Translational pharmacology of the mGluR2-preferring agonist LY2812223 in animal and human brain

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Running Title: Pharmacology of an mGlu2-Preferring Receptor Agonist in Native Tissue

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List of non-standard abbreviations: EAAT1 (Excitatory Amino Acid Transporter 1), FLIPR (Fluorescence Imaging Plate Reader), GPCR (G protein coupled receptors), HTRF (Homogeneous Time Resolved Fluorescence), IPSC (Induced Pluripotent Stem Cells), KO
(Knock Out), mGlur (metabotropic glutamate receptor), NHP (non-human primate), PD (pharmacodynamic), RFU (relative fluorescence units), RGT (rat glutamate transporter), RTN (reticular nucleus), VB (ventrobasal complex), WT (wild type)
Abstract

LY2812223 was identified via structure-activity studies arising from the potent mGlu2/3 receptor agonist LY354740 as an mGlu2-preferring agonist. This pharmacology was determined using cells stably transfected cells either containing the human mGlu2 or mGlu3 receptor. We extended the pharmacological evaluation of LY2812223 to native brain tissues derived from relevant species used for preclinical drug development as well as human post mortem brain tissue. This analysis was conducted to ensure pharmacological translation from animals to human subjects in subsequent clinical studies. A GTP-γ-[35S] functional binding assay, a method for measuring Gi-coupled signaling which is inherent to the Group 2 mGlu receptors, was used to evaluate LY2812223 pharmacology of native mGlu receptors in mouse, rat, non-human primate, and human cortical brain tissue samples. In native tissue membranes, LY2812223 unexpectedly acted as a partial agonist across all species tested. Activity of LY2812223 was lost in cortical membranes collected from mGlu2 knock-out (KO) mice, but not those from mGlu3-KO, providing additional support for mGlu2-preferring activity. Other signal transduction assays were used for comparison to the GTP binding assay (cAMP, calcium mobilization, and dynamic mass redistribution). In ectopic cell line-based assays, LY2812223 displayed near maximal agonist responses at the mGlu2 receptor across all assay formats while at the mGlu3 receptor it showed no functional agonist activity except in the cAMP assay. In native brain slices or membranes that express both mGlu2 and mGlu3 receptors, LY2812223 displayed unexpected partial agonist activity which may suggest a functional interplay between these receptor subtypes in the brain.
Introduction

Glutamate is a ubiquitous neurotransmitter that binds to and activates the glutamate receptor family of receptors made up of ion channels (GluR1-4) and metabotropic G protein coupled receptors (mGlu1-8) (O'Neil et al., 2010). The metabotropic glutamate receptors belong to the superfamily of G protein coupled receptors that are characterized by their distinct single peptide structure that passes through the plasma membrane in a serpentine seven transmembrane orientation. mGlu receptors belong to the class C GPCRs which contrast in overall structure from the class A rhodopsin-like and the class B peptide binding type of GPCRs by their large N-terminal extracellular glutamate binding domain. The mGlu receptors fall into 3 groups based on sequence similarity (Group 1: mGlu1,5; Group 2: mGlu2,3; Group 3: mGlu4,6,7,8). More recently mGlu2 and mGlu3 have been characterized pharmacologically and physiologically using selective pharmacological ligands and transgenic knockout mice. Therapeutic interest in the group 2 receptors has derived from their distribution in key brain regions and behavioral activity in rodent models suggesting utility in pain, anxiety, depression, and psychosis (Li et al., 2015; Filippisa et al., 2014).

