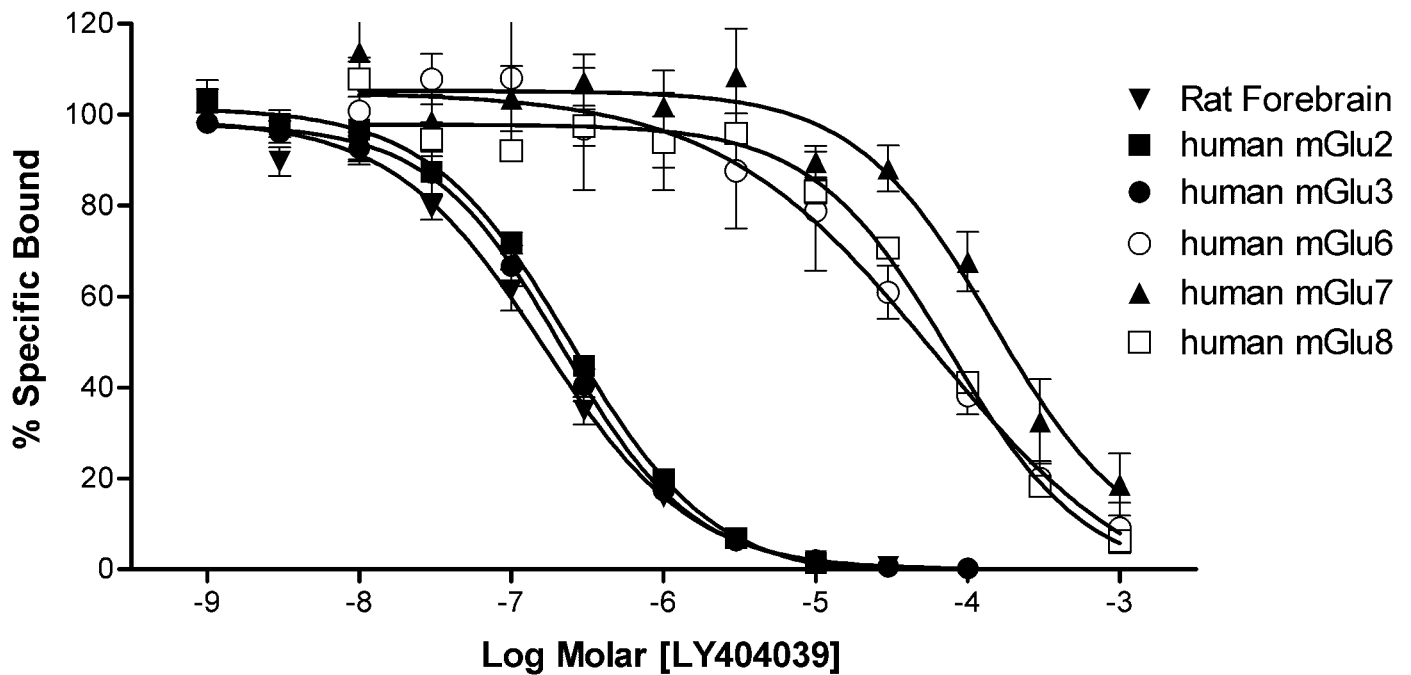
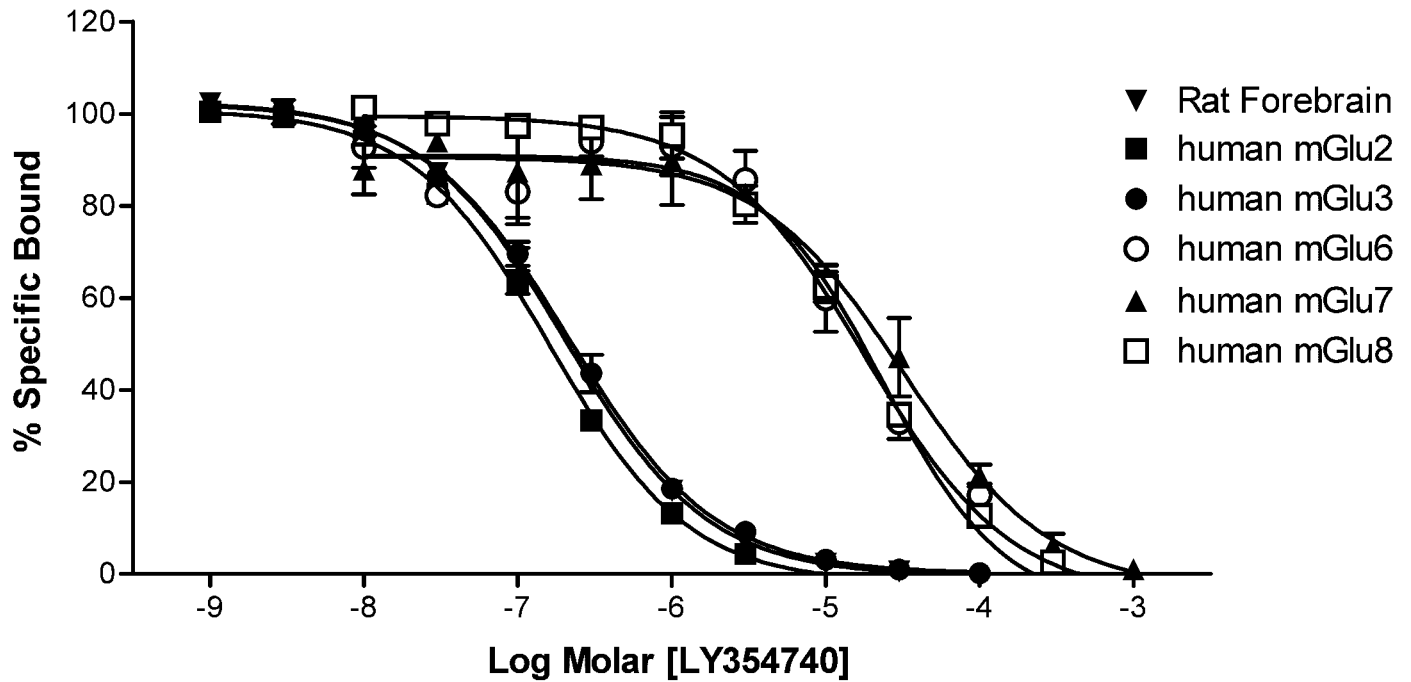


