Phenyl-tetrazolyl Acetophenones: Discovery of Positive Allosteric Potentiatiors for the Metabotropic Glutamate 2 Receptor

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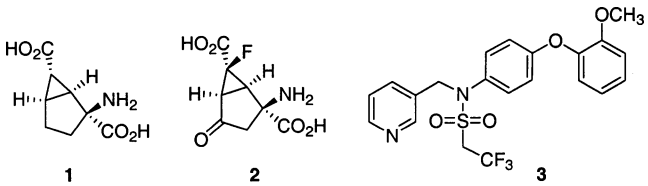
Herein we disclose the discovery of a new class of positive allosteric potentiators of the metabotropic glutamate receptor 2 (mGlu2), phenyl-tetrazolyl acetophenones, e.g. 1-(2-hydroxy-3-propyl-4-{4-[4-(2*H*-tetrazol-5-yl)phenoxy]butoxy}phenyl) ethanone (**4**). These potentiators were shown to have no effect in the absence of glutamate as well as no effect at mGlu3 or the other mGlu receptors. The compounds were also evaluated in rodent models with potential relevance for schizophrenia, and **4** was shown to have activity in the inhibition of ketamine-induced norepinephrine release and ketamine-induced hyperactivity. This represents the first example of the efficacy of mGlu2 receptor potentiators in these models.

Introduction

Glutamate is the transmitter of the large majority of fast excitatory synapses in the CNS and plays an important role in a wide variety of CNS functions. Glutamate activates both ionotropic glutamate (iGlu) receptors, which are glutamate-gated ion channels, as well as the metabotropic glutamate receptors (mGlu) which are a family of G-protein-coupled receptors. Eight subtypes of the mGlu receptors have been identified which fall into three main groups.[1] Group I consists of mGlu1 and -5, which have mainly been shown to be stimulatory. Groups II (mGlu2 and -3) and III (mGlu4, -6, -7, -8), however, are often concentrated presynaptically and generally inhibit neurotransmission. Therefore, agents targeting the mGlu receptors may have utility in a variety of clinical conditions.[2−4] Specifically, there has been evidence implicating mGlu receptors, especially mGlu2, as a potential target for the treatment of schizophrenia.[5]

The physiological importance of group II mGlu receptors has been shown by the efficacy of rigid glutamate analogues such as (1*S*,2*S*,5*R*,6*S*)-2-aminobicyclo[3.1.0]-hexane 2,6-dicarboxylic acid (**1**, LY354740)[6,7] and (1*R*, 2*S*,5*S*,6*S*)-2-amino-6-fluoro-4-oxobicyclo[3.1.0]hexane 2,6-dicarboxylic acid (**2**, MGS0028)[8] in both animal models as well as human clinical trials.[9,10]



1 **2** **3**

Due to the high degree of sequence homology between group II mGlu receptors, especially at the glutamate binding site, selective agonists for mGlu2 over mGlu3 have not, as yet, been discovered. Therefore, another strategy for selectivity involves the discovery of allosteric modulators that do not bind at the glutamate binding site.[11] Lilly has recently disclosed compounds such as *N*-(4-(2-methoxyphenoxy)phenyl)-*N*-(2,2,2-trifluoroethylsulfonyl)pyrid-3-ylmethylamine (**3**, LY487379) as positive allosteric potentiators of the mGlu2 receptor.[12,13] This paper details the discovery of a novel class of mGlu2 selective potentiators with efficacy in rodent models with relevance for schizophrenia.

Results and Discussion

High throughput screening of compounds was carried out using a Ca^{2+} flux functional (FLIPR384) assay using a stable cell line coexpressing human mGlu2 receptors coupled to a promiscuous G-protein (Gα16). Receptor activity was detected by changes in [Ca^{2+}], measured using the fluorescent, Ca^{2+} sensitive dye fura-2.[14] First, an EC_{10} (0.5 μM) of glutamate was added to the cell line followed immediately by the test compound at varying concentrations. The response was then compared to a response using a saturating amount of glutamate (1 mM) to give both an EC_{50} and a percent potentiation (the response normalized to the maximum response of glutamate alone). The same experiment was carried out in the absence of glutamate to test whether the compound was truly a positive allosteric modulator.