LY2812223, an mGlu2-preferring agonist, has recently been described that provides a unique selective orthosteric ligand for target validation and preclinical studies (Monn et al., 2015). The pharmacology of this ligand was initially evaluated using recombinant receptors expressed in mammalian cell lines where it exhibited maximally efficacious human mGlu2 receptor mediated responses in both cAMP and Ca2+ FLIPR formats, but only partial agonist (cAMP) or no agonist (Ca2+ FLIPR) responses in cells expressing human mGlu3 receptors. Moreover, behavioral efficacy was observed in rodents indicating central mGlu2 engagement. However, the pharmacology was not assessed across several signaling assays in different animal
species typically used in the path towards clinical development including non-human primate and human brain tissue. The cell line constructs were engineered to optimize ligand discovery and development and may misreport the pharmacology measured in native tissue assays. For example, the cell lines used in the drug screening campaign and ligand optimization involved co-expression of each of the mGlu family members along with RGT (rat glutamate transporter to reduce extracellular glutamate), and $G_{\alpha{15}}$ (promiscuous G protein to allow signaling through calcium mobilization). These highly engineered cell lines over-express the receptor proteins, may not express brain cell-related protein partners, and may lack contextually-relevant signaling proteins. Here we report the pharmacological profiling of LY2812223 in several cell line signaling assays as well as native tissue assays derived from rodent, primate, and human brain to address these potential discrepancies. Through this effort, we discovered unexpected partial agonist pharmacology across all species in all assays tested and thus show reasonable fidelity of translational pharmacology in animals and human brain tissue.
Materials and Methods

Materials:

GTP-γ-[35S] was purchased from PerkinElmer, Melville NY. LY2812223 ((1R,2S,4R,5R,6R)-2-Amino-4-(1H-1,2,4-triazol-3-ylsulfanyl)bicyclo[3.1.0]hexane-2,6-dicarboxylic acid), LY379268 ((1R,4R,5S,6R)-4-Amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid), LY404039 ((1R,4S,5S,6S)-4-Amino-2-thiabicyclo[3.1.0]hexane-4,6-dicarboxylic acid 2,2-dioxide) and LY341495 ((2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid) were synthesized by Eli Lilly & Co.

GTP-γ-[35S] Binding Studies (GTPγS):

The measurement GTP-γ-[35S] binding (GTP) to native membranes was performed as previously described (DeLapp et al., 1999) with the following modifications. Transgenic mice lacking mGlu2 (mGlu2−/−) or mGlu3 (mGlu3−/−) receptors were generated as previously described (Linden et al., 2005; Wright et al., 2013). All other fresh frozen tissues were purchased ABS (Analytical Biological Services, Inc). Membranes were made using pre-cooled 10mls of sucrose buffer (10mM HEPES, 1mM EGTA, 1mM DTT, 10% sucrose, and 1 tablet/50ml of Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN). Each tissue sample was homogenized in a glass homogenizer for 15 strokes on ice then the homogenate was centrifuged at 800 x g at 4°C for 15 minutes. The supernatant was placed into a new tube then centrifuged at 27,000 x g for 20 minutes at 4°C. The pellet was re-suspended in 15ml of buffer containing 10mM HEPES, 10mM MgCl2, 1mM DTT, and 0.5mM EDTA (pH 7.4), and centrifuged at 27,000 x g for 20 minutes at 4°C. The pellet was re-suspended in 1.6ml of suspension buffer and
the protein concentration measured using the Bradford method with BSA standards. Samples were aliquoted and stored at -80°C.

The assay buffer contained 20mM HEPES, 100mM NaCl, 5mM MgCl₂ and was adjusted to pH 7.4 with 5M NaOH, then stored at 4°C. The assay was initiated with the addition of 100µl of diluted membrane (5µg/well final concentration) with GDP (1µM) into a 96 well plate. Subsequently, 50µl of 400µM L-glutamic acid solution or buffer solution was added to each well and mixed. 50µl of GTP-γ-S [³⁵S] was then added to reach a final concentration of 500pM and then allowed to incubate for 120 minutes at room temperature. Twenty microliters of 3% NP40 was added, the solution mixed well and incubated for 30 minutes at room temperature. Twenty microliters of anti-Gᵢ antibody was then added and mixed well and left to incubate for 60 minutes at room temperature followed by 50µL anti-rabbit SPA beads (Final incubation volume is 290µl). The plate was covered with sealing tape and vortexed for 10-15 seconds and incubated at room temperature for 3 hours before centrifugation for 10 minutes at 184 x g. The amount of radioactive GTP bound to the membrane was measured using a Wallac MicroBeta (³⁵S, 1 min/well)(PerkinElmer, Melville, NY).

