Discovery of subtype-selective metabotropic glutamate receptor ligands using functional HTS assays

Mark A. Varney and Carla M. Suto

Recent research in the area of metabotropic glutamate receptors suggests that subtype-selective modulators of individual family members could be promising candidates for a variety of nervous system disorders including pain, neurodegeneration and numerous psychiatric diseases. Relatively few subtype-selective metabotropic glutamate receptor ligands have been identified. This article will address how HTS can play an important role in identifying structurally novel ligands as drug leads and research tools.

Mark A. Varney and *Carla M. Suto
Merck Research Laboratories, 505 Coast Boulevard South, La Jolla CA 92037, USA
*tel: +1 858 452 5892
fax: +1 858 452 9279
e-mail: carla_suto@merck.com

▼ Glutamate is the principal transmitter of fast synaptic excitatory synapses in the mammalian CNS and plays an important role in a wide variety of CNS functions. Many of these actions of glutamate are mediated through ionotropic glutamate receptors that are glutamate-gated cation channels, sub-divided into α-amino-3-hydroxy-5-methylisoazoleproprionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptor classes. However, in the mid-1980s, a group of glutamate receptors termed metabotropic glutamate receptors (mGluRs) was discovered that could directly couple to second messenger systems through G proteins. It is now clear that mGluRs provide a mechanism whereby glutamate can 'fine-tune' activity at the same synapses at which it can elicit fast synaptic responses.

To date, eight mGluRs have been cloned and functionally expressed (Table 1) and they are classified into three groups based on their degree of shared sequence homologies, similar coupling mechanisms and pharmacological properties[1]. Group I mGluRs include mGluR1 and mGluR5 that couple to phospholipase C, resulting in phosphoinositide hydrolysis and elevation of intracellular Ca^{2+} levels by Ca^{2+} mobilization from intracellular stores. Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7 and mGluR8) mGluRs inhibit cAMP formation when heterologously expressed in mammalian cells[2]. In addition, electrophysiological data from tissue slices and recombinant systems support a widespread modulatory (in general, inhibitory) action of mGluRs on voltage-gated Ca^{2+} channels and K^+ channels, as well as an activation of non-selective cation channels[3].

Except for mGluR6, which is essentially only expressed in the retina, the mGluRs are widely expressed throughout the CNS. This, along with the heterogeneity of family members and their modulatory actions, makes the mGluRs attractive targets to the biotechnological and pharmaceutical industries. Many of the existing mGluR ligands have a low potency and limited selectivity (Table 1). Localization and mutant mouse studies have provided some insight into the role of mGluRs in the CNS. However, the availability of highly selective and potent ligands will be invaluable in further understanding the roles of individual mGluR subtypes in normal physiology and in pathophysiology.

Assays used for HTS against mGluRs

Traditional radioligand-binding assays have not been widely used for characterizing mGluRs, nor for HTS against mGluRs, because of the absence of commercially available potent and selective radioligands. Furthermore, radioligand-binding assays only measure the binding properties of compounds that interact at the same site or modulate the affinity of the ligand-binding site. For these reasons, functional screening assays are preferred over traditional radioligand-binding assays. Table 2 lists functional assays that have been or could, in theory, be used for HTS against mGluRs.