Figure 3

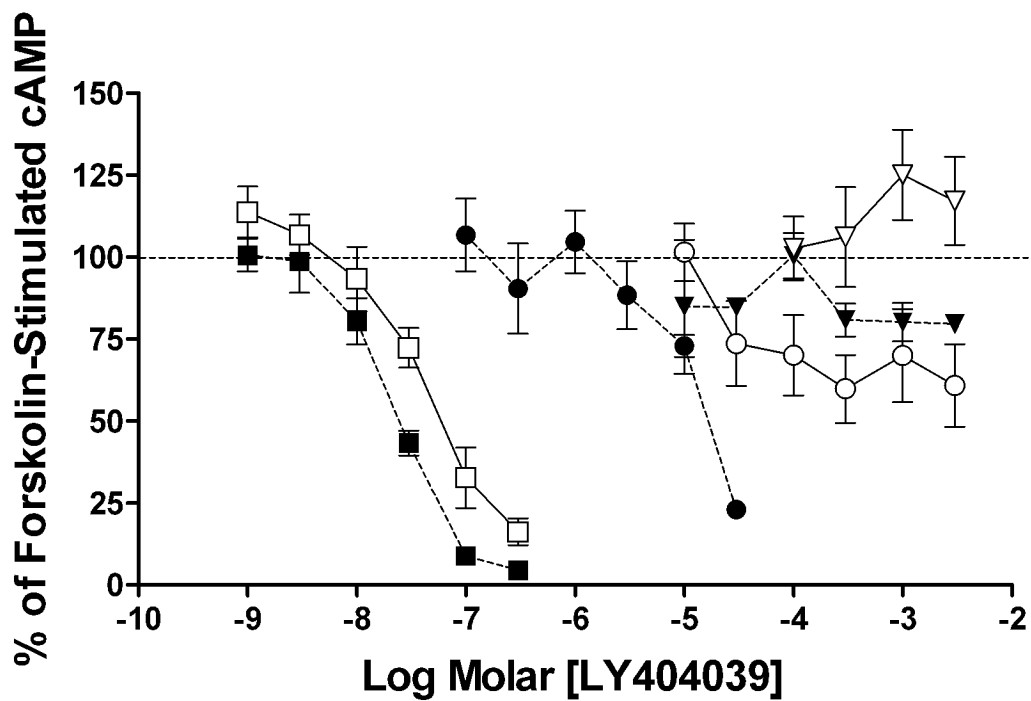
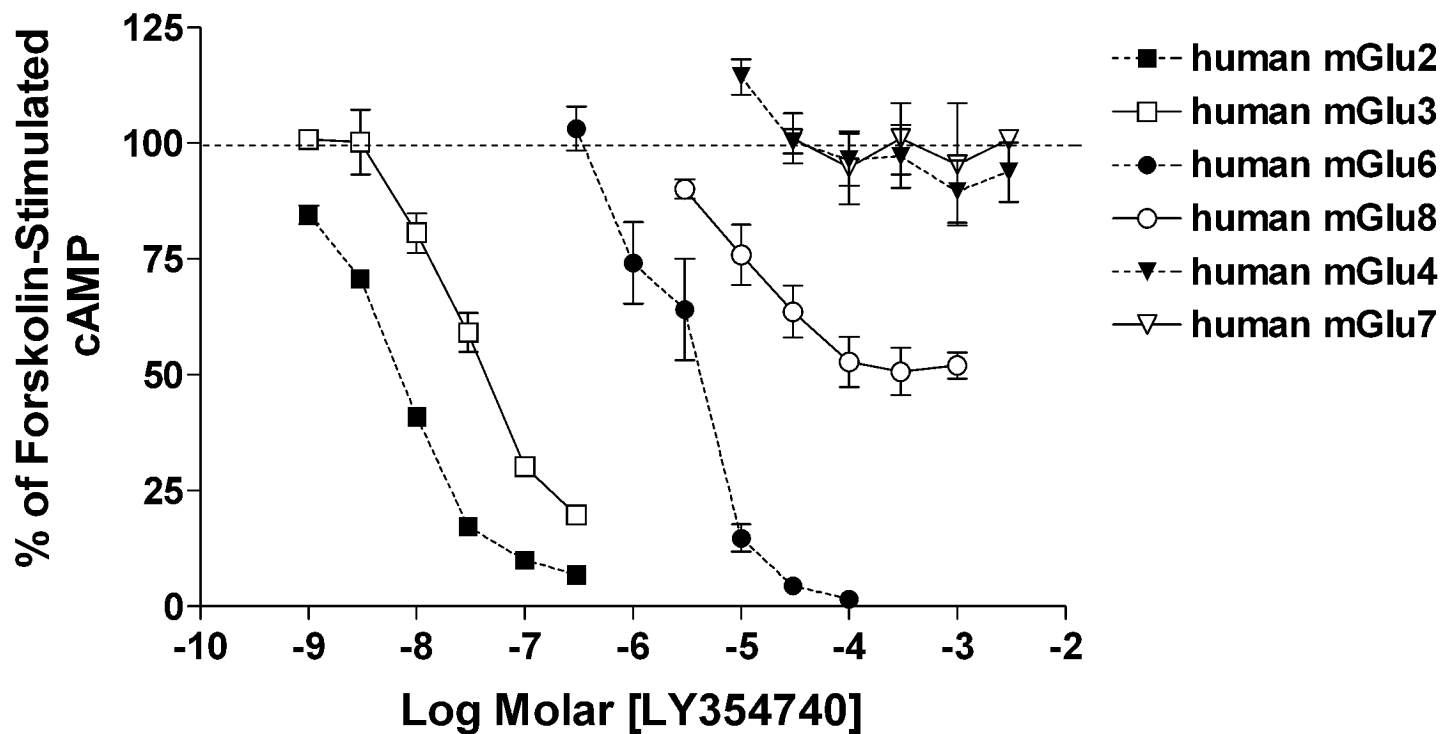