**Calcium Mobilization Assays:**

AV12 cells stably expressing the rat glutamate transporter (EAAT1), Gᵢ₁₅ subunit, and either the human mGlu2 or mGlu3 receptors (mGlu2-AV12, mGlu3-AV12) were created as previously described (Schoepp et al., 1997). Gᵢ₁₅ expression allows these Gᵢ-coupled receptors to signal through the phospholipase C pathway, resulting in ability to measure receptor activation via the calcium flux assay. Twenty-four hours before assay, cells were plated at 85,000 (mGlu2) or 115,000 (mGlu3) cells/well into 96-well, black-walled, poly-D-lysine-coated plates and
Compounds were tested in 10-pt concentration response curves using 3X serial dilution. Intracellular calcium levels were monitored before and after the addition of compounds using Fluo-3 AM dye (Thermo Fisher Scientific) in a FLIPR instrument (Molecular Devices, Sunnyvale, CA). The maximal response (EC$_{max}$) was defined using 100µM glutamate. Compound effect was measured as max minus min peak heights in relative fluorescent units (RFUs) corrected for basal fluorescence in the absence of glutamate. Agonist effects were quantified as % stimulation induced by compound alone relative to the maximal glutamate response. All data were calculated as relative EC$_{50}$ values using a four-parameter logistic curve fitting program (Prism v6.05 GraphPad Software Inc., La Jolla, CA).

**cAMP Assays:**

Twenty-four hours before assay the human mGlu2-AV12 and mGlu3-AV12 cell lines were plated at a density of 8-10,000 cells/well (mGlu2) or 6-8,000 cells/well (mGlu3) in tissue culture treated, 96-well, half-area black plates and incubated in medium containing 250µM (mGlu2) or 125µM (mGlu3) glutamine. Compounds were tested in 10-pt concentration response curves using 3X serial dilution. The final reaction mixture contained 1µM forskolin (Sigma F6886) and up to 25µM of compound. Reactions were incubated at 37˚C for 20 minutes. DCG-IV was used as a positive control. Cell lysates were assayed using cyclic AMP cell-based assay kit (Cisbio Assays) for visualization of signal at room temperature for 1 hour. The HTRF signal (ratio of fluorescence at 665 to 620 nM) was detected with an EnVision plate reader (PerkinElmer). Raw data were converted to pmol/well of cAMP with a cAMP standard curve generated for each experiment. Relative EC$_{50}$s were calculated from the top-bottom range of the
concentration response curve using a four-parameter logistic curve fitting program (Prism v6.05 GraphPad Software Inc.).

**Impedance Assay:**

Human mGlu2-AV12 and mGlu3-AV12 cells were grown in the appropriate growth media prior to plating and harvested when between 50-80% confluent. Prior to plating, CellKey 96-well plates (Molecular Devices) were coated with poly-d-lysine (10 mg/ml for 2 hours). Cells were plated at 200,000 cells/well in 150μl of growth media in the afternoon. Following plating, cells were allowed to attach for 20 minutes at room temperature then placed in an 37°C incubator and grown overnight. The next morning, each plate was placed on the CellKey instrument and washed 3 times with assay buffer (DMEM/F12 media containing 20mM HEPES) and then incubated at 37°C incubator for 1 hour. After this incubation, the cell plate and compound plate were placed back into the CellKey instrument for compound addition. Data were analyzed by Graphpad software.