1359-6446/00/$ – see front matter PII: S1359-6446(00)01499-9

Table 1. Characteristics of metabotropic glutamate receptors

	Group I	Group II	Group III
Members	mGluR1, mGluR5	mGluR2, mGluR3	mGluR4, mGluR6, mGluR7, mGluR8
Effector	Increase in phospholipase C	Decrease in adenylyl cyclase	Decrease in adenylyl cyclase
G-protein	$G\alpha_{q/11}$	$G\alpha_{i/o}$	$G\alpha_{i/o}$
Selective agonists	DHPG [(*S*)-3,5-dihydroxyphenylglycine][31] CHPG [(*S*)-2-chloro-5-hydroxyphenylglycine] (mGluR5 > mGluR1)[32] *Z-CBQA [(Z)-1-amino-3-[2′-(3′,5′-dioxo-1′,2′,4′-oxadiazolidinyl]cyclobutane-1-carboxylic acid] (mGluR5 > mGluR1)[33]	APDC [(2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate][34] *LY354740 [(1*S*,2*S*,5*R*,6*S*)-(+)-2-aminobicyclo[3.1.0.]hexane-2,6-dicarboxylic acid][35]	L-AP4 [(*S*)-4-phosphono-2-aminobutyric acid][36] PPG [(*R*,*S*)-4-phosphono-phenylglycine][37] L-SOP [(*S*)-serine-*O*-phosphate][36]
Selective antagonists	4CPG [(*S*)-4-carboxyphenylglycine][38] CPCCOEt [7-hydroxyiminocyclopropan-[*b*]chromen-1a-carboxylic acid ethyl ester] (mGluR1 > mGluR5)[14] SIB1757 [6-methyl-2-(phenylazo)-pyridin-3-ol] (mGluR5)[11] MPEP [2-methyl-6-(phenylethynyl)-pyridine] (mGluR5)[12]	LY341495 [(2*S*,1′*S*,2′*S*)-2-(9-xanthylmethyl)-2-(2′-carboxy-cyclopropyl)glycine][39] MCCG [(2*S*,3*S*,4*S*)-2-methyl-2-(carboxycyclopropyl)glycine][40] EGLU [(2*S*)-α-ethylglutamic acid][41]	MAP4 [(*S*)-2-amino-2-methyl-4-phosphonobutanoic acid][16] CPPG [(*RS*)-α-cyclopropyl-4-phosphonophenylglycine][42] MSOP [(*RS*)-α-methyl-4-phosphonophenylglycine][43]
Radioligands	[^{3}H]-quisqualate	*[^{3}H]-DCG IV [(2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxy-cyclopropyl)glycine][22] *[^{3}H]-LY341495 (Ref. 44) *[^{3}H]-LY354740 (Ref. 45)	[^{3}H]-L-AP4 (Ref. 46) [^{3}H]-CPPG [(*RS*)-α-cyclopropyl-4-phosphonophenylglycine]

*Compound is not commercially available.

Functional assays offer several advantages for HTS. Cell-based assay systems provide a more physiological environment in which to evaluate receptor function. Compounds can be identified that interact at novel sites for which no known ligand exists. The signaling events that occur downstream of receptor activation can also be analyzed and assay protocols can be established that can differentiate between the activity of agonists, antagonists or allosteric modulators. For HTS, it is common practice to establish a cell line that stably expresses the heterologous recombinant target of interest rather than using a transient expression system. Alternatively, a cell type can be identified that endogenously expresses the receptor of interest, such as astrocytes that express mGluR5 (Ref. 4). However, in the latter case, the selectivity of compounds cannot be addressed by performing control experiments on non-transfected cells. When screening against recombinant receptors, it is important to verify that the compounds of interest interact with native receptors, and with the same receptor subtype across different species, including the human homologs[5].

Ca^{2+}-based HTS assays

As described above, stimulation of Group I mGluRs activates $G\alpha_{q/11}$, resulting in the hydrolysis of phosphoinositides by phospholipase C and the concomitant release of Ca^{2+} from intracellular stores. Receptor activation can be monitored using several methods including measuring the levels of inositol phosphates (IP), cytidine monophosphate-phosphatidate (CMP-PA) or intracellular Ca^{2+}. The IP and CMP-PA assays are multi-step, radioactive end-point assays that are not convenient for HTS, although a 96-well plate version of the IP assay protocol has been described[6]. Because of its simplicity and sensitivity, the measurement of intracellular Ca^{2+} levels using fluorescent indicators is better suited to HTS.