The effect of these compounds were confirmed and further characterized in the [^{35}S]-GTPγS binding assay.[13] The same assay was used to determine the selectivity of the compound against mGlu3 receptors. This binding assay was conducted using membranes from hmGlu2 or hmGlu3 receptor-expressing stable cell lines prepared as previously described. Glutamate (1 μM) was used as an agonist, and the activity was measured in the presence or absence of compound. Nonspecific binding was determined by addition of 10 μM unlabeled GTPγS. In this manner 1-(2-hydroxy-3-propyl-4-{4-[4-(2*H*-tetrazol-5-yl)phenoxy]butoxy}phenyl)-ethanone (**4**) was identified as an mGlu2 selective potentiator (Figure 1), which had an EC_{50} of 0.348 μM with 31% potentiation at hmGlu2 and an EC_{50} of 0.435 μM with 100% potentiation with rat brain membranes. In the absence of glutamate, compound **4** displayed no

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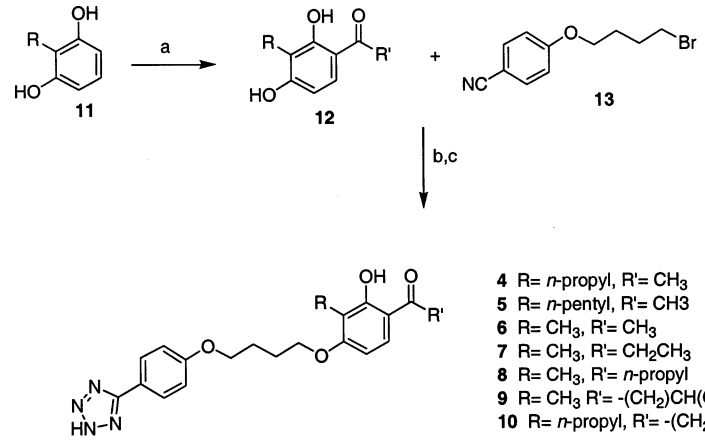
Scheme 1[a]



	hmGlu2 EC_{50} (μM)[b]
4 R= *n*-propyl, R'= CH_3	0.348 ± 0.149
5 R= *n*-pentyl, R'= CH3	>10
6 R= CH_3, R'= CH_3	3.365 ± 1.649
7 R= CH_3, R'= CH_2CH_3	0.380 ± 0.017
8 R= CH_3, R'= *n*-propyl	0.304 ± 0.098
9 R= CH_3 R'= $-(CH_2)CH(CH_3)_2$	0.229 ± 0.059
10 R= *n*-propyl, R'= $-(CH_2)CH(CH_3)_2$	0.421 ± 0.097

[a] Reagents and conditions: (a) R′COCl, $AlCl_3$, CH_2Cl_2, 0 °C, (b) K_2CO_3, acetone, 45 °C, (c) Bu_2SnO, $TMSN_3$, toluene, reflux. [b]Value represents mean ((SEM) of three or more experiments.