**Rat Cortical Synaptosomes cAMP Assay:**

Rat cortical synaptosomes were prepared essentially as described by Dunkley et al. (Dunkley et al., 1988). Ten-week old Sprague-Dawley rats were sacrificed by decapitation, the brains surgically removed, and the cortex collected and rinsed several times in ice-cold homogenizing buffer (320mM sucrose, 1mM EDTA, 5mM Tris, pH 7.4). The tissue was cut into small pieces, homogenized using 10 strokes in a Dounce glass homogenizer, and centrifuged at 1600 x g for 10 minutes at 4°C. The supernatant was layered over the top of a three-step Percoll gradient (3, 15, 23%), and spun at 48,000 x g for 5 minutes at 4°C. Fractions F2-F4 were collected and pooled, diluted to 35-40mls with ice-cold homogenizing buffer, and centrifuged at 31,000 x g for 30 minutes at 4°C. The pellet was re-suspended in 35-40mls of washing buffer.
(140mM NaCl, 5mM KCl, 1mM MgCl₂, 1.2mM NaH₂PO₄, 5mM NaHCO₃, 10mM glucose, and 10mM HEPES, pH 7.4), pelleted at 27,000 x g for 10 minutes at 4°C, and re-suspended in 1-2mls of washing buffer. Protein concentration was determined using the Pierce Coomassie Plus assay. Synaptosomes were diluted to 200µg/mL in washing buffer and stored on ice. Compounds were solubilized in 0.1N NaOH as 10mM stocks, stored at -20°C, and serially diluted in assay buffer supplemented with 20mM HEPES buffer at the start of each experiment. The reversal of forskolin-stimulated cAMP production was conducted using the same HTRF methodology as described for recombinant cells. In this case, each well contained 5 µg of purified synaptosomes, the reaction was conducted for 1 hour at 37°C, and the incubation with cAMP-d2 conjugate and anti-cAMP cryptate conjugate was conducted for 2 hours prior to signal detection.

**Electrophysiology:**

The methods for these experiments are described in detail previously (Rorick-Kehn et al., 2007). Briefly, coronal brain slices 350 µm in depth containing the RTN and VB nuclei of the thalamus were prepared from 12-16 day old male Sprague-Dawley rats. Slices were placed into a submersion-type recording chamber mounted on an upright microscope and continuously superfused (2-3 ml/min) with oxygenated extracellular recording solution containing: 124mM NaCl, 26mM NaHCO₃, 3mM KCl, 10mM glucose, 2.3mM CaCl₂, 1.3mM MgCl₂, 0.4% ascorbic acid, pH = 7.3, osmolarity = 300 mOsm/liter). The whole-cell variant of the patch-clamp technique was used for recording current signals from VB thalamic neurons. Patch pipettes filled with a solution containing: 130mM CsMeSO₃, 4mM CsCl, 5mM MgCl₂, 10mM EGTA, 5mM Na₂ATP, 1mM Na₂GTP; pH adjusted to 7.3 with CsOH, osmolarity of 300 mOsm/liter. Current signals were amplified, low-pass filtered at 5kHz, digitized with an analog-to-digital
converter and collected onto a computer hard-drive. Series resistance (10-30MΩ) compensation was monitored and recordings displaying >30% change in resistance were not included in subsequent analyses. Inhibitory Postsynaptic Currents (IPSCs) were evoked from VB neurons (holding potential -20 mV) using a stimulator triggering a constant current unit to deliver single stimulation pulses (100µs, 200-2000µA) to bipolar stimulating electrodes positioned proximal (50-200µm) to recording electrode. For all experiments, the GABA<sub>A</sub> receptor-mediated responses were isolated from glutamatergic γ-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptor-dependent responses by addition to the extracellular solution of LY300168 (20µM) and DL-AP5 (50µM) which are selective antagonists of these receptors, respectively. In addition, possible confounding effects of GABA<sub>B</sub> receptor-mediated responses was eliminated by including the selective GABA<sub>B</sub> receptor antagonist, SCH50911 (10µM) in the extracellular recording solution.
Results

The *in vitro* pharmacology of the novel mGlu2-prefering agonist, LY2812223, was investigated along with the control pharmacologically balanced mGlu2/3 agonist, LY379268, in cell lines stably expressing the human mGlu2 and mGlu3 subtypes across several signaling assays (Fig 1, Table 1). GTP-γ-[35S] binding assesses direct Gᵢ protein activation by the mGlu subtypes, an event proximal to receptor stimulation. The FLIPR assay measures downstream intracellular calcium mobilization coupled through an artificially expressed calcium-coupling G protein, Gᵤ₁₅. The cAMP assay measures Gᵢ-mediated decreases in the cyclic nucleotide formation following activation of the signaling enzyme, adenylyl cyclase. The CellKey assay measures impedance changes through plate sensors which are general cellular activation signals.

As previously observed in the mGlu2 receptor expressing cell line, LY2812223 and the control pharmacologically balanced, maximally efficacious mGlu2/3 agonist LY2379268 generated agonist concentration response curves with efficacies in the range of 80-100% (100% being defined as a saturating glutamate response). In contrast, LY2812223 was not active at the cell line expressing mGlu3 receptors, except in the highly amplified cAMP assay where it displayed a partial agonist response. Under identical conditions LY379268 elicited full agonist responses.

