Psychopharmacology (2005) 179: 271–283
DOI 10.1007/s00213-004-2099-9

ORIGINAL INVESTIGATION

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# Metabotropic glutamate 2 receptor potentiators: receptor modulation, frequency-dependent synaptic activity, and efficacy in preclinical anxiety and psychosis model(s)

Received: 28 June 2004 / Accepted: 5 November 2004 / Published online: 17 February 2005


**Abstract** *Rationale:* To increase subtype selectivity and provide a novel means to alter receptor function, we discovered and characterization potentiators for the metabotropic glutamate 2 receptor (mGlu2). *Methods and results:* A class of 3-pyridylmethylsulfonamides (e.g., 3-MPPTS; 2,2,2-trifluoro-*N*-[3-(2-methoxyphenoxy)phenyl]-*N*-(3-pyridinylmethyl)-ethanesulfonamide) were found to be potent, subtype-selective potentiators of human and rat mGlu2. The sulfonamides increased agonist potency in functional assays but did not displace orthosteric radiolabeled antagonist or agonist binding to cloned mGlu2 receptors. Rather, the modulators increased the affinity of most of the orthosteric agonists including glutamate, DCG-IV (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxylcyclopropyl)glycine), and LY354740 (1*S*,2*S*,5*R*,6*S*-2-aminobicyclo[3.1.0]hexane-2,6-bicaroxylate monohydrate). In striatal brain slices, LY354740 inhibited evoked excitatory postsynaptic potentials (EPSPs) equally well following either a low- (0.06 Hz) or high (4 Hz)-frequency stimulation of corticostriatal afferents. In contrast, the mGlu2 potentiator cyPPTS (2,2,2-trifluoro-*N*-[3-(cyclopentyloxy)phenyl]-*N*-(3-pyridinylmethyl)-ethanesulfonamide) inhibited striatal EPSPs only at higher frequencies of stimulation (2 and 4 Hz). Several sulfonamides including 4-MPPTS, 4-APPES (*N*-[4-(4-carboxamidophenoxy)phenyl]-*N*-(3-pyridinylmethyl)-ethanesulfonamide hydrochloride monohydrate) and/or CBiPES *N*-[4′-cyano-biphenyl-3-yl)-*N*-(3-pyridinylmethyl)-ethanesulfonamide hydrochloride) were tested in mGlu2/3 agonist-sensitive rodent model(s) of anxiety and psychosis. As seen with LY354740, both 4-MPPTS and 4-APPES were efficacious in a rat fear-potentiated startle paradigm. Likewise in mice, CBiPES attenuated a stress-induced hyperthermia and PCP-induced hyperlocomotor activity. Furthermore, CBiPES mediated alteration in PCP-induced hyperlocomotor activity was sensitive to mGlu2/3 antagonist pretreatment. *Conclusions:* Taken together, the data indicate mGlu2 receptor potentiators have a unique use-dependent effect on presynaptic glutamate release, and show efficacy in several mGlu2/3-sensitive animal models of psychiatric disorders.

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## Introduction

Our current understanding of the significant roles played by various ionotropic (iGlu) and metabotropic (mGlu) glutamate receptors is due in no small part to the discovery, biochemical characterization and in vivo effects of group- and subtype-selective agents for these receptors. An example of this is found with the Group II mGlu selective agonists such as LY354740 (1*S*,2*S*,5*R*,6*S*-2-aminobicyclo[3.1.0]hexane-2,6-bicaroxylate monohydrate), and LY379268 (1*R*,4*R*,5*S*,6*R*-4-amino-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid) (Monn et al. 1999; Monn et al. 1997). Since the discovery of these peripherally active and potent mGlu2 and -3 receptor agonists, well over 100 publications have appeared utilizing these compounds to investigate the role of group II mGlu receptors in neurotransmission. Many of these have focused on the psychopharmacological actions of the mGlu2/3 agonists in animal models and more recently in human clinical trials.

For instance, LY354740 and/or LY379268 have been shown to be efficacious in anxiety models such as: fear-induced potentiated startle (Helton et al. 1998; Monn et al. 1997; Tizzano et al. 2002; Walker and Davis 2002), elevated plus maze (Ferris et al. 2001; Helton et al. 1998;

Monn et al. 1997), stress-induced hyperthermia and monoamine release (Spooren et al. 2002; Swanson et al. 2004), and lactate-induced anxiety (Shekhar and Keim 2000). Most significantly, the mGlu2/3 agonists are anxiolytic in human paradigms such as potentiated startle (Grillon et al. 2003) and $CO_2$-induced panic attacks (Schoepp et al. 2003). Similarly, the mGlu2/3 agonists have shown activities that are characteristic of the atypical antipsychotics. LY354740 and/or LY379268 are active in reversing psychostimulant (e.g., PCP and amphetamine)-mediated locomotor (Cartmell et al. 1999, 2000a,b,c; Clark et al. 2002; Kim and Vezina 2002; Swanson and Schoepp 2002) and rearing behaviors, as well as increasing the release of prefrontal cortex dopamine and other limbic nuclei monoamines (Cartmell et al. 2000d,e, 2001; Lorrain et al. 2003).

Clearly, this strongly supports the hypothesis that group II mGlu agonists play a key role in regulating anxiety responses and may also be effective in psychosis, but the relative contribution of mGlu2 and mGlu3 receptors in these actions have not always been clear. Indeed, there has been little success in the identification of orthosteric (i.e., glutamate site) compounds that show selectivity for mGlu2 versus mGlu3 receptors that could be utilized to pharmacologically differentiate these receptors. However, the combination of knockout mice and the group II agonists has begun to provide some insights to these questions. In utilizing mGlu2 knockout mice, the reversal of PCP-induced locomotor activity with LY354740 appears dependent upon mGlu2 receptors (Spooren et al. 2000). However, developmental or compensatory alterations in neuronal circuitry cannot be ruled out as an explanation. Thus the lack of efficacy with mGlu2/3 agonists in mGlu2 knockout mice cannot eliminate the possibility that mGlu3 activation plays a key role when combined with mGlu2 activation in wild-type animals.

Recently, several highly subtype-selective allosteric modulators of mGlu receptors have been described. This includes positive allosteric modulators of the Group I receptors mGlu1 (Knoflach et al. 2001; Wichmann et al. 2002) and mGlu5 (Goudet et al. 2004; O'Brien et al. 2003; Williams et al. 2003), and a group III receptor mGlu4 (Maj et al. 2003; Marino et al. 2003; Mathiesen et al. 2003). All of these modulators have a unique pharmacological action of potentiating the responses of agonists such as glutamate without activating the receptors alone. Similarly, recent reports have described positive allosteric modulators of the mGlu2 receptor discovered at Lilly Research Laboratories (Johnson et al. 2003; Schaffhauser et al. 2003). The present report further describes some of the in vitro biochemical, electrophysiological, and behavioral effects of a number of mGlu2 potentiators within this chemical series.

## Materials and methods

### Materials

$^3$H-DCG-IV (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxylcyclopropyl)glycine) was obtained from Tocris (Ellisville, MO) at a specific activity of 18.3 Ci/mmol. Unlabelled DCG-IV, and 1*S*,3*R*-ACPD (1-amino-1,3-cyclopentanedicarboxylic acid) were also obtained through Tocris. Diazepam and PCP (phencyclidine) were obtained from RBI-Sigma (Natick, MA, USA). 4-MPPTS (LY487379; 2,2,2-trifluoro-*N*-[4-(2-methoxyphenoxy)phenyl]-*N*-(3-pyridinylmethyl)-ethanesulfonamide), 3-MPPTS (2,2,2-trifluoro-*N*-[3-(2-methoxyphenoxy)phenyl]-*N*-(3-pyridinylmethyl)-ethanesulfonamide), cyPPTS (2,2,2-trifluoro-*N*-[3-(cyclopentyloxy)phenyl]-*N*-(3-pyridinylmethyl)-ethanesulfonamide), 4-APPES (*N*-[4-(4-carboxamidophenoxy)phenyl]-*N*-(3-pyridinylmethyl)-ethanesulfonamide hydrochloride monohydrate), and CBiPES (*N*-[4′-cyano-biphenyl-3-yl)-*N*-(3-pyridinylmethyl)-ethanesulfonamide hydrochloride) were synthesized at Lilly Research laboratories as previously described (Barda et al. 2004; Johnson et al. 2003). LY354740, LY379268, and LY341495 ((αS)-α-amino-α-[(1*S*,2*S*)-2-carboxycyclopropyl]-9*H*-xanthene-9-propanoic acid) were synthesized at Lilly Laboratories (Eli Lilly, Indianapolis, IN). Compounds diazepam, 4-MPPTS and 4-APPES were prepared for injection intraperitoneally or subcutaneously (s.c.) by suspending in a 5% ethanol, 0.5% carboxy-methylcellulose, 0.5% Tween 80. CBiPES was prepared for intraperitoneal injection by suspending in 25% β-cyclodextran. Compounds LY354740, LY379268, and LY341495 were prepared in sterile water.