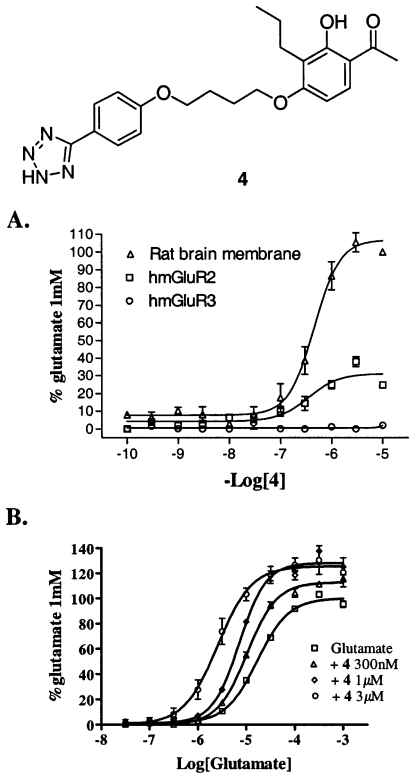


Figure 1. Effect of Glutamate-induced GTPγS binding. (A) Dose response of **4** after addition of 1 μM glutamate in GTPγS binding in hmGlu2, rat brain membranes and hmGlu3 (n = 3). (B) Shift of dose response curve of glutamate with hmGlu2 using varying concentrations of **4** (n = 2). Data displayed as a percentage of the maximal glutamate response at 1 mM.

activity. Also, compound **4** displayed no activity at hmGlu3 in the presence or absence of glutamate. In a similar fashion, compound **4** was found to be selective against the other mGlu receptors (-1, -4, -5, -7, -8) as well as NMDA, AMPA and kainate receptors (data not shown).[15] In the GTPγS assay, **3** (LY487379) displayed activity of 1.7 μM with a 52% level of potentiation at hmGlu2.[13] Therefore, compound **4** represents a significant increase in potency.

Initial SAR investigations around compound **4** showed that the acidic proton on the tetrazole as well as the phenol moiety on the acetophenone were necessary for

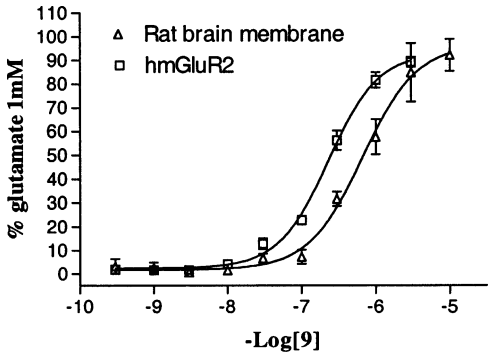


Figure 2. Effect of glutamate-induced GTPγS binding. Dose–response of **9** after addition of 1 μM glutamate with hmGlu2 and rat brain membranes (n = 3). Data displayed as a percentage of the maximal glutamate response at 1 mM.

activity. For example methylation of either the tetrazole or phenol in **4** led to inactive compounds (data not shown). In an attempt to improve upon the low level of potentiation of compound **4** with hmGlu2, we investigated a number of derivatives of **4**. Straightforward modification of the acetophenone (R and R′ in Scheme 1) indicated that one larger aliphatic group (propyl or isobutyryl) was optimal for activity. Larger groups than these, however, were detrimental. For example, **5** and **6**, with methyl or pentyl groups respectively in place of propyl, were much less potent than **4**. However, potency could be regained and improved with incorporation of larger aliphatic groups at R′. These SAR investigations led to isobutyryl derivative **9** which showed an increase in potency (EC_{50} of 0.229 μM vs 0.348 μM) and more importantly potentiaton comparable to the maximal response of glutamate at the hmGlu2 receptor (89% vs 31% for compound **4**) (Figure 2). As with compound **4**, **9** had no activity in the absence of glutamate and displayed a similar selectivity profile. Interestingly, when two larger aliphatic groups were incorporated around the acetophenone, as in compound **10**, potency was diminished.