Fluorometric imaging plate readers such as the SpeedReader (SIBIA Neurosciences, La Jolla, CA, USA)[7] and the Fluorometric Imaging Plate Reader (FLIPR; Molecular Devices, Sunnyvale, CA, USA)[8] have been developed that can rapidly detect changes in intracellular Ca^{2+} levels by monitoring cell permeant ion-sensitive fluorescent indicators such as fura-2 or fluo-3. These readers are integrated with automated liquid handling systems

Table 2. Potential functional screening assays for metabotropic glutamate receptors

		mGluRs			
Technology	**Assay**	**Group I**	**Group II**	**Group III**	**Issues relating to HTS**
Activation of endogenous $G\alpha_{q/11}$	PI hydrolysis	✓	✗	✗	Radioactive assay. Multi-step protocol. Low–medium throughput. End-point assay.
	Ca^{2+} assay	✓	✗	✗	Simple protocol. Medium–high throughput. Kinetic information. Multiple tests possible.
	Transcription-based assay (Ca^{2+} response element)	✓	✗	✗	Simple protocol. High throughput. End-point assay. Not yet published for mGluRs.
Activation of endogenous $G\alpha_{i/o}$	cAMP decrease	✗	✓	✓	Multi-step protocol. Low throughput. End-point assay.
	Transcription-based assay (cAMP response element)	✗	✓	✓	Simple protocol. High throughput. End-point assay. Not yet published for mGluRs.
	[^{35}S]-GTPγS binding	✗	✓	✓	Radioactive assay. Simple protocol. High throughput. End-point assay.
Activation of exogenous $G\alpha_{15}$/$G\alpha_{16}$/chimeric G proteins	Ca^{2+} assay	✓	✓	✓	Simple protocol. Medium–high throughput. Kinetic information. Multiple tests possible.
	Transcription-based assay (Ca^{2+} response element)	✓	✓	✓	Simple protocol. High throughput. End-point assay. Not yet published for mGluRs.

and enable real-time measurement of receptor-mediated activity in living cells. They are well-suited for HTS because of their rapid (1 s) detection of functional modulations of receptors and their ability to simultaneously analyze all the wells in a 96- or 384-well microtiter plate. Furthermore, unlike end-point assays, Ca^{2+} assays can be set up to differentiate between agonist and antagonist activities of compounds in the same well.

Group I mGluRs

Ca^{2+} measurements have been used to characterize the pharmacology of cells stably expressing recombinant human mGluR1 and mGluR5, which compares well with that obtained from IP measurements in the same cells[9,10]. These cell lines have been used in a system that incorporates a proprietary fluorescence imaging plate reader (SpeedReader), automated liquid handling, plate washing and a robotic plate transfer and tracking system to perform Ca^{2+}-based HTS (Refs 9,10).

Using this system, SIB1757 (Fig. 1a), a selective antagonist at mGluR5, was identified from a random compound library[11]. SIB1757 inhibited mGluR5 with an IC_{50} of 0.3 μM while showing no antagonist activity at other mGluR subtypes at concentrations of 100 μM[11]. Further medicinal chemistry efforts led to the identification of SIB1893 (Fig. 1b)[11] and, in collaboration with Novartis Pharma AG (Basel, Switzerland), the highly potent antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP; Fig. 1c)[12]. Using this Ca^{2+}-based assay, these antagonists were shown to non-competitively inhibit mGluR5 (Ref. 11). The amino acid residues to which these compounds bind were later identified using chimeric receptor and point mutation studies[13], and it was confirmed that these compounds interact outside of the glutamate binding site (i.e. by non-competitive mechanism). Interestingly, the non-competitive nature of the antagonism is similar to that observed for the mGluR1-selective antagonist, 7-hydroxyiminocyclopropan[*b*]chromen-1a-carboxylic acid ethyl ester (CPCCOEt)[14]. These findings highlight some of the advantages of using a functional screen over a traditional radioligand-binding screen for novel lead identification and characterization.