A chemogenomic approach was used to further validate the selectivity of LY2812223 (Fig. 2, Table 2). LY2812223 was active as a partial agonist in membranes derived from brain cortex from the WT and mGlu3-KO mouse but not in mGlu2-KO membranes employing GTP-γ-[35S] binding as the functional readout. In contrast, the dual mGlu2/3 agonist LY379268 was active in both KO systems (66-76% efficacy).
Furthermore, the pharmacology was assessed subsequently in rat brain cortex membrane preparations (Fig. 3, Table 3). Both LY2812223 and LY379268 fully displaced the mGlu2/3 agonist radioligand, [3H]-LY459477, with IC$_{50}$ values of 114nM and 15nM, respectively, indicating that each was binding to the same glutamate orthosteric domain with high affinity (Fig. 3, Table 3). In rat cortical brain membrane preparations, LY2812223 demonstrated partial agonist (37% efficacy) compared to the full agonist response of LY379268 in the GTP-γ-[35S] binding assay (93% efficacy). This phenomenon was similarly observed in the rat cortical synaptosome preparation measuring changes in cAMP production where LY2812223 behaved as a partial agonist (63% efficacy) compared to the full agonist response of LY379268 (90% efficacy). LY2812223 also displayed a modest suppression of GABAergic synaptic transmission in the ventrobasal (VB) thalamus, consistent with a partial agonist effect on mGlu2/3 receptors.

LY2812223 and LY379268 were finally assessed by the GTP-γ-[35S] binding assay in cortical membranes derived from both non-human primates (NHP) and human post mortem brain samples (Fig. 4, Table 4). As was observed in rodent brain tissue preparations, LY2812223 elicited partial, and LY379268 maximal agonist responses in both NHP and human brain membranes respectively.
Discussion

LY2812223 is a recently described novel, potent and maximally efficacious mGlu2-preferring agonist (Monn et al., 2015). The pharmacological selectivity and efficacy was initially defined primarily in recombinant cell lines stably expressing the mGlu family of receptors. However, the pharmacology of this drug candidate was not evaluated previously in native brain tissue preparations derived from species typically utilized to test compounds for toxicological, pharmacokinetic, biomarker and behavioral studies. A functional GTP-$\gamma$-[^35S] binding assay was employed that has been optimized to measure GPCR activation or inhibition in brain membranes derived from multiple species (DeLapp et al., 1999; Porter et al., 2002; Overk et al., 2010). Comparisons to other signaling pathways were also assessed using mechanistically diverse signal transduction assays.

Pharmacological selectivity across species has been observed for other GPCR ligands creating unique challenges for these targets (Chan et al., 2008). Generally, GPCR drug screening is performed against the human recombinant receptor stably expressed at high levels in a clonal mammalian or insect cell line. The hits are then counter screened against other family member subtypes and a broad range of common GPCRs, ion channels, kinases, etc. to determine the selectivity profile. As physiologic assessment is done primarily in rodents, the hits are also counter screened against the rodent homolog of the target receptor. Due to cost and need for efficiency, rarely are hits also evaluated in functional assays across species used in subsequent drug development studies, unless issues arise. Here we have developed a facile functional GTP binding assay using brain membranes that provides pharmacological assessments across a variety of species reducing downstream drug development risk. Our results revealed an unexpected partial agonist-like pharmacology for LY2812223 in cortical tissues which were tightly
conserved with respect to both potency and efficacy across rat, mouse, monkey and human cortical brain tissues. These results provide confidence that studies across species, such as those involving behavioral efficacy, pharmacokinetic parameters, biomarker discovery, and toxicology would not be confounded based on pharmacologic disparities across species.