The syntheses of compounds **4**–**10** were accomplished in a straightforward manner (Scheme 1). Beginning with commercially available or readily accessed 2-substituted resorcinols **11**, these were reacted with an acid chloride in the presence of aluminum trichloride to give

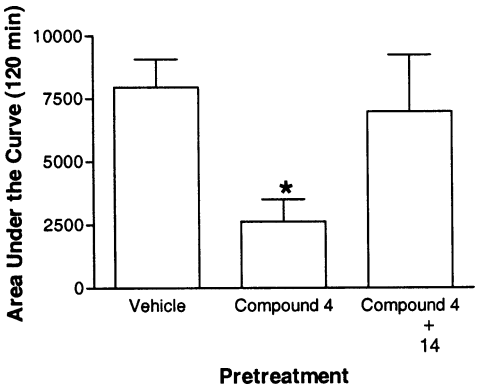


Figure 3. Compound **4** inhibits ketamine-evoked norepinephrine (NE) release. Summary data showing the area under each 120-min time-course curve. Ketamine (25 mg/kg, sc) produced a significant increase in ventral hippocampus NE release. Compound **4** (100 nmol, given icv 30 min before ketamine challenge) significantly reduced the increase in NE. This inhibitory effect was reversed by cotreatment with the group II mGlu receptor selective antagonist **14** (3 mg/kg, sc). Data are normalized as a percentage of baseline NE and are expressed as mean ± SEM. Baseline NE levels were estimated to be 15 ± 1 fg/mL of sample. $n = 4-8$/group. Data were analyzed by a one-way ANOVA. *$P < 0.05$ compared to vehicle and compound **4** + LY341495 treatment groups.

acetophenone derivatives **12**. These were then alkylated selectively using alkyl bromide **13** with potassium carbonate in acetone to give the cyano precursor to the desired compounds. Acetophenones **4**−**11** were generated via a tin-catalyzed tetrazole formation using trimethylsilyl azide in good yield.

To study the in vivo effect of this new class of mGlu2 receptor potentiators, two rodent models with potential relevance for schizophrenia were examined. Previous work has demonstrated that nonselective mGlu2/3 agonists, such as **1** and **2**, are efficacious in these animal models.[6−8] In both of these models, no additional mGlu2 receptor agonists were added, and the efficacy of these compounds in potentiating the response of endogenous glutamate was examined.

First, a neurochemical readout of efficacy was established, the inhibition of ketamine-induced norepinephrine release in rodents.[16] It has been shown that, upon acute challenge, ketamine activates release of norepiniphrine as well as glutamate, dopamine and serotonin. It is not clear which of these responses contribute to the behavioral aberrations caused by ketamine, but recent studies have implicated the importance of glutamate neurotransmission, especially recent papers describing the activity of **1**.[17−19] Due to poor brain penetration, mGlu2 receptor potentiator **4** was dosed intracerebroventricular (icv). As displayed in Figure 3, **4** shows activity at a single dose of 100 nmoles. To confirm that the response was mediated through the mGlu2 receptor, it was shown that coapplication with an mGlu2/3 antagonist (2*S*,1′*S*,2′*S*)-2-(9-xanthylmethyl)-2-(2′-carboxycyclopropyl)glycine (**14**, LY341495),[20] effectively blocked the response of **4**.

On the basis of these positive results in a pharmacodynamic readout, a behavioral model was also examined, the inhibition of ketamine-induced hyperactivity. To evaluate the effects of **4** on ketamine hyperactivity, rats were habituated to individual activity chambers (Med Associates Inc., Georgia, VT) for 60 min. Following

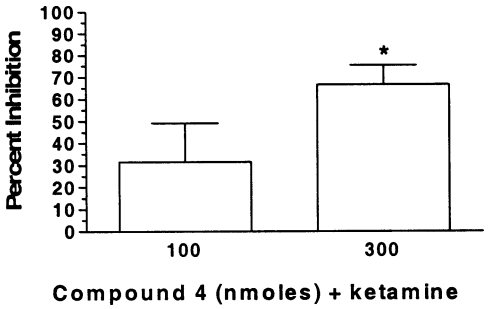


Figure 4. Modulation of ketamine hyperactivity in rats by the mGlu2 potentiator (**4**). Subjects were dosed with **4** (100 and 300 nmoles, icv) or vehicle (icv) 30 min before receiving sc injections of ketamine (25 mg/kg) or vehicle. Activity data (% inhibition) are presented as the group mean (±S. E. M.) recorded for the total duration of the 120 min test period. Data were analyzed by a one-way ANOVA followed by Dunnett's *t*-tests; *$p < 0.05$ compared to vehicle/ketamine-treated rats.

habituation, subjects were injected with **4** (icv) or vehicle (icv) and returned to the chambers. After a 30-min pretreatment period, rats were dosed with either ketamine (25 mg/kg, sc) or vehicle (sc) and activity was recorded in 10 min intervals for 120 min.