Group II and Group III mGluRs

Assays that measure Ca^{2+} are relatively straightforward to develop for Group I mGluRs. However, in recombinant expression systems, the Group II and Group III mGluRs do not stimulate phospholipase C, but are negatively coupled to adenylate cyclase (Table 1). Similar to other $G\alpha_{i/o}$-coupled receptors[15], Group II and Group III

mGluRs stimulate phospholipase C when the receptors are co-expressed with promiscuous ($G\alpha_{15}$ or $G\alpha_{16}$) or chimeric ($G\alpha_{qi}$, $G\alpha_{qo}$ or $G\alpha_{qz}$) G proteins[16–18]. The pharmacological profiles of mGluR2 and mGluR4 determined using $G\alpha_{qi}$ and $G\alpha_{qo}$, respectively, correlate well with those generated by measuring inhibition of cAMP formation in cells stably expressing these receptors[16]. Therefore, the use of chimeric or promiscuous G proteins can be combined with automated sample handling and fluorometric imaging to develop HTS systems for $G\alpha_{i/o}$-coupled receptors[19].

Similar to other functional assays, there are several issues relating to Ca^{2+}-level measurements to consider when establishing this assay as a high-throughput screen. Changes in Ca^{2+} levels are not always linear with changes in receptor activity. Therefore, although the rank order of potency should be the same between different functional assays, absolute agonist and/or antagonist potencies determined from Ca^{2+} measurements could differ from those of other assays. In addition, agonist efficacies might not be the same between assays, as this depends on the receptor reserve (that is, there might be a greater number of receptors than are actually required to evoke a full response, so called 'spare receptors'). Thus, a compound that appears to be a partial agonist at a receptor when measured by one assay could be detected as a full agonist in the Ca^{2+} assay (or vice versa), provided it activates a sufficient number of receptors to elicit a saturating Ca^{2+} response.

Receptor desensitization could also influence results between assays. A Ca^{2+} signal might be a more sensitive assay for receptors that desensitize rapidly, compared with an end-point assay such as the IP or CMP-PA assay. In addition, some agonists can evoke different levels of receptor desensitization for the same receptor. Consequently, the Ca^{2+} assay might not detect differences in efficacy between two agonists that could be observed in end-point assays.

Because of these considerations with functional screens, it is important to reiterate that results from HTS assays should always be confirmed in independent assay systems and, when possible, also using native receptors. These secondary assays could typically be IP (Group I mGluRs) or cAMP measurements (Group II or Group III mGluRs) in recombinant or native tissues[11,12].