All procedures involving the use of animals were routinely reviewed by the animals care and use committee and followed the NIH guidelines of 2003.

### Intracellular calcium release in clonal cell lines expressing the human mGlu2 receptors

The potentiators were tested in cell lines that expressed each of the eight human mGlu receptor, mGlu1a, mGlu2, mGlu3, mGlu4a, mGlu5a, mGlu6, mGlu7a, or mGlu8a, in an AV-12 (ATCC-CRL9595) cell background that also stably expresses the rat glutamate aspartate transporter (EAAT-1 or GLAST). With the group II and III receptors (mGlu2/3/4/6/7/8), the promiscuous G protein Gα15 was also stably expressed. This allowed receptor activation to stimulate phospholipase C, increasing inositol triphosphate formation, and subsequently a transient rise of intracellular calcium. Quantitation utilized to be determined using a calcium-sensitive dye and a Fluorometric Imaging Plate Reader (FLIPR) as described in Johnson et al. (2003).

### Radioligand binding of $^3$H-DCG-IV to the cloned human mGlu2 receptor

In order to determine the impact of allosteric modulators of agonist binding, the methods of Cartmell et al. (1998) were utilized with minor modifications. Briefly, mGlu2 receptors were labeled with $^3$H-DCG-IV (spec. act. 18.3 Ci/mmol) and affinity of various orthosteric agonists determined in the presence or absence of 3-MPPTS, in a scintillation proximity assay (SPA) method. Cell mem-

brane homogenates containing the mGlu2 receptor were prepared as described (Cartmell et al. 1998) and 25 μg of protein incubated in a 50 mM Tris–HCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$ pH 7.0 buffer at a total volume of 200 μl. Incubations included 0.25 nM $^3$H-DCG-IV, 1.0 mg of WGA-SPA beads (Amersham, Arlington Heights, IL). Each well contained either buffer, varying concentrations of orthosteric agonist, and/or 3 μM 3-MPPTS. Non-specific binding was defined with 10 μM LY354740. The affinity constants for orthosteric agonists, with and without 3-MPPTS present, were calculated using the Cheng and Prusoff equation ($K_i$=$IC_{50}$/(1+[$^3$H-DCG-IV]/$K_d$)) (Cheng and Prusoff 1973) and the $IC_{50}$ from a four-parameter fit of the displacement curves. A two-way analysis of variance (ANOVA) followed by a contrast analysis post-hoc comparison was performed using the program JMP (SAS Institute, Cary, NC).

## Striatal slices: preparation and recording procedures

Coronal striatal slices were prepared from young (14–22 days old) male Sprague–Dawley rats. Animals were sacrificed and their brains were removed and immersed in a cold (~2°C) $NaHCO_3$–buffered saline solution: 126 mM NaCl, 3 mM KCl, 1.5 mM $MgCl_2$, 1.25 mM $Na_2PO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose; pH=7.4, osmolarity=300±5 mosM. The brains were blocked and 400-μm-thick coronal sections were cut through the rostrocaudal extent of the striatum using a Vibroslice (Campden Instruments, London, England). Slices then were placed into the continuously oxygenated (95% $O_2$:5% $CO_2$) $NaHCO_3$–buffered saline solution warmed to 32°C for 30 min and then maintained at room temperature. After at least 1 h of incubation, individual slices were transferred to a recording chamber mounted on an upright microscope (BX50WI, Olympus Corporation) and continuously superfused (2–3 ml/min) with the oxygenated saline solution maintained at 30±0.2°C. Differential interference videomicroscopy (Dodt and Zieglgansberger 1990) was used to visualize medium spiny neurons in the striatum.

The whole-cell variant of the patch-clamp technique was used for voltage recording from striatal spiny neurons. Patch pipettes were fabricated from thin-walled borosilicate glass and had resistances of 1–4 MΩ when lowered into the $NaHCO_3$–buffered extracellular solution. The pipette solution contained: 130 mM $K^+$ gluconate, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES, 2 mM $Na_2ATP$, 0.3 mM $Na_2GTP$; pH 7.3, osmolarity of 290–300 mosM. Voltage-dependent $Na^+$ currents were blocked by inclusion of QX314 (1 mM) in the patch pipette solution. γ-Aminobutyric acid (GABA) synaptic transmission was eliminated using the selective antagonists of $GABA_A$ and $GABA_B$ receptors, bicuculline methiodide (1–2 μM) and SCH59011 (2 μM), 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, Axon Instruments, Foster City, CA). Series resistance (10–30 MW) 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 stimulation pulses (100 μs, 50–500 μA) delivered using bipolar electrodes, positioned ~200 μm from the recorded neuron. The effects of stimulation frequency was measured using 0.06, 1.0, 2.0, and 4.0 Hz stimulation protocols. For the 0.06 Hz stimulation protocol, ten stimuli were delivered. For other stimulation protocols, 30 stimuli were delivered. An average response to all stimuli was calculated for each neuron in response to each stimulation protocol during control and drug conditions.

## Fear-potentiated startle paradigm

The methods of Tizzano et al. (2002) were utilized. Briefly, male Sprague–Dawley rats weighing 325–400 g were purchased from Harlan Sprague–Dawley (Cumberland, IN) and given a 1-week acclimation period before testing. Rats were individually housed with food and water without restriction in an animal room on a 12-h light/dark cycle. The fear-potentiated startle paradigm was conducted over 3 consecutive days. All 3 days begin with a 5-min adaptation period before the trial starts. On day 1 (baseline startle) after the adaptation period, each animal received 30 trials of 120 dB auditory noise (for 50 ms) and the mean startle amplitude ($V_{max}$ or the peak voltage recorded through transducers located in the center of the platform) used to randomly assign animals to groups such that each group had a similar mean startle before conditioning began. Day 2 consisted of conditioning the animals. Each animal received 0.5 mA of shock for 500 ms preceded by a 5-s presentation of light (15 W), which remained on for the duration of the shock. Ten presentations of the light and shock were administered with an inter-trial interval of 10 s. Twenty-four hours after conditioning, the rats were administered vehicle or compound by the route and pretreatment time indicated. Effects of treatment(s) on baseline startle were determined from ten trials of acoustic startle (120 dB), non-light paired, presented at the beginning of the test session. None of the treatments were found to alter the unpaired startle amplitude in these experiments. This was followed by 20 random trials of the noise alone or noise preceded by the 5-s presentation of light. Excluding the first ten trials, the startle response amplitudes for each trial condition was averaged for each animal. Data were presented as the difference between light/noise and noise-alone. Differences in startle response amplitudes were analyzed by JMP statistical software using a one-way analysis of variance (ANOVA). Group differences were considered to be significant at $p<0.05$.

### Stress-induced hyperthermia in mice

The ability of stress to induce a transient rise in body temperature in DBA/2 mice as previously described (Borsini et al. 1989) was monitored as follows. A total of ten male DBA/2 mice were implanted with radiotelemetric transmitters (model # TA10TA-F40; Data Sciences International) into the peritoneal cavity and allowed at least 1 week to recover from surgery. Mice were single-housed and maintained on free food and water under a 12-h light–dark cycle with light onset at 0600 h. All studies were conducted between 1100 and 1700 h. The same mice were used in all the studies reported with dose group assignments counterbalanced across experiments. At least 3 days separated test days.