As in the case with mGlu2/3 agonists, the mGlu2 selective potentiator **4** inhibits the effect of ketamine at a 300 nmoles icv dose (Figure 4). Also, as outlined above, no additional mGlu2 receptor agonist is needed; compound **4** potentiates the response of the endogenous glutamate.

In conclusion, phenyltetrazolyl acetophenones such as **4** have been shown to be positive allosteric potentiators of the mGlu2 receptor. Combined with the in vitro data, the in vivo data suggests that these compounds modulate glutamate-mediated stimulation of mGlu2 receptors and therefore have potential therapeutic value for the treatment of schizophrenia. This represents the first examples of mGlu2 receptor potentiators with activity in these models. Likewise, these compounds are the most potent mGlu2 receptor potentiators reported to date. Further studies as well as additional structure activity relationships of this class of compounds will be reported in due course.

Experimental Section

General Procedures. All reactions were performed under a positive pressure of dry nitrogen within glassware that had been oven dried prior to use unless otherwise indicated. All solvents were obtained from ACROS or Aldrich as anhydrous and used without further purification. NMR spectra were obtained on a Bruker 500 MHz spectrometer and are recorded in ppm (δ) downfield of TMS ($\delta = 0$). Flash column chromatography was performed with silica gel (Merck, 230−420 mesh). Purity was ascertained via Shimadzu HPLC and combustion analysis (Prevalere, Inc., Whitesboro, NY). Mass spectra were obtained on a Waters LC-MS.

1-(2-Hydroxy-3-propyl-4-{4-[4-(2*H*-tetrazol-5-yl)phenoxy]-butoxy}phenyl)ethanone (4). Potassium carbonate (5.70 g, 41.2 mmol) was added to a stirred solution of 1-(2,4-dihydroxy-3-propylphenyl)ethanone (Aldrich, 4 g, 20.6 mmol) and 4-(4-bromobutoxy)benzonitrile (6.30 g, 24.7 mmol) in acetone (400 mL) at 45 °C. The reaction mixture was stirred for 16 h, then the acetone was removed in vacuo. The residue was then mixed with dichloromethane (500 mL) and water (500 mL). The organic layer was separated, dried over $MgSO_4$, and then concentrated in vacuo to give a residue that was purified via column chromatography on silica gel (eluting 5−50% ethyl acetate/hexanes) to give 5.76 g (76%) of 4-[4-(4-acetyl-3-

hydroxy-2-propylphenoxy)butoxy]benzonitrile as a white solid. 4-[4-(4-Acetyl-3-hydroxy-2-propylphenoxy)butoxy]benzonitrile (5.76 g, 15.7 mmol), trimethylsilyl azide (3.61 g, 4.16 mL, 31.3 mmol) and dibutyltin oxide (586 mg, 2.4 mmol) were dissolved in toluene (130 mL) and heated to reflux for 16 h. The reaction mixture was then cooled to room temperature and applied directly to a silica gel column (eluting first with 20% ethyl acetate/hexanes followed by 10% MeOH/dichloromethane) to give 6.09 g of 1-(2-hydroxy-3-propyl-4-{4-[4-(2*H*-tetrazol-5-yl)phenoxy]butoxy}phenyl)ethanone (95%) as a white solid. Compound was >95% pure by HPLC and NMR analysis. ^{1}H NMR (DMSO-d_6, 500 MHz), δ 12.85 (s, 1H, O*H*), 7.95 (d, 2H), 7.80 (d, 1H), 7.11 (d, 2H), 6.66 (d, 1H), 4.18−4.14 (m, 4H), 2.58−2.51 (m, 5H), 1.95−1.90 (m, 4H), 1.49−1.44 (m, 2H), 0.86 (t, 3H). MS (ESI): 411.2 $(M + H)^+$,Calcd 411.2. HRMS calcd for $C_{22}H_{27}N_4O_4$ $(M + H)^+$: 411.2032. Found: 411.2000. Anal. ($C_{22}H_{26}N_4O_4 \cdot H_2O$) C, H, N.