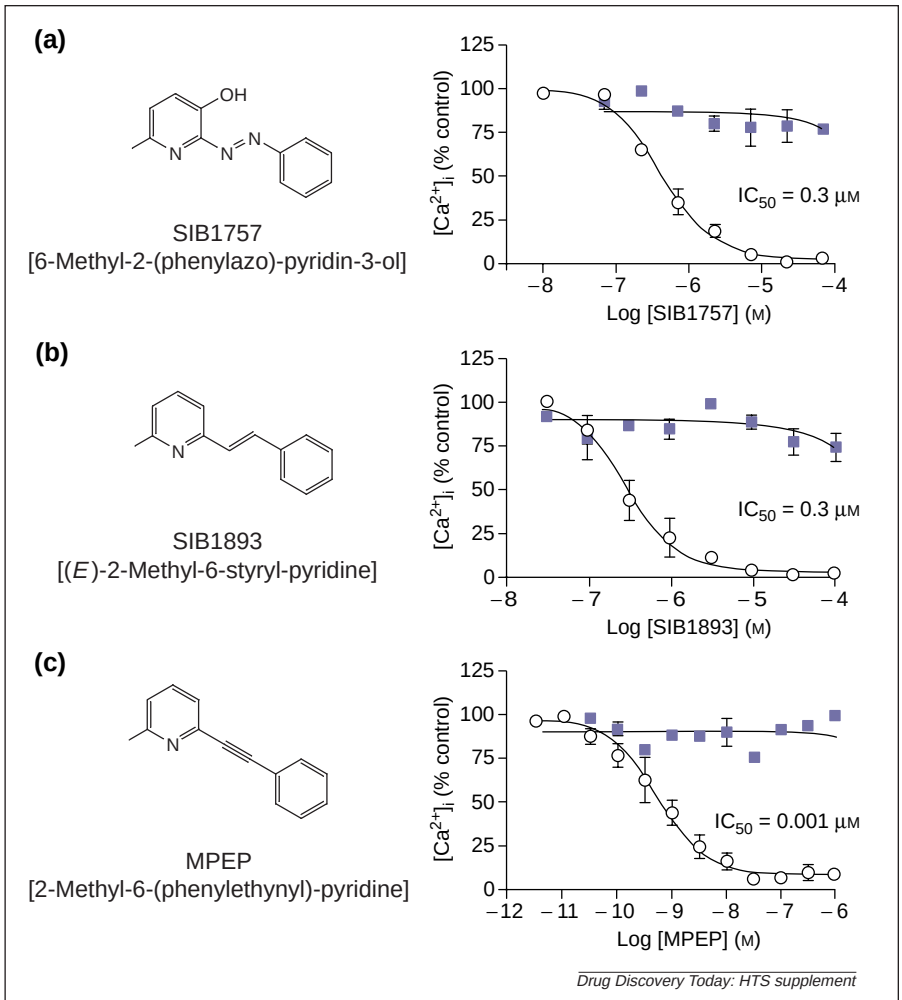

Figure 1. SIB1757 (a) was identified from a random HTS library as an inhibitor of glutamate-evoked Ca^{2+} signals in cells stably expressing human mGluR5 (–○–) with an IC_{50} of 0.3 μM, and negligible activity at human mGluR1 (–■–). Medicinal chemistry efforts yielded the analog, SIB1893 (b), which was also selective for mGluR5 (–○–) over mGluR1 (–■–), and the highly potent mGluR5 antagonist, MPEP (c), which inhibited glutamate-evoked Ca^{2+} signals with an IC_{50} of 1 nM at mGluR5 (–○–), while retaining a high level of selectivity over mGluR1 (–■–).

[^{35}S]-GTPγS binding: a functional radioligand-binding assay

Activated mGluRs transduce their signals across the plasma membrane by interacting with G proteins, stimulating the exchange of GDP for GTP from the G protein α-subunit. This guanine nucleotide exchange provides a direct measurement of receptor-dependent G protein activation and can be assayed using radiolabeled GTP or GTP analogs. In particular, the GTP analog guanosine 5′-O-(3-[^{35}S]thiotriphosphate) ([^{35}S]-GTPγS), which has a high affinity for G proteins and is resistant to GTPase activity, has been useful for studying $G\alpha_{i/o}$-coupled receptors, including mGluR2 and mGluR4 (Refs 20–22).

A high correlation has been established between cAMP measurements and [^{35}S]-GTPγS binding[21], and between [^{3}H]-DCG-IV

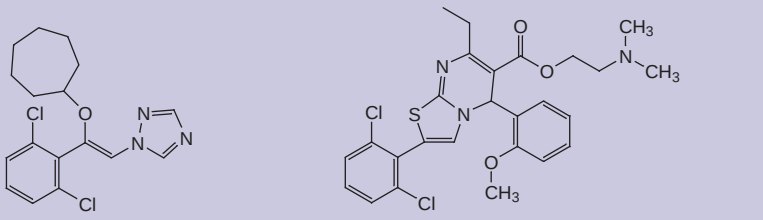

Figure 2. Structures of selective mGluR2 antagonists identified by [^{35}S]-GTPγS binding: the heterocyclic enol ethers, typified by Ro645229, and one of the more potent thiazolo-pyrimidine derivatives.

binding and [^{35}S]-GTPγS binding[22] using cell lines expressing mGluR2, indicating that the pharmacology observed with [^{35}S]-GTPγS binding is consistent with other assays. Furthermore, [^{35}S]-GTPγS binding assays have several advantages over second messenger assays. As they are radioligand-binding assays (albeit functional), they are rapid, easy to automate and amenable to HTS, especially when combined with FlashPlate (NEN, Boston, MA, USA) or scintillation proximity assay (SPA; Amersham Pharmacia Biotech, Piscataway, NJ, USA) technology. However, disadvantages of the assays are that they use radioactivity and are end-point assays. Moreover, because of the slower guanine nucleotide exchange rate of $G\alpha_q$-coupled receptors, it is difficult to measure robust [^{35}S]-GTPγS binding with Group I mGluRs (but see Ref. 23).