On each test day, mice received subcutaneous administrations of vehicle or CBiPES and remained in their home cages under low-light conditions for a baseline assessment period of 50 min. Following the baseline period, the room lights were turned on and mice were placed into a holding tub that contained soiled rat shavings for 30 min. Body temperature was recorded in 5-min intervals over the entire test period. Data were analyzed via a mixed ANOVA with dose group as the between-group factor and time as the repeated-measures factor. In cases of a significant main effect or interaction, a simple effects analysis was conducted to determine the source of the differences.

### Activity assessment after PCP treatment

The procedure of Cartmell et al. (1999) was modified for mice and utilized. Specifically, male ICR mice were group-housed (maximum of eight mice per cage) under standard laboratory conditions with unrestricted access to food and water (12 h light/dark cycle). Mice were injected intraperitoneally with compounds and the spontaneous locomotor activity monitored for a 45-min acclimation period in a transparent, plastic shoe-box cages of dimensions 45×25×20 cm, with ~1 cm depth of wood chips as bedding and a metal grill on top of the cage. Subsequently, the mice were given a subcutaneous injection of PCP (7.5 mg/kg) (Sigma, St. Louis, MO, USA) or sterile water (1 ml/kg), and returned to the shoe-box cages for immediate locomotor activity monitoring for 60 min. Motor monitors (Hamilton Kinder, San Diego, CA, USA) consisted of a rectangular rack of 12 photobeams arranged in an 8×4 formation. Shoe-box cages were placed inside this rack, enabling the activity of the mice to be monitored. The rack was positioned at a height of 3 cm, which allowed the detection of PCP-induced movements of the body of the mice. The number of beam breaks was quantitated for a 60-min period after PCP administration. Statistical analysis of behaviors was carried out using the Graphpad PRISM statistical program (Graphpad, San Diego, CA). Data were analyzed by a one-way ANOVA, and the post-hoc comparisons for each dose group versus control or PCP alone and compound were made using Newman–Keuls multiple comparison test ($p<0.05$ for significance).

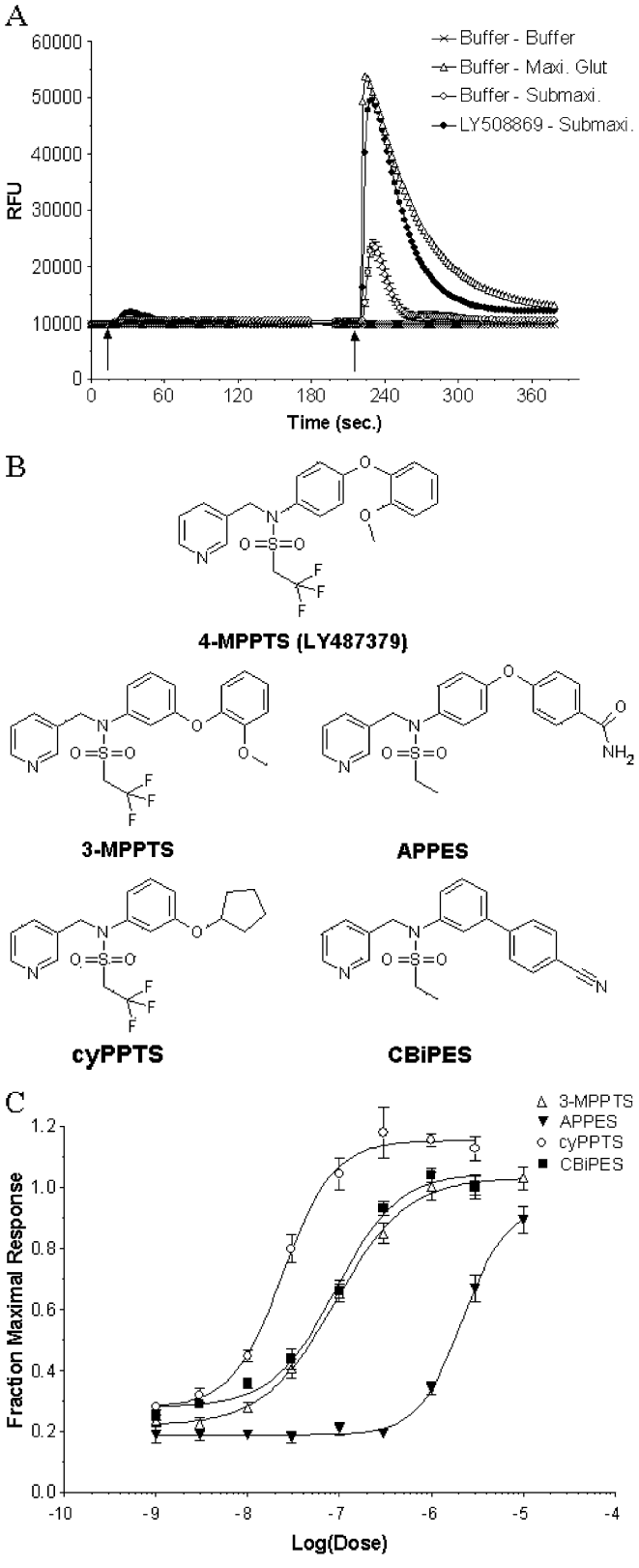
## Results

### Effects in a functional assay with cell lines expressing the human mGlu receptors

As shown in Fig. 1a, when given alone 3-MPPTS did not induce a significant response in cell lines expressing mGlu2 receptor. However, with the subsequent addition of glutamate at a submaximally effective concentration, a significantly elevated rise in intracellular calcium was evident. Thus a dose-dependent potentiation for 3-MPPTS, cyPPTS, 4-APPES, and CBiPES (Fig. 1c) was established with $EC_{50}$ values for potentiation of 154±13 nM (mean± SE, $n$=45), 24.0±0.6 nM ($n$=3), 2,260±195 nM ($n$=3), and 92.8±18.4 nM ($n$=3), respectively (Fig. 1b). When these potentiators were similarly tested in the other mGlu cell lines for either agonist, potentiator or antagonist activity, no activity was seen up to 30 μM in the mGlu1/3/4/5/6/7/8 cell lines. Furthermore, these compounds did not alter the response seen by activating either constitutively expressed adrenergic or purinergic receptors (data not shown), indicating a response specific to the mGlu2 receptor. Addition of the potentiator 3-MPPTS with varying concentrations of glutamate or the mGlu2 agonist 1S,3R-ACPD, resulted in a parallel left shift in the agonists dose–response curves without a significant change in the maximal response (Fig. 2a). Fitting a curve to the ratio of $EC_{50}$ values versus the concentration of potentiators (Fig. 2b) (Christopoulos and Kenakin 2002) resulted in a equilibrium constant for potentiation ($K_{pt}$) of 144±43 nM (mean± SE, $df$=12, $R^2$=0.92) and an $\alpha$ potentiation value (a measure of the degree of positive modulation) of 3.31±0.03.