1-(2-Hydroxy-3-methyl-4-{4-[4-(2*H*-tetrazol-5-yl)-phenoxy]-butoxy}-phenyl)-3-methyl-butan-1-one (9). Isobutyryl chloride (1.25 g, 1.3 mL, 10.4 mmol) was added to a stirred solution of 2-methylresorcinol (1 g, 8.0 mmol) and aluminum trichloride (1.39 g, 10.4 mmol) in dichloromethane (40 mL) at 0 °C. The reaction was allowed to warm to room temperature, then stirred for 16 h. It was then quenched by addition of 1 N aqueous HCl. The organic layer was separated, dried over $MgSO_4$ and then concentrated in vacuo to give a residue that was purified via column chromatography on silica gel (eluting 5−60% ethyl acetate/hexanes) to give 946 mg (57%) of 1-(2,4-dihydroxy-3-methyl-phenyl)-3-methyl-butan-1-one as a white solid. Potassium carbonate (398 mg, 2.88 mmol) was added to a stirred solution of 1-(2,4-dihydroxy-3-methyl-phenyl)-3-methyl-butan-1-one (300 mg, 1.44 mmol) and 4-(4-bromo-butoxy)-benzonitrile (403 mg, 1.58 mmol) in acetone (20 mL) at 45 °C. The reaction mixture was stirred for 16 h, then the acetone was removed in vacuo. The residue was then mixed with dichloromethane (100 mL) and water (100 mL). The organic layer was separated, dried over $MgSO_4$ and then concentrated in vacuo to give a residue that was purified via column chromatography on silica gel (eluting 5−50% ethyl acetate/hexanes) to give 378 mg (69%) of 4-{4-[3-hydroxy-2-methyl-4-(3-methyl-butyryl)-phenoxy]-butoxy}-benzonitrile as a white solid. 4-{4-[3-Hydroxy-2-methyl-4-(3-methyl-butyryl)-phenoxy]-butoxy}-benzonitrile (257 mg, 0.67 mmol), trimethylsilyl azide (155 mg, 0.18 mL, 1.3 mmol) and dibutyltin oxide (25 mg, 0.10 mmol) were dissolved in toluene (12 mL) and heated to reflux for 16 h. The reaction mixture was then cooled to room temperature and applied directly to a silica gel column (eluting first with 20% ethyl acetate/hexanes followed by 10% MeOH/dichloromethane) to give 242 mg of 1-(2-hydroxy-3-methyl-4-{4-[4-(2*H*-tetrazol-5-yl)-phenoxy]-butoxy}-phenyl)-3-methyl-butan-1-one (85%) as a white solid. Compound was >95% pure by HPLC and NMR analysis. ^{1}H NMR (DMSO-d_6, 500 MHz), δ 13.03 (s, 1H), 7.95 (d, 2H), 7.85 (d, 1H), 7.10 (d, 2H), 6.65 (d, 1H), 4.18-4.13 (m, 4H), 2.85 (d, 2H), 2.17-2.14 (m, 1H), 2.00 (s, 3H), 1.95-1.93 (m, 4H), 0.93 (d, 6H). MS (ESI): 425.2 $(M + H)^+$, calcd 425.2. HRMS calcd for $C_{23}H_{29}N_4O_4$ $(M + H)^+$: 425.2189. Found: 425.2162. Anal. ($C_{23}H_{28}N_4O_4 \cdot H_2O$) C, H, N.