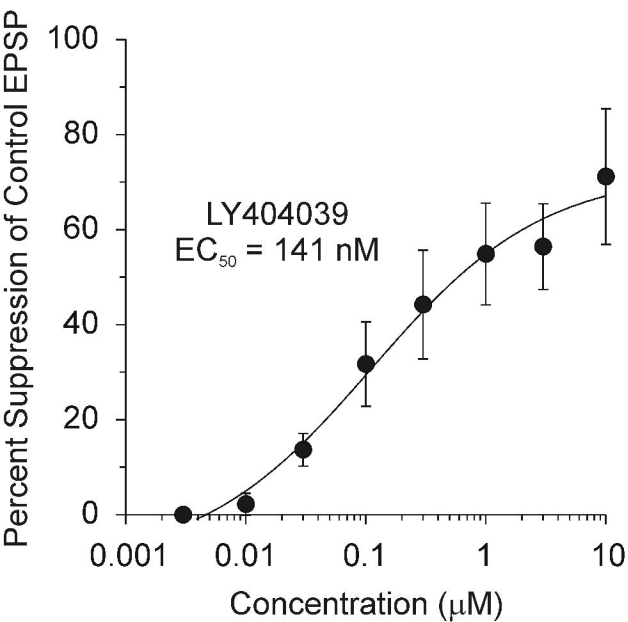
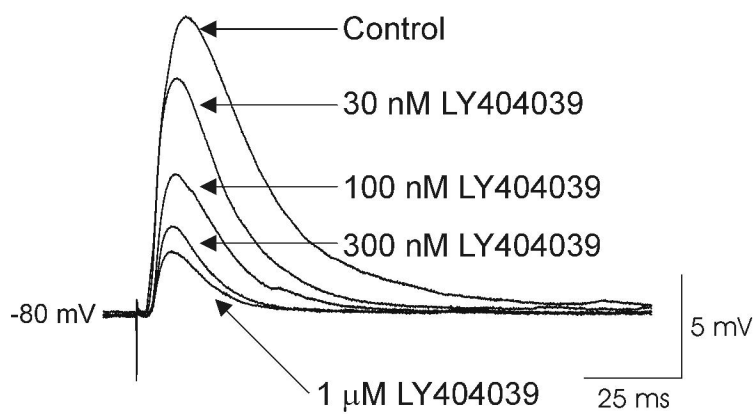