### Effects on orthosteric agonist radioligand binding

In the mGlu2 clonal cell line, the agonist, $^3$H-DCG-IV, specifically bound to the glutamate site of the mGlu2 receptor as previously reported (Cartmell et al. 1998). In competition experiments, the mGlu2 potentiator, 3-MPPTS, did not inhibit the binding of $^3$H-DCG-IV (IC50 >30 μM), clearly indicating that 3-MPPTS does not directly interact with the mGlu2 receptor glutamate-recognition site contained within the amino-terminus. Indeed, the potentiator increased the binding of lower concentrations of $^3$H-DCG-IV. Saturation experiments with and without the potentiator indicated a modest but significant two-fold decrease in the $K_d$ for $^3$H-DCG-IV without a significant change in the $B_{max}$ value [Fig. 2c: buffer 21.8±3.0 ($n$=3), 3-MPPTS 22.4±3.0 ($n$=3) pmol/mg protein; $p>0.05$, Student's $t$-test]. This is in agreement with the results reported by Schaffhauser et al. (2003). When displacement competition curves were examined in $^3$H-DCG-IV-labelled binding sites, 3-MPPTS similarly increased the affinity of most orthosteric agonists for the cloned human mGlu2 receptor, including glutamate, 1S, 3R-ACPD, DCG-IV, and LY354740. However, there was no apparent change in affinity for the most potent mGlu2/3 agonist examined LY379268, nor the potent mGlu2/3 antagonist LY341495 (Table 1). The reason that




◀ **Fig. 1** Allosteric potentiators of the human mGlu2 receptor. **a** Representative transient increases in intracellular calcium levels as measured with Fluo-3 dye in a clonal cell line expressing the mGlu2 receptors (see Materials and methods). *Arrows* indicate the time that the indicated compounds (1, 10 μM glutamate and/or 10 μM 3-MPPTS) or buffer was added. **b** Chemical structures of some of the *N*-methylpyridine sulfonamide mGlu2 potentiators. **c** Representative dose–response determinations of the mGlu2 potentiators in a cell line expressing the human receptor. The concentration of the potentiators were varied and the subsequent response to 1 μM glutamate plotted. A four-parameter least-squares curve fitting routine was employed to determine the resulting $IC_{50}$ as indicated: 3-MPPTS 154 nM, 4-APPES 2,260 nM, cyPPTS 24.0 nM, and CBiPES 92.8 nM

of the orthosteric agonists examined only LY379268 did not show an apparent increase in affinity is unclear, but may relate to the sensitivity of the assay and the higher affinity of LY379268 for mGlu2 receptor.

## Frequency-dependent modulation of glutamatergic corticostriatal synaptic transmission by cyPPTS

Previous immunocytochemical (Petralia et al. 1996) and electrophysiological (Lovinger and McCool 1995) studies have suggested that mGlu2 receptors are located presynaptically on cortical glutamatergic afferents to striatal medium spiny neurons. Activation of these presynaptic receptors with selective group II agonists can suppress excitatory corticostriatal synaptic transmission (Lovinger and McCool 1995; Picconi et al. 2002). By virtue of its allosteric mechanism of action, cyPPTS would be expected to preferentially suppress corticostriatal synaptic responses during periods of enhanced, but not normal glutamate release, when the neurotransmitter would be able to diffuse away from the synapse and activate presynaptic mGlu2 receptors. In order to test this hypothesis, the effects of cyPPTS on excitatory postsynaptic potentials (EPSPs) in striatal neurons evoked in response to different frequencies of stimulation of glutamatergic cortical inputs were measured in a corticostriatal slice preparation. During conditions of low-frequency stimulation (0.06 Hz, $n$=5), cyPPTS (0.3–1.0 μM) did not alter EPSP amplitude. This contrasted with the marked reduction in EPSP amplitude produced by the selective mGlu2/3 agonist, LY354740 (0.03–1.0 μM), confirming the lack of agonistic properties of cyPPTS (Fig. 3a–c). In response to higher frequencies of stimulation (1.0–4.0 Hz), cyPPTS suppressed corticostriatal EPSPs evoked in a frequency- [$F(3,48)=20.1$, $p<0.0001$, $n$=5] and concentration-dependent manner [$F(2,48)=3.2$, $p<0.05$, $n$=5] (Fig. 3a and c). For example, 1 μM cyPPTS suppressed EPSP amplitudes by 19.5±4.9, 29.7±2.9, and 33.4±1.6% in response to 1, 2, and 4 Hz stimulation, respectively. The receptor selectivity of the frequency-dependent effect of cyPPTS was tested using the selective mGlu2/3 receptor antagonist, LY341495. Application of LY341495 (0.3 μM) alone did not alter the amplitude of corticostriatal EPSPs (data not shown), but did block the suppressive effects of cyPPTS in response to 4.0 Hz stimulation (Fig. 4a–c). The frequency-dependent effects of cyPPTS on cortically evoked EPSPs occurred in the absence of changes in resting membrane potential or input resistance of striatal neurons, supporting a presynaptic mode of action.

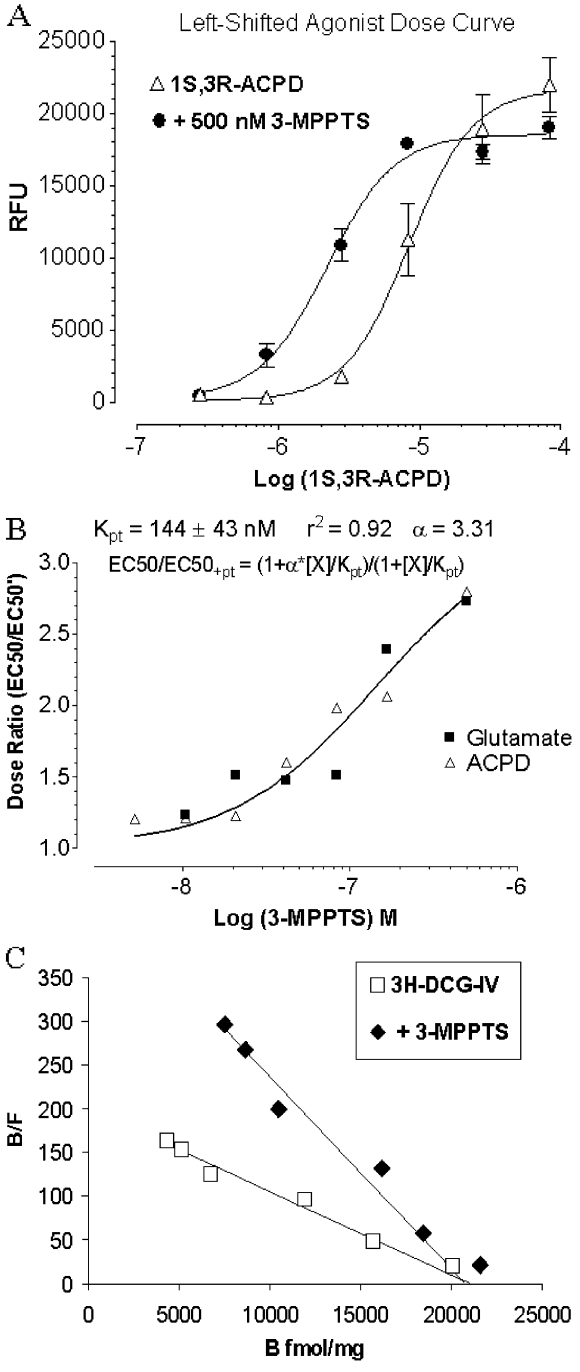



**Fig. 2** Allosteric potentiators of the human mGlu2 receptor: in vitro biochemical characteristics. **a** Example leftward shift in the orthosteric agonists dose–response curve for the calcium response in a mGlu2 expressing cell line. The approximately threefold parallel shift in the dose–response curve for 1*S*,3*R*-ACPD, with no apparent change in the maximal response. **b** *Graph* of the ratios of $EC_{50}$ values determined for glutamate and 1*S*,3*R*-ACPD at varying concentrations of the mGlu2 potentiator 3-MPPTS. A least-square fit of the data indicated an apparent affinity constant for potentiation ($K_{pt}$) of 144 nM and a maximal degree of potentiation ($\alpha$) of 3.31. **c** Scatchard plot transformation of the saturation binding with $^3$H-DCG-IV with and without 3 μM 3-MPPTS in a human mGlu2 receptor expressing cell line. Addition of the mGlu2 potentiator resulted in a decreased $K_d$ (see Table 1) but no change in the $B_{max}$

**Table 1** Effects of 3-MPPTS on agonist affinity

| Agonist | Control $K_i$ (nM) | +3-MPPTS[a] $K_i$ (nM) | *p* Value[b] | Fold increase in affinity |
|---|---|---|---|---|
| Glutamate | 7,284+1,152 (9) | 4,806+531 (9) | 0.014 | 1.51 |
| 1*S*,3*R*-ACPD | 6,827+214 (3) | 3,447+409 (3) | 0.010 | 1.98 |
| DCG-IV[c] | 100+3 (3) | 46.9+0.9 (3) | 0.005 | 2.13 |
| LY354740 | 16.3+1.6 (3) | 9.21+0.64 (3) | 0.034 | 1.77 |
| LY379268 | 2.45+0.06 (3) | 2.71+0.09 (3) | 0.69 | 0.90 |
| LY341495 | 2.31+0.63 (3) | 2.65+0.19 (3) | 0.44 | 0.87 |

Values are mean±SEM (*n*).
[a]3 μM 3-MPPTS added.
[b]Listed are the *p*-values from a two-way ANOVA (interaction term $F(1,5)=2.56$, $p=0.044$) followed by a contrast analysis comparing the $K_i$s with 3-MPPTS versus Control for each orthosteric ligand.
[c]Values for $^3$H-DCG-IV are the $K_d$ obtained from saturation experiments.