1-(2-Hydroxy-3-methyl-4-{4-[4-(2*H*-tetrazol-5-yl)-phenoxy]butoxy}phenyl)ethanone (5). Prepared in a similar fashion as outlined for compound **4** using 1-(2,4-dihydroxy-3-methylphenyl)ethanone. ^{1}H NMR (DMSO-d_6, 500 MHz), δ 12.85 (s, 1H, −O*H*), 7.95 (d, 2H), 7.80 (d, 1H), 7.11 (d, 2H), 6.66 (d, 1H), 4.18−4.14 (m, 4H), 2.57 (s, 3H), 2.01 (s, 3H), 1.95−1.90 (m, 4H). MS (ESI): 382.4 $(M + H)^+$, Calcd 382.4. Anal. ($C_{20}H_{22}N_4O_4 \cdot H_2O$) C, H, N.

1-(2-Hydroxy-3-pentyl-4-{4-[4-(2*H*-tetrazol-5-yl)phenoxy]-butoxy}phenyl)ethanone(6). Prepared in a similar fashion as outlined for compound **4** using 1-(2,4-dihydroxy-3-pentylphenyl)ethanone. ^{1}H NMR (DMSO-d_6, 500 MHz), δ 12.84 (s, 1H, −O*H*), 7.98 (d, 2H), 7.82 (d, 1H), 7.13 (d, 2H), 6.65 (d, 1H), 4.17−4.14 (m, 4H), 2.58−2.52 (m, 5H), 1.97−1.91 (m, 4H), 1.55−1.34 (m, 6H), 0.87 (t, 3H). MS (ESI): 438.6 $(M+H)^+$, Calcd 438.5. Anal. ($C_{24}H_{30}N_4O_4 \cdot H_2O$) C, H, N.

1-(2-Hydroxy-3-methyl-4-{4-[4-(2*H*-tetrazol-5-yl)-phenoxy]-butoxy}-phenyl)-propan-1-one (7). Prepared in a similar fashion as outlined for compound 9 using propanoic acid chloride. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 12.70 (s, 1H), 7.95 (d, 2H), 7.83 (d, 1H), 7.13 (d, 2H), 6.66 (d, 1H), 4.18−4.13 (m, 4H), 2.04 (q, 2H), 1.99 (s, 3H), 1.97−1.92 (m, 4H), 1.10 (t, 3H). MS (ESI) 396.7 $(M^+ + H)$, Calcd 396.5. Anal. ($C_{21}H_{24}N_4O_4 \cdot H_2O$) C, H, N.

1-(2-Hydroxy-3-methyl-4-{4-[4-(2*H*-tetrazol-5-yl)-phenoxy]-butoxy}-phenyl)-butan-1-one (8). Prepared in a similar fashion as outlined for compound **9** using butyryl chloride ^{1}H NMR (DMSO-d_6, 500 MHz), δ 13.02 (s, 1H), 7.96 (d, 2H), 7.85 (d, 1H), 7.14 (d, 2H), 6.66 (d, 1H), 4.18−4.15 (m, 4H), 2.98 (t, 2H), 2.00 (s, 3H), 1.96−1.93 (m, 4H), 1.67−1.63 (m, 2H), 0.95 (t, 3H). MS (ESI): 411 $(M + H)^+$, Calcd 411.2. Anal. ($C_{22}H_{26}N_4O_4 \cdot H_2O$) C, H, N.