Drug discovery is currently experiencing a paradigm shift to include direct assessments of target engagement and subsequent activity within the target tissue, in this case cortical membranes, to properly interpret pharmacodynamic (PD) effects and efficacy both pre-clinically and clinically (Durham and Blanco, 2015). By doing so, data linking target engagement to PD effects and preclinical efficacy should enhance the clinical experiment outcome. To bridge this gap, we relied on the GTP binding assay to study the functional in vitro pharmacology of LY2812223 in native cortical membranes from several species. However, because native brain cortical tissue expresses both mGlu2 and mGlu3 subtypes, mGlu2 and mGlu3 knock out mice were used to assess selectivity. Consistent with the mGlu2-preferring agonist activity of LY2812223 in heterologous cell systems, GTP binding signal was lost in the mGlu2-KO mice, but not in the mGlu3-KO mice indicating that LY2812223 prefers the mGlu2 receptor subtype as measured by the GTP binding assay. In a recent autoradiographic study using a highly selective group-2 mGlu radioligand, [³H]LY459477, as well as mGlu2-, mGlu3-, and mGlu2/3 double-KO mice it was shown that mGlu2 and mGlu3 have a significant overlap in expression, particularly in cortical and hippocampal regions (Wright et al., 2013). We found that both LY379268 and LY2812223 completely displaced LY459477 binding in native rat cortical membranes, observations which are unsurprising given that these molecules exhibit similar affinity for both mGlu2 and mGlu3 subtypes (Monn et al., 1999, Monn et al., 2015). The observation of maximal agonist efficacy for LY379268 (a full agonist at both mGlu2 and mGlu3 receptors) but partial
agonist responses for LY2812223 (a full agonist at mGlu2 but low efficacy agonist at mGlu3) in tissues derived from WT mouse, rat, monkey and human is curious and may point to a functional interplay between mGlu2 and mGlu3 receptors in the cortex. While speculative, it is possible that this functional interaction arises from a physical mGlu2-mGlu3 heterodimer. Hetero- and homo-dimerization of mGlu receptors has been well documented with mGlu2-mGlu3 heterodimers being a preferred pairing (Doumazane et al., 2010). Partial agonist responses in native tissues where both of these receptors are expressed could conceivably be consistent with results from studies showing that hetero-dimerization can modify specificity and efficiency of signaling compared to homo-dimerized receptors through inter-subunit interactions and rearrangements (Kammermeier, 2012; Brock et al., 2007). If an mGlu2-mGlu3 heterodimer exists in the cortex, LY379268 would be expected to simultaneously bind to and activate both subunit proteins, whereas LY2812223 would be expected to bind to both subunits, but activate only one half of the dimeric complex. Possible effects of LY2812223 on native mGlu3 receptors were further studied using an electrophysiology rat brain thalamic slice preparation which express mGlu3 receptors on the synaptic terminals of reticular thalamic nucleus (RTN) GABAergic afferents and has been shown to reduce GABAergic transmission in ventrobasal thalamic relay neurons (Baez et al., 2005). Consistent with partial agonist effect on mGluR3-mediated cAMP, LY2812223 produced a submaximal effect on RTN GABAergic synaptic transmission. By comparison, the full mGlu2/3 agonist, LY404039 produced a greater reduction GABAergic transmission.

While the GTP binding assay can indicate target engagement in native tissue, this occurs close to the receptor and does not provide any information into any downstream signaling events that may occur following activation of the receptor. To address this concern we looked at mGlu2
and mGlu3 signaling by LY2812223 using several downstream signaling assays. Potency values for LY2812223 are in the low nM range and thus similar across the different methods at the mGlu2 and mGlu3 receptors. However the percent efficacy was assay dependent which may be due to different receptor expression levels in cells utilized in the cAMP assay versus FLIPR assay, less efficient coupling of human mGlu2 receptors to Gαq (Ca2+, FLIPR) compared to Gi/o (cAMP) and the measurement of either an accumulated, amplified signal (cAMP, FLIPR) or an direct 1:1 binding relationship (GTP-γ-binding). LY2812223 was a maximally efficacious agonist in the cAMP, calcium mobilization and impedance assays in cells expressing mGlu2 receptors, though in the GTP assay it exhibited high, but not maximal agonist efficacy. Of note, partial agonism by LY2812223 was only observed at mGlu3 receptors when measured using an amplified (cAMP) assay format. In native membranes from KO mice, LY2812223 produced no discernable agonist response in mGlu2(-/-) underlining the requirement for high receptor reserve to observe an in vivo mGlu3 functional effect. In native brain slice preparation derived from the ventroposteriomedial and ventroposteriolateral relay neurons known to express predominantly mGlu3 receptors, a modest signal was detected (Fig. 3). Given the lack of mGlu2/3 radiotracer binding in this region in mGlu3 KO mice (Wright et al., 2013), a clear explanation for this weak and submaximal agonist response (65.7 + 8.2% of control at 30 mM) is not evident. It is conceivable that, similar to what is observed in the ectopically expressed mGlu3 cAMP assay where LY2812223 exhibited a weak partial (60%) agonist response (Monn et al., 2015), a high level of receptor reserve in these mGlu3-containing thalamic neurons might be amplifying an otherwise weak mGlu3 agonist response in this tissue, though this remains a point of speculation.