## The efficacy of an mGlu2 potentiator in a potentiated startle anxiety model

Previous work has shown that mGlu2/3 orthosteric agonists such as LY354740 and LY379268 are efficacious in a rat fear-potentiated startle paradigm (Helton et al. 1998; Monn et al. 1997; Tizzano et al. 2002; Walker and Davis 2002). In a previous report (Johnson et al. 2003), we showed that a mGlu2 potentiator, 4-MPPTS, was also efficacious in this anxiety model, and that the efficacy could be prevented by pretreatment of an mGlu2/3 antagonist. As shown in Fig. 5a, we have replicated this finding by demonstrating significant, dose-related activity similar to that seen with diazepam, with a dose as low as 0.1 mg/kg (s.c.) of 4-MPPTS showing significant efficacy. 4-APPES similarly showed anxiolytic-like activity in this model at a 0.1-mg/kg (s.c.) dose. In separate experiments with 4-MPPTS and 4-APPES, we found that while 4-APPES is ten-fold less potent in vitro as an mGlu2 potentiator and has a lower brain/plasma ratio than 4-MPPTS, 4-APPES does have higher plasma exposure levels and a longer half live in rats (S. Swanson, personal communication). Importantly, the described fear-potentiated startle paradigms has consistently been very sensitive in showing efficacy for mGlu2/3 agonists such as LY354740 and LY379268 (D.D. Schoepp, personal communication). Thus, it might be expected that lower doses and lower exposure levels of the mGlu2 potentiators would be required to show efficacy in this behavioral paradigm than other models of anxiety or psychosis.

## The efficacy of an mGlu2 potentiator in a stress-induced hyperthermia anxiety model

Previous work has shown that the mGlu2/3 agonist LY 354740 is efficacious in stress-induced hyperthermia models (Spooren et al. 2002), albeit requiring doses higher than those needed to show efficacy in the rat fear-potentiated

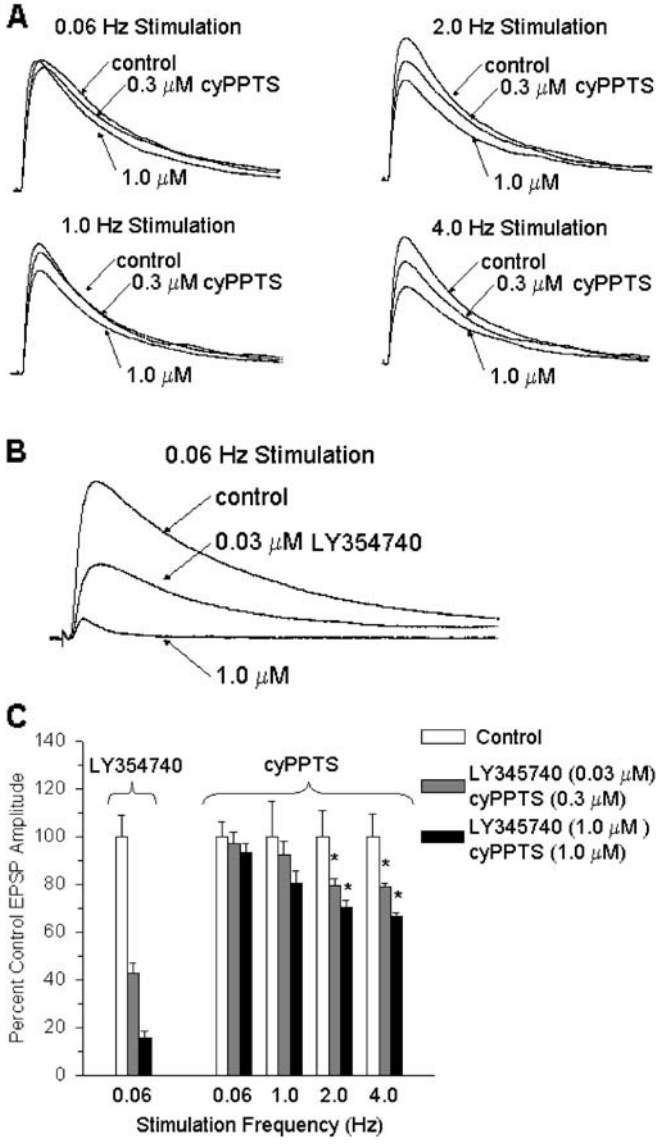



**Fig. 3** Frequency- and concentration-dependent suppression of corticostriatal EPSPs by cyPPTS. **a** Responses from an individual striatal neuron to 0.06, 1.0, 2.0, and 4.0 Hz stimulation of glutamatergic inputs before and after application of cyPPTS (0.3–1.0 μM). **b** Responses from an individual striatal neuron to 0.06 Hz stimulation before and after application of LY345740 (0.03, 1.0 μM). Each trace in **a** and **b** represents the average of ten (0.06 Hz) or 30 (1.0–4.0 Hz) responses. **c** *Bar graph* plotting the average responses expressed as a percent of control values of striatal neurons to different frequencies of stimulation of corticostriatal afferents during control conditions and in the presence of LY354740 (0.03–1.0 μM) and cyPPTS (0.3–1.0 μM). *$p<0.05$, significant differences from control responses at a given stimulation frequency for cyPPTS using a two-way ANOVA with Dunnett's multiple comparison test; $p<0.001$ significant differences from control responses for LY354740 using a one-way ANOVA with Dunnett's multiple comparison test

startle model described above. As such, the pharmacodynamic properties of 4-MPPTS or 4-APPES were insufficient to test the hypothesis that mGlu2 potentiators would also be efficacious in this anxiety model. As such, a more optimized molecule with superior pharmacodynamic properties (CBiPES) was tested at higher doses. Following a dose of 25 and 50 mg/kg, CBiPES produced a transient hypothermic response with the 50-mg/kg dose significantly