1-(2-Hydroxy-3-propyl-4-{4-[4-(2*H*-tetrazol-5-yl)-phenoxy]-butoxy}-phenyl)-3-methyl-butan-1-one (10). Prepared in a similar fashion as outlined for compound 4 using 1-(2,4-Dihydroxy-3-propyl-phenyl)-3-methyl-butan-1-one. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 13.03 (s, 1H), 7.97 (d, 2H), 7.85 (d, 1H), 7.15 (d, 2H), 6.65 (d, 1H), 4.17−4.14 (m, 4H), 2.85 (d, 2H), 2.51 (t, 2H), 2.17−2.12 (m, 1H), 1.94−1.92 (m, 4H), 1.48−1.43 (m, 2H), 0.94 (d, 6H), 0.86 (t, 3H). MS (ESI) 452.5 $(M^+ + 1)$, Calcd 452.6. Anal. ($C_{25}H_{32}N_4O_4 \cdot H_2O$) C, H, N.

In Vitro Binding Studies. Materials. Glutamate, GDP, probenecid and GTPγS were obtained from Sigma Chemical (St Louis, MO). [^{35}S]GTPγS and 14 were obtained from Tocris (Ellisville, MO).

Membrane Preparation. hmGlu2 and hmGlu3 receptor-expressing stable cell lines were grown to confluence in a T-225-cm^2 flask and washed twice with ice-cold PBS. The cells were then scraped with a cell scraper in phosphate-buffered saline and harvested by centrifugation (200*g*) using a tabletop centrifuge. The cell pellet was homogenized in hypotonic buffer A (20 mM HEPES and 10 mM EDTA, pH 7.4) using a Polytron homogenizer (Brinkmann, Westbury, NY). The homogenate was centrifuged at 40 000*g* for 20 min. The resulting pellet was washed once in the same buffer and once with buffer B (20 mM HEPES and 0.1 mM EDTA, pH 7.4). At the last centrifugation, the pellet was resuspended in buffer B and the homogenate was aliquoted and stored at −80 °C at a protein concentration of approximately 1 mg/mL. Protein measurement was determined with the Bio-Rad detergent-compatible protein assay kit using bovine serum albumin as standard. Rats (250−300 g) were decapitated; the whole brain was removed, placed on ice, and homogenized in 6 volumes (w/v) of 10% sucrose at 4 °C using a glass-Teflon homogenizer. The homogenate was centrifuged at 1000*g* for 10 min, and the supernatant was centrifuged at 40 000*g* for 20 min at 4 °C. The supernatant was removed and the pellet was resuspended in buffer C (5 mM HEPES−KOH, pH 7.4). The homogenate was freeze−thawed twice before being centrifuged at 40 000*g* for 20 min. Finally, the resulting pellet was resuspended in buffer C, aliquoted and stored at −70 °C until used.

[^{35}S]GTPγS Binding Assay. Membranes were thawed and homogenized in 25 mL of a 20 mM HEPES containing 0.1 mM EDTA, pH 7.4, and centrifuged at 40 000*g* for 20 min. The pellet was resuspended in assay buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl and 3 mM $MgCl_2$ at a final protein concentration of 0.5 mg/mL (hmGlu2 and hmGlu3 receptors) or 0.1 mg/mL (rat brain). In a 96-well plate (Beckman Coulter, Fullerton, CA), test compounds and glutamate were added along with 5 μM GDP, membrane (10 μg/well for rat brain and 50 μg for recombinant mGlu receptors), and 0.05 nM [^{35}S]GTPγS to achieve a total volume of 0.5 mL in assay buffer. The plate was incubated at 30 °C for 1 h, then the assay was terminated by rapid filtration over Unifilter GF/B plate using a 96-well cell harvester (Brandel, Gaithersburg, MD). The plate was rinsed three times with ice-cold assay buffer, dried, and 50 μL of Microscint 20 was added to each well. The plate was counted in a Topcount scintillation counter (PerkinElmer Life Science). Each experiment was performed using triplicate samples per data point and then

repeated on separate occasions to obtain a total of three determinations. Data were normalized to the response obtained with 1 mM glutamate. The curves were fitted to a four-parameter logisitic equation giving EC_{50} values, Hill coefficient, and maximal effect using Prism (GraphPad Software, San Diego, CA).

More detailed information concerning the in vitro[13,14] and in vivo[16,19] assays described can be found in the appropriate references.

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