Figure 4

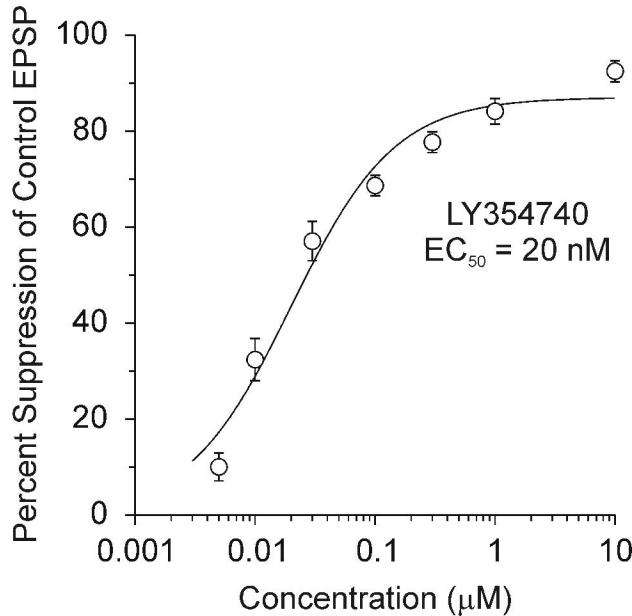
A.



B.



C.



D.