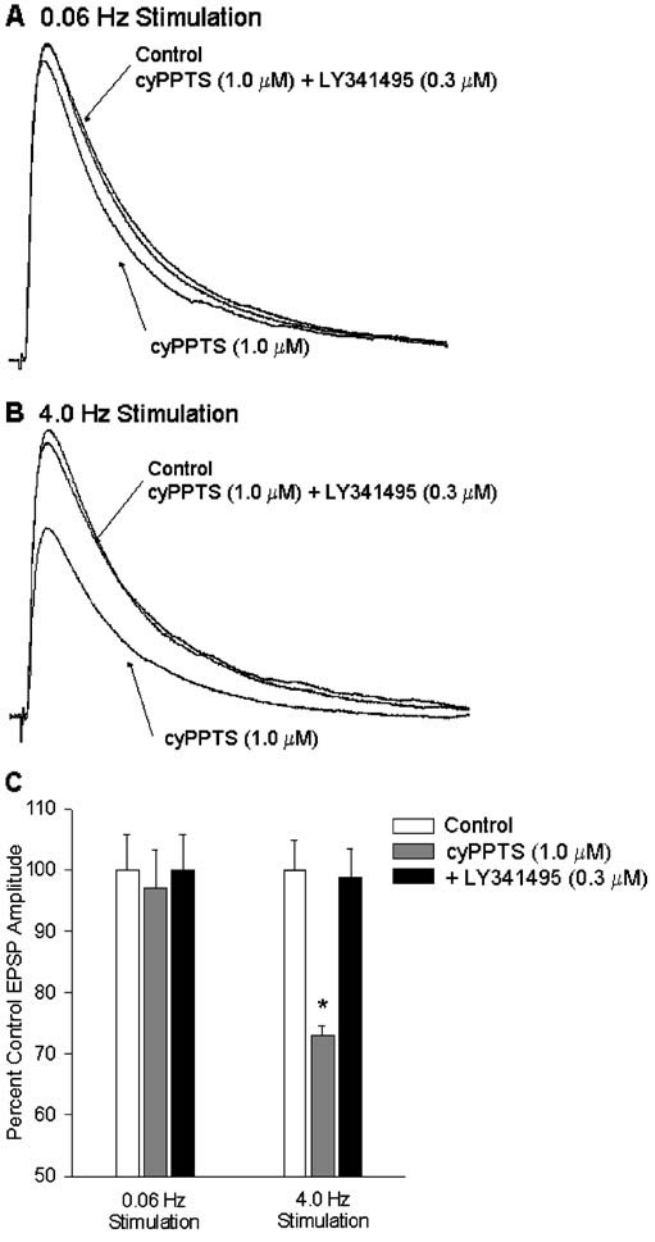



**Fig. 4** LY341495 blocks the frequency-dependent suppression of EPSPs in striatal neurons by cyPPTS. Responses from an individual striatal neuron to 0.06 and 4.0 Hz stimulation of glutamatergic inputs during control conditions and in the presence of 1.0 μM cyPPTS alone and in combination with 0.3 μM LY341495. Each trace in **a** and **b** represents the average of 10 (0.06 Hz) or 30 (1.0–4.0 Hz) responses. **c** *Bar graph* plotting the average responses expressed as a percent of control values of striatal neurons to 0.06 and 4.0 Hz stimulation of corticostriatal afferents during control conditions and in the presence of cyPPTS (1.0 μM) alone and in combination with LY341495 (0.03 μM). *$p<0.001$, significant differences from control responses using a one-way ANOVA followed by Dunnett's multiple comparison test

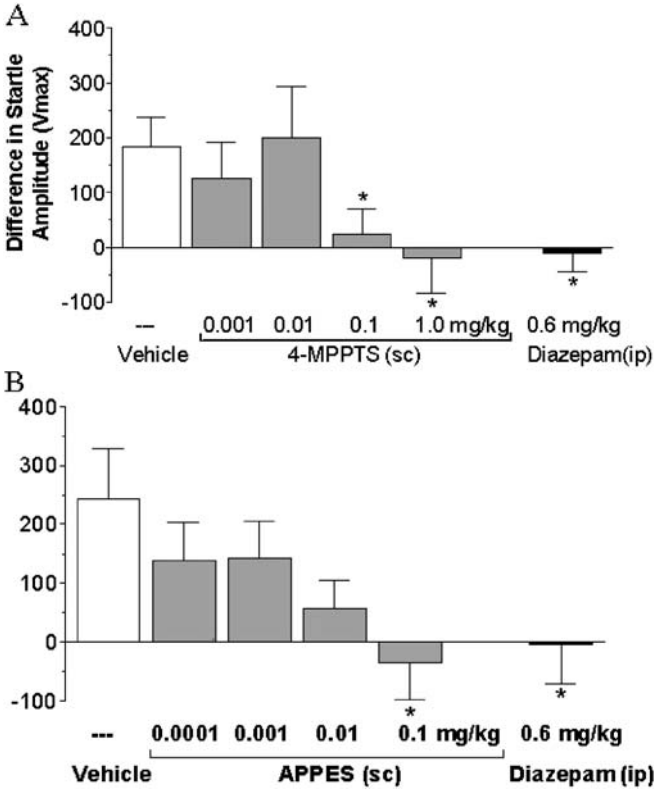



**Fig. 5** Response of mGlu2 potentiators in the rat potentiated startle response anxiety model. Rats were trained to associate a light cue to a foot shock, and the auditory-induced startle response with and without the light cue measured subsequently (see Materials and methods). **a** 4-MPPTS (0.1 and 1.0 mg/kg, s.c.) showed a significant decrease in the potentiated startle response. Note the similar magnitude of response seen with either the mGlu2 potentiator or the anxiolytic diazepam (0.6 mg/kg, i.p.). **b** 4-APPES also induced a significantly decreased the potentiated startle response (0.1 mg/kg, s.c.). *$p$<0.05, significantly different than Vehicle control (ANOVA followed by Dunnett's multiple comparison test)

differing from vehicle controls over the first 15 min post-dosing period [data not shown; Dose × Time interaction, $F(10,35)=3.18$, $p<0.01$]. Analysis of the post-stress data indicated that only a main effect of Time was present [$F(11,77)=12.21$, $p<0.001$]. CBiPES at 25 and 50 mg/kg doses did not significantly reduce the hyperthermic response to the stressor [Dose, $F(2,7)=0.30$, $p=0.75$ or Dose×Time, $F(22,77)=0.86$, $p=0.64$].

A second experiment was conducted to determine whether a 100-mg/kg dose of CBiPES would have anxiolytic effects upon stress-induced hyperthermia. Due to the transient hypothermic effects observed in the first study, a 50-min baseline period was incorporated in order to allow for normal thermoregulation to be reached. Additionally, as the peak stress response occurred within the first 15 min, a 30-min post-stress period was recorded. Figure 6a shows the effects of 100 mg/kg of CBiPES on baseline and post-stress body temperature. Significant main effects of both Dose and Time were found during the baseline drug assessment period [Fig. 6a; $F(1,7)=28.97$, $p<0.001$ and $F(9,63)= 2.60$, $p<0.01$, respectively]. Additionally, the Dose × Time interaction proved significant [$F(9,63)=12.16$, $p<0.001$] and simple effects analyses on each time period indicated that CBiPES produced significant hypothermia over the first 35 min, relative to vehicle controls, following drug administration. CBiPES reduced the hyperthermic effects of stress as indicated by a significant Dose × Time interaction [Fig. 6b; $F(11,77)=18.59$, $p<0.001$]. Simple effects analysis determined that 100 mg/kg CBiPES reduced stress-induced hy perthermia over the first 20 min following stress induction.

## The efficacy of an mGlu2 potentiator in a PCP-induced locomotor activity model

Previous work has shown that activation of mGlu2 and/or mGlu3 receptor by orthosteric agonists decrease spontaneous locomotor activity seen when mice are introduced to a novel environment. As seen in Fig. 7a, treatment with either an mGlu2/3 agonist LY379268 (30 mg/kg, i.p.) or the mGlu2 potentiator CBiPES (100 mg/kg, i.p.) sig-

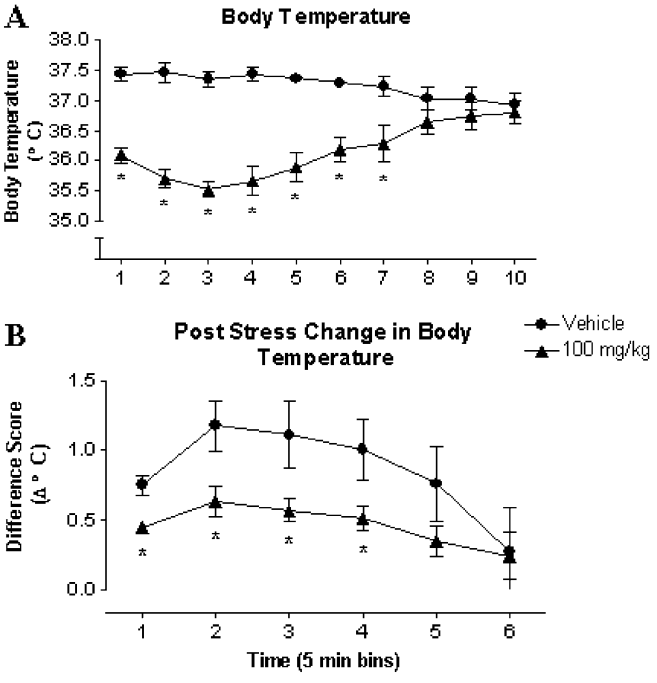



**Fig. 6** Effect of CBiPES on basal body temperature and stress-induced hyperthermia in DBA/2 mice. **a** Vehicle or 100 mg/kg s.c. CBiPES was administered and the basal body temperature monitored with previously implant telemetry probes for 50 min. A two-way ANOVA indicated a significant interaction with treatment and time [$F(9,63)=12.16$, $p<0.001$] and simple effects analyses on each time period indicated that CBiPES produced significant hypothermia over the first 35 min. **b** Mice were subjected to a stressor of increased light and soiled rat bedding and the rise in body temperature monitored. CBiPES reduced the stress-mediated hyperthermic effects as indicated by a significant Dose×Time interaction [$F(11,77)=18.59$, $p<0.001$]. Simple effects analysis determined that 100 mg/kg CBiPES reduced stress-induced hyperthermia over the first 20 min following stress induction. Values graphed are the mean±SEM ($n$= 8–10). *$p$<0.05, significantly different than vehicle control at the corresponding time interval (two-way ANOVA, followed by post-hoc comparison of interaction levels)