Taken together, these studies lend confidence that the predominant pharmacological profile of LY2812223 is best described as an mGlu2-preferring receptor agonist with likely very
low partial mGlu3 agonist activity. This pharmacology is unique in this regard compared to other agonist ligands within this structural class. Additional studies are warranted in native tissues and animal behavioral models sensitive to mGlu2 pharmacology to better understand the influence of brain region/cell specific co-expressed receptors and accessory proteins on the ultimate pharmacology of therapeutic ligands. These studies also support following a translational drug development program in which multiple species may be used to assess efficacy, pharmacokinetics, toxicology, and biomarker development.

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*Conducted experiments and data analysis:* Tu, Schober, Quets, Xiao, Watt, Siuda, Sanger

*Wrote or contributed to the writing of the manuscript:* Felder, Schober, Nisenbaum, Heinz, McKinzie, Prieto, Monn, Quets
References


potent and highly selective group II metabotropic glutamate receptor agonist in cells expressing human glutamate receptors. Neuropharmacology 36: 1-11.


Figure Legends

Fig. 1. **Comparison of agonist-stimulated functional responses between different *in vitro* assay methodologies.** Left: human mGlu2 receptors. Right: human mGlu3 receptors. The human mGlu2 and mGlu3 receptors were stably expressed in AV12 cells. Agonist compounds used were: LY2812223 (panels A and B) and the mGlu2/3 full agonist LY379268 (panels C and D). Responses were expressed as percentage of maximal stimulation activity in presence of 100 µM of the orthosteric agonist glutamate. Stimulation curves were analyzed by nonlinear regression analysis, and are the results of independent experiments performed in duplicate. The symbols and error bars are expressed as the mean ± SEM, respectively.

Fig. 2. **Agonist functional GTP-γ-S-[35S] binding to native tissue cortical membranes derived wild-type, mGlu2 and mGlu3 knockout mice** Tissue were collected from wild-type (closed circle), mGlu2-KO (open circle), mGlu3-KO (closed square) mice. Agonists LY2812223 (panel A) and LY379268 (panel B) results are shown. Data shown are the result of 2-4 independent experiments. The symbols and error bars are expressed as the mean ± SEM, respectively.

Fig 3. **Comparison between LY2812223 and LY379268 in rat brain** Radioligand binding from rat cortical membranes are shown in the top left (panel A) and functional GTP-γ-S-[35S] binding shown on the top right (panel B). Rat synaptosomes are depicted on the bottom left (panel C) and the effects of LY404039 and LY2812223 on mGlu3-mediated modulation of GABAergic synaptic inputs from rat brain slices are shown on the bottom right (panel D).

Fig. 4. **Agonist functional GTP-γ-S-[35S] binding to native tissue cortical membranes derived Non-human Primate (NHP) and Human** Tissue were collected from human postmortem cortex (open triangle), and NHP (closed triangle). Agonists LY2812223 (panel A) and
LY379268 (panel B) results are shown. Data shown are the result of 2-4 independent experiments. The symbols and error bars are expressed as the mean ± SEM, respectively.
Table 1. Summary of agonist response data for LY2812223 and LY379268 in hmGlu2 and hmGlu3 expressing cells

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<td>Emax ± SEM (%)</td>
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<td>LY2812223</td>
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<td>cAMP</td>
<td>5.6 ± 0.5</td>
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<td>$Ca^{2+}$</td>
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<td>$Ca^{2+}$</td>
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Table 2. **Summary of GTPγS binding in mouse cortical tissues for agonist response to LY2812223 and LY379268**