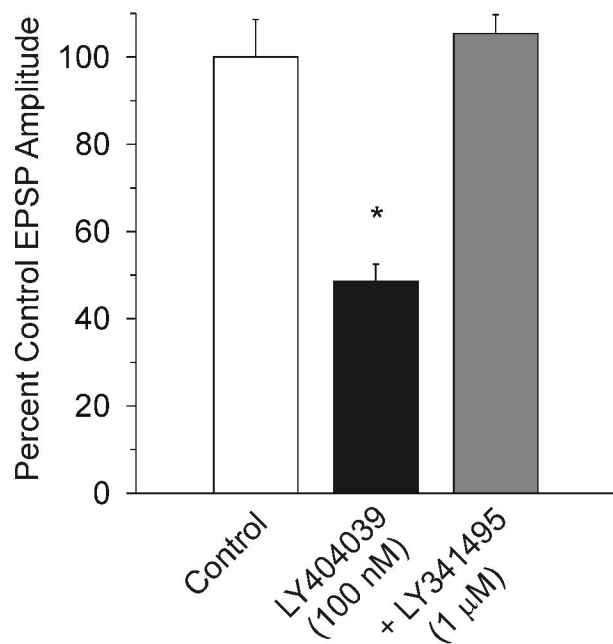


Figure 5

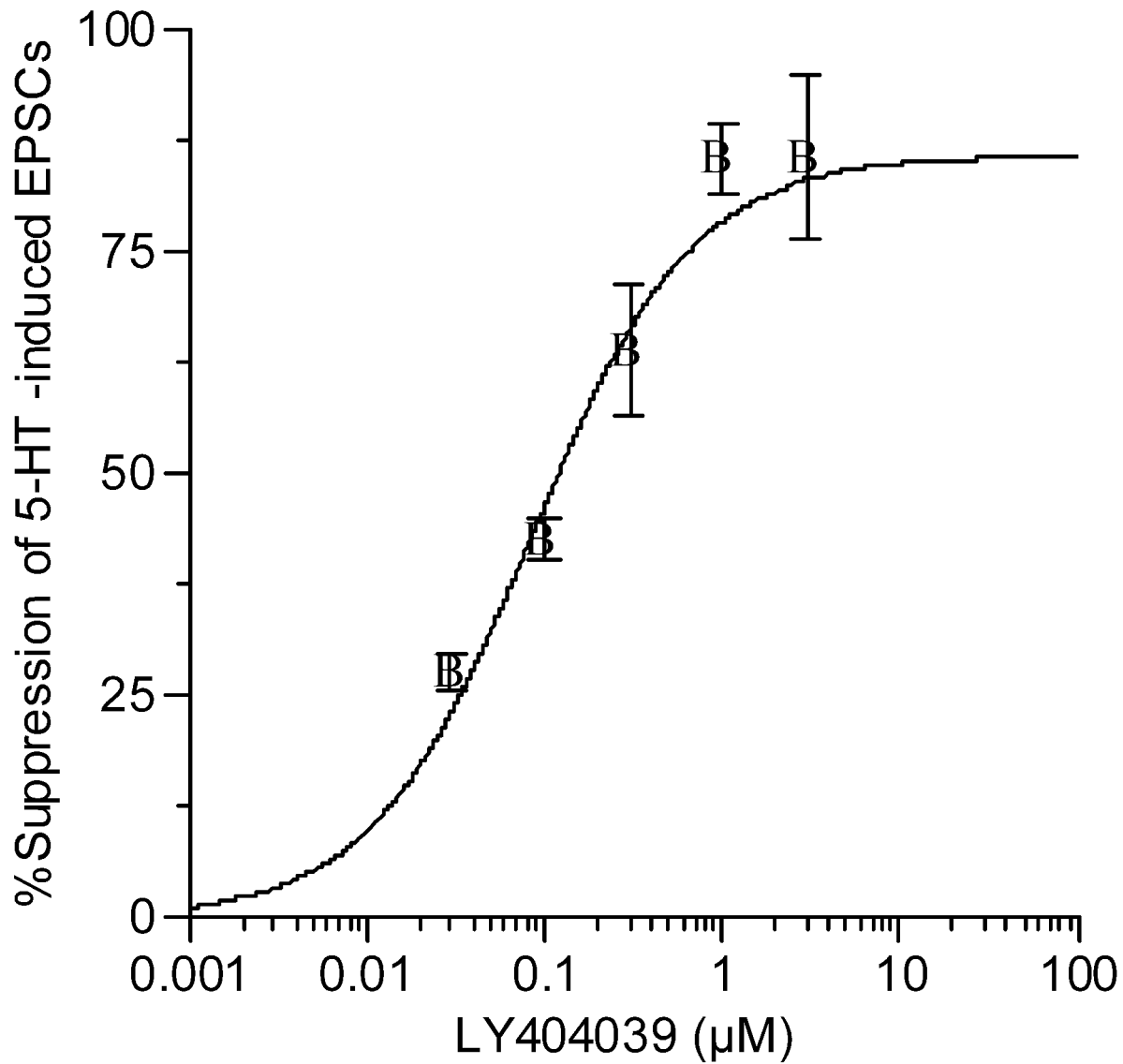


Figure 6