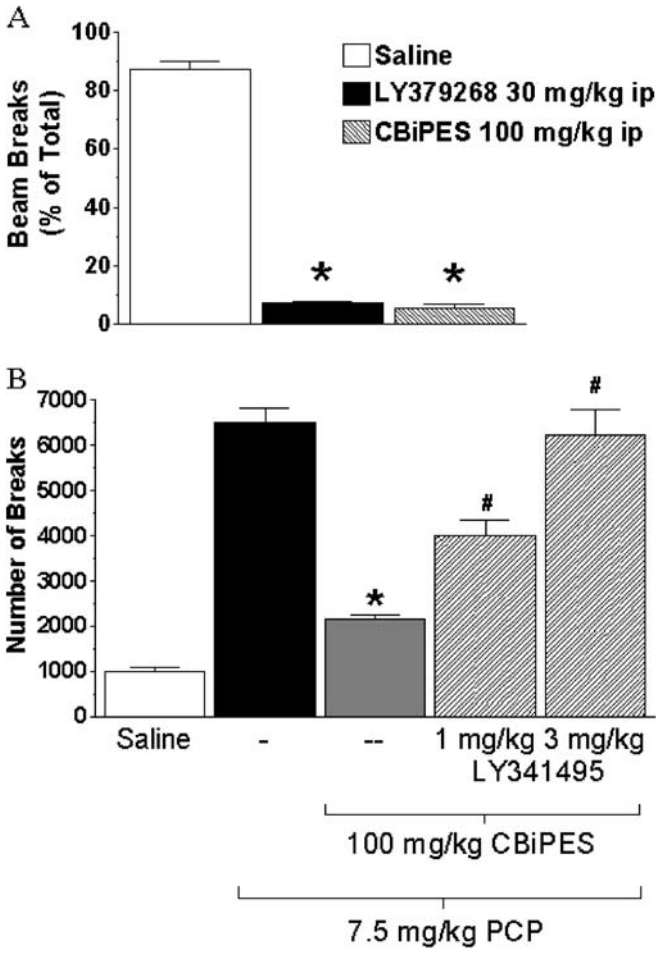



**Fig. 7** Effect on spontaneous or PCP-induced locomotor activity in mice with mGlu2 potentiators treatment. **a** Either the mGlu2/3 agonist LY379268 (30 mg/kg, i.p.) or the mGlu2 potentiator CBiPES (100 mg/kg, i.p.) was administered, and the spontaneous locomotor activity measured during a 45-min acclimation period. Note the significant decrease in locomotor activity as measured by photobeam breaks with either the mGlu2/3 agonists or mGlu2 potentiator. Graphed are the mean±SEM ($n$=8–10) for the total number of beam breaks in the 45-min period as a percentage of the total beam breaks for all treatment groups combined. *$p$<0.05, significantly different than vehicle control (ANOVA followed by Newman–Keuls comparison). **b** Pretreatment with CBiPES (100 mg/kg, i.p.) significantly attenuated the hyperlocomotor activity seen with PCP (7.5 mg/kg, i.p.) treatment. When LY341495 (1.0 or 3.0 mg/kg, s.c.) was injected 30 min prior to 3-MPPTS, a dose-dependent reversal of the mGlu2 potentiator response was evident. Graphed are the mean± SEM ($n$=8–10). *$p$<0.05, significantly different than PCP-treated group (ANOVA followed by Newman–Keuls comparison). #$p$<0.05, significantly different than CBiPES/PCP-treated group (ANOVA followed by Newman–Keuls comparison)

nificantly decreased spontaneous locomotor activity. Following administration of PCP (7.5 mg/kg, i.p.), mice typically exhibit a hyperlocomotor behavior that is evident in the total number of beam breaks (Fig. 7b). Previous work has shown that mGlu2/3 agonists effectively prevent this behavior, which has been hypothesized to model some of the symptoms of psychosis (Cartmell et al. 2000a,c; Tomita et al. 2000). As seen in Fig. 7b, the mGlu2-selective potentiator CBiPES significantly decreased the PCP-induced hyperlocomotor activity in PCP-treated mice. Most significantly, the response appears to be mGlu2-dependent as it could be prevented by the pretreatment of the orthosteric antagonist LY341495. This is similar to the electrophysiological responses described above in the corticostriatal pathway.

## Discussion

The present studies were designed to evaluate the in vitro biochemical and in vivo behavioral properties of a novel class of mGlu2 receptor positive allosteric modulators. The biochemical actions of these compounds have a number of distinct properties from those of previously discovered orthosteric ligands for the group II mGlu receptors, such as LY354740 and LY379268. Clearly, the chemical structures of these modulators are substantially different than the constrained or substituted amino acid analogs. Thus, not surprisingly, the potentiators do not directly bind to the glutamate site of the mGlu2 receptor that is contained within the shell-like structure formed by the large extracellular amino-terminus of the class C GPCRs. Specifically, 3-MPPTS does not significantly displace an agonist radioligand, $^{3}$H-DCG-IV. Previously it has been reported (Johnson et al. 2003; Schaffhauser et al. 2003) that activity in chimera receptor constructs with similar mGlu2 potentiators is dependent upon a portion of the transmembrane region of the receptor. The Group I antagonists, such as MPEP and CPCCOEt (Brauner-Osborne et al. 1999; Gasparini et al. 2002) (Kuhn et al. 2002), and the more recently reported $GABA_B$ (Urwyler et al. 2001), mGlu1 and mGlu5 potentiators (Knoflach et al. 2001; O'Brien et al. 2003; Wichmann et al. 2002; Williams et al. 2003), are all suggested to interact within the TM regions of their respective receptors as well.

Cross-reactivity screen has demonstrated that this class of sulfonamides can provide a high degree of selectivity for the mGlu2 receptor over the other cloned mGlu receptors (present work and Johnson et al. 2003). For example, no significant agonist, potentiator, or antagonist activity was seen in an identical functional assay format (i.e., intracellular calcium) against mGlu1 or mGlu3-8 receptors expressed in the same cell line (AV-12) as the mGlu2 receptor. Equally important, these allosteric modulators do not potentiate the responses of adrenergic or purinergic receptors that are present and responsive in the mGlu2/AV-12 cell line (data not shown). Rather, the potent potentiation seen with 3-MPPTS, 4-APPES, cyPPTS, and CBiPES for the mGlu2 receptor responses occurs only in cellular or neuronal preparations known to contain this receptor. Also, the potentiation of mGlu2 responses can be illustrated in native tissue systems, such as the inhibition of corticostriatal mediated EPSPs (Figs. 3, 4). Taken together, these results indicate that these allosteric modulators are highly selective for the mGlu2 receptor.