<table>
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<tr>
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<td></td>
<td>EC₅₀ ± SEM (nM)</td>
<td>Emax ± SEM (%)</td>
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<td>EC₅₀ ± SEM (nM)</td>
<td>Emax ± SEM (%)</td>
</tr>
<tr>
<td>WT</td>
<td>23.3 ± 4.2</td>
<td>30.5 ± 6.0</td>
<td>6.48 ± 1.09</td>
<td>1.2 ± 0.3</td>
<td>36.8 ± 8.5</td>
<td>53 ± 6</td>
<td>76.5 ± 7.2</td>
<td>107 ± 2</td>
</tr>
<tr>
<td>mGlu2 (-/-)</td>
<td>N/D</td>
<td>N/D</td>
<td>8.51 ± 1.8</td>
<td>5.1 ± 2.4</td>
<td>X%</td>
<td>7%</td>
<td>65.5 ± 5.5</td>
<td>93 ± 7</td>
</tr>
<tr>
<td>mGlu3 (-/-)</td>
<td>46.4 ± 7.3</td>
<td>20.6 ± 2.0</td>
<td>6.19 ± 1.4</td>
<td>2.8 ± 1.6</td>
<td>60.6 ± 9.9</td>
<td>86 ± 15</td>
<td>75.6 ± 7.5</td>
<td>120 ± 7</td>
</tr>
</tbody>
</table>
Table 3. **Summary of pharmacology in rat cortical tissue**

<table>
<thead>
<tr>
<th></th>
<th>[³H]-LY459477 Binding</th>
<th>GTPγS Binding</th>
<th>Inhibition of Forskolin-Stimulated cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ki ± SEM (nM)</td>
<td>EC₅₀ ± SEM (nM)</td>
<td>EC₅₀ ± SEM (nM)</td>
</tr>
<tr>
<td></td>
<td>N=4</td>
<td>Emax ± SEM (%)</td>
<td>Emax ± SEM (%)</td>
</tr>
<tr>
<td>LY2812223</td>
<td>114.3 ± 17.4</td>
<td>40.1 ± 13.3</td>
<td>16.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37.3 ± 8.4</td>
<td>62.9 ± 4.1</td>
</tr>
<tr>
<td>LY379268</td>
<td>14.8 ± 0.5</td>
<td>7.6 ± 1.9</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>92.8 ± 4.4</td>
<td>96.4 ± 7.8</td>
</tr>
</tbody>
</table>
Table 4. Summary of GTPγS binding agonist response data for LY2812223 and LY379268 in monkey and human cortical membranes

<table>
<thead>
<tr>
<th></th>
<th>LY2812223</th>
<th></th>
<th>LY379268</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC$_{50}$ ± SEM (nM)</td>
<td>Emax ± SEM (%)</td>
<td>EC$_{50}$ ± SEM (nM)</td>
<td>Emax ± SEM (%)</td>
</tr>
<tr>
<td>Monkey</td>
<td>13.5 ± 1.39</td>
<td>39.8 ± 5.7</td>
<td>4.31 ± 0.67</td>
<td>101.5 ± 10.1</td>
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<tr>
<td>Human</td>
<td>7.57 ± 2.73</td>
<td>49.2 ± 10.6</td>
<td>5.73 ± 1.34</td>
<td>74.9 ± 12.3</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2

A. LY2812223 (n=3-4)

B. LY379268 (n=3-4)
Fig. 3

A. Rat Cortical Membranes (n=4)

\[
\text{[H]-LY459477 Bound (\% of B_0)}
\]

- LY2812223 (K_i = 110.8 nM)
- LY379268 (K_i = 14.7 nM)

B. Rat Cortical Membranes (n=4)

\[
\text{GTP[\gamma]S Binding (% of Control)}
\]

C. Rat Cortical Synaptosomes (n=3)

\[
\text{% of 379268 inhibition}
\]

D. Rat Thalamic Brain Slices (n=5)

\[
\text{Percent Control IPSC Amplitude}
\]

- LY404039
- LY2812223

Log [Compounds], M

Log [Compounds], M

Compound Concentration (mM)
Fig. 4