The mechanism by which a potentiator modulates its GPCR target remains somewhat theoretical. As implied in their categorization as potentiators, these sulfonamide allo-

steric modulates are dependent upon orthosteric agonist-mediated activation to induce a rise in intracellular calcium. For instance, 3-MPPTS does not induce a significant calcium response without some level of stimulation from an orthosteric-site agonist such as 1*S*,3*R*-ACPD (Fig. 1). As such, the positive allosteric modulation produced by these compounds is revealed as a leftward shift in the dose response of orthosteric agonists, apparently increasing the potency of the agonists. Parmentier et al. (2002) have recently hypothesized that potentiators acting at class C GPCRs, such as mGlu2, would increase both the apparent potency and/or maximal effect of an agonist. In the present work, the allosteric modulators did not appear to significantly increase the maximal effect in the recombinant human mGlu2 cells (Fig. 2a). Notably, experiments indicate that this is not the result of instrument limitations or depletion of intracellular calcium stores. Rather, the degree of receptor reserve may be masking the potentiator's ability to induce a supramaximal response in this functional assay. For this discussion, receptor reserve is defined simply as activation of less than 100% of the receptors required to see a maximal response. Indeed, previous experiments with this cell line show a large mGlu2 receptor reserve in this cell line (unpublished results), making a potentiator-mediated supramaximal response difficult to determine in this system. In the aforementioned model of modulator actions (Parmentier et al. 2002), potentiators were predicted to increase the affinity of orthosteric agonists. Certainly, the described sulfonamides appear to increase the affinity of mGlu2 agonists in functional assays, giving a leftward shift in the dose–response curve of agonists in the intracellular calcium (Fig. 2b). More directly, when 3-MPPTS was combined with the radioligand agonist, $^{3}$H-DCG-IV, a significantly higher affinity for glutamate was found (Fig. 2c and Table 1). However, limitations of the assay may limit the sensitivity to see changes in the apparent number of receptors that bind.

The functional consequences of positive allosteric modulators of native mGlu2 receptors were initially evaluated at the synaptic level by assessing their effects on glutamaterigc corticostriatal neurotransmission. Previous studies have demonstrated that mGlu2/3 receptor agonists can suppress glutamatergic excitatory responses in striatal neurons (Kahn et al. 2001). On the basis of the mechanistic studies from the clonal cell lines, it was postulated that the potentiators would preferentially attenuate corticostriatal EPSPs during periods of enhanced, but not normal glutamate release, when the neurotransmitter would be expected to diffuse away from the synapse and activate perisynaptic mGlu2 receptors. This hypothesis was tested by measuring the effects of cyPPTS on corticostriatal EPSPs in response to low and high frequencies of stimulation when glutamate concentrations at perisynaptic mGlu2 receptors would be expected to be correspondingly low and high, respectively. As predicted, results showed that cyPPTS displayed a marked frequency-dependent modulation of corticostriatal transmission with significant effects observed only in response to high-frequency stimulation (Figs. 3, 4). In contrast, the orthosteric agonist, LY354740, showed a similar inhibition of striatal EPSPs in response to either low- or high-frequency stimulation. These results suggest that cyPPTS acted to sensitize the naturally occurring mGlu2 receptor-mediated presynaptic inhibition of glutamate release by altering synaptic transmission only when the levels of glutamate were significantly elevated by the higher frequency stimulation. These data suggest that potentiators would have an ability to uniquely modulate excessive synaptic glutamate release while exerting little effect on normal or basal release. Consequently, a potentiator, with a more state-dependent mode of action, may provide a safer, more easily tolerated, and/or more efficacious means to treat disorders associated with glutamatergic hyperactivity compared to orthosteric agonists which may induce undesirable side effects or tolerance, as a consequence of continuous GPCR activation and/or desensitization.

These compounds have proven to be very useful pharmacological tools in defining the relative significance of mGlu2 receptor activation compared with the other closely related receptors, such as mGlu3. To date, it has often been very difficult to definitively determine the relative contribution of mGlu2 over mGlu3 receptors partially due to the lack of systemically active, potent, and selective agents for the mGlu2 receptor. The compounds 3-MPPTS, 4-APPES, and CBiPES appear to be very selective for the mGlu2 receptor, bioavailable after subcutaneous or intraperitoneal dosing, and show efficacy in animal models linked to mGlu2 receptor activation. The efficacy in three purportedly mGlu2-sensitive animals models of anxiety or psychosis was examined by utilizing these novel pharmacological tools.

The mGlu2/3 agonists have previously been reported to be efficacious in several models of anxiety or psychosis. In fear-potentiated startle response paradigms, mGlu2/3 agonists such as LY354740 has demonstrated efficacy in both rodents (Helton et al. 1998) and humans (Grillon et al. 2003). Similarly, the present (Fig. 5a and b) and previous results (Johnson et al. 2003) show that mGlu2-positive allosteric modulators effectively reverse the fear-potentiated startle response in rats to a similar extent as the mGlu2/3 agonists. Most interestingly, the degree of efficacy with the mGlu2 potentiators was similar to that of diazepam, but no effects on motor coordination or sedative responses have been seen with orders of magnitude higher doses (data not shown). In a second model of anxiety, the stress-induced hyperthermic response in mice, another mGlu2 potentiator (CBiPES) with improved pharmacokinetic/pharmacodynamic parameters was examined. Previous work has shown that mGlu2/3 agonists, such as LY354740, are efficacious in this animal model of anxiety as well (Spooren et al. 2002), though typically higher doses (e.g., 10 mg/kg, s.c.) are required to show significant efficacy. Similarly, CBiPES was efficacious in the SIH anxiety model, limiting the degree of hyperthermia seen with an acute stressor in mice.

One commonly utilized animal model of psychosis is reversal of PCP-induced hyperlocomotor activity. Atypical antipsychotics are particularly effective in this model, and

many of the behavioral consequences of PCP intoxication appear to be very similar to those seen in psychosis (for reviews, see Halberstadt 1995; Javitt and Zukin 1991). Previously, mGlu2/3 agonists have shown efficacy in both rat and mouse models of PCP-induced locomotor responses (Cartmell et al. 1999, 2000a,b,c; Clark et al. 2002). In addition, reports utilizing mGlu2 KO animals and an mGlu2/3 agonist suggested that the efficacy was dependent upon the mGlu2 receptor (Spooren et al. 2000). In support of the significance of mGlu2 receptors in this behavioral paradigm, the selective mGlu2 potentiator CBiPES decreased the PCP-induced locomotor effects in mice (Fig. 7b). As seen in the slice experiments, this response remains dependent on orthosteric agonist stimulation of the mGlu2 receptor, as the response could be blocked with the mGlu2/3 antagonist LY341495 (Fig. 7b). It should be noted that the potentiator also appeared to limit the spontaneous locomotor activity (Fig. 7a) associated with an exploration in a novel environment as has also been previously reported with mGlu2/3 agonists and is absent in mGlu2 knockout mice (Klodzinska et al. 1999; Spooren et al. 2000).

To summarize, the present work describes subtype-selective potentiators of the mGlu2 receptor. These compounds are allosteric modulators interacting within the TM region of the mGlu2 receptor. These unique compounds appear to act by increasing the affinity of glutamate-site agonists. Thus, both in vitro and in vivo, responses still require some activation with orthosteric agonists such as glutamate. Yet mGlu2 potentiators are effective in several previous behavioral models where the mGlu2/3 agonists are active, including model(s) of anxiety (fear-induced potentiated startle and stress-induced hyperthermia) and psychosis (PCP-induced hyperlocomotor activity). Given the frequency-dependent results in corticostriatal slices, this implies that a certain inhibitory tone from presynaptic mGlu2 receptors occurs during these behavioral paradigms. These unique compounds are the first positive allosteric modulators reported for the mGlu2 receptor and should serve as useful in vitro and in vivo tools in the characterization of mGlu2 receptor-mediated responses.

**Acknowledgements** The authors wish to thank the following individuals at Lilly Research Laboratories who contributed significantly to the scientific design and results: Sherri Andis, Mary Jo Chamberlain, Darrell S. Coleman, Kelly I. Griffey, John C. Hart, Steven S. Henry, Bryan G. Johnson, Gerard M. Kelly, Mark A. Muhlhauser, Grant M. Vaught, Zhao-Qing Wong, and Jin Zhai.

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