Two different structural series of mGluR2-selective antagonists have been reported recently. These compounds were identified using [^{35}S]-GTPγS binding to rat mGluR2-transfected cell membranes. The first, a series of heterocyclic enol ethers typified by Ro645229 (Fig. 2)[24], non-competitively inhibited agonist-stimulated [^{35}S]-GTPγS binding at mGluR2 with an IC_{50} of 0.1 μM, with no activity observed at ionotropic glutamate receptors, or mGluR1, mGluR3, mGluR4 and mGluR5. The second series, thiazolo-pyrimidine derivatives (Fig. 2)[25], was less potent, inhibiting agonist-stimulated [^{35}S]-GTPγS binding at mGluR2 with IC_{50} values of ≈1.0 μM, but also showed weak cross-reactivity at NMDA and AMPA receptors[25].

Transcription-based assays

Activation of Group II and Group III mGluRs decrease forskolin-stimulated cAMP levels. Typically, cAMP levels are measured by radioimmunoassay (RIA), which is a multi-step assay requiring large numbers of cells and the use of radioactive materials. As such, RIAs have not been readily amenable to automated HTS approaches. An alternative approach to a high-throughput functional assay has been transcription-based assays that respond to receptor-mediated changes in intracellular messengers [Ca^{2+}/protein kinase C (PKC) or cAMP], which in turn activate DNA regulatory sequences attached to reporter genes[26].

There have been several reports on the use of transcription-based assays for G protein-coupled receptors (GPCRs), although no reports specifically using mGluRs. Assays have been developed for receptors that alter cAMP levels using a cAMP-responsive element (CRE) coupled to reporter genes[26–28]. Likewise, assays have also been developed for receptors that increase phospholipase C activity, using Ca^{2+}/PKC-sensitive regulatory elements such as c-fos, intracellular adhesion molecule 1 (ICAM-1) or nuclear factor of activated T cells (NFAT) coupled to reporter genes[26–29]. In addition, the Ca^{2+}-sensitive protein, apoaequorin, can be used as a reporter for GPCR-mediated Ca^{2+} signaling when co-expressed in cell lines stably expressing the receptor of interest[30]. Thus, co-expression of chimeric or promiscuous G proteins with apoaequorin might provide the basis for a high-throughput bioluminescent screen for $G\alpha_{i/o}$-coupled receptors[30] such as Group II or Group III mGluRs.

Conclusions

The interest in mGluRs as potential therapeutic targets and the lack of subtype-selective mGluR ligands have driven the pharmaceutical industry to search for novel lead molecules using HTS assays. The discovery by HTS of the SIB1757 series of highly selective, non-competitive mGluR5 antagonists and the Roche series of selective mGluR2 antagonists supports the use of functional high-throughput screens for mGluR drug discovery efforts. The use of fluorescent Ca^{2+}-sensitive dyes, [^{35}S]-GTPγS binding assays and possibly transcription-based assays in HTS should facilitate the discovery of additional subtype-selective mGluR ligands that could interact at the ligand-binding site or at other modulatory sites for which no known ligands currently exist. The identification of such novel compounds should aid in the pharmacological characterization of this therapeutically relevant class of receptors, but also in the generation of potential drug leads.

References

1 Conn, P.J. and Pin, J.P. (1997) Pharmacology and functions of metabotropic glutamate receptors. *Annu. Rev. Pharmacol. Toxicol.* 37, 205–237

2 Pin, J.P. and Duvoisin, R. (1995) The metabotropic glutamate receptors: structure and functions. *Neuropharmacology* 34, 1–26

3 Anwyl, R. (1999) Metabotropic glutamate receptors: electrophysiological properties and role in plasticity. *Brain Res. Brain Res. Rev.* 29, 83–120

4 Miller, S. *et al.* (1995) Growth factor upregulation of a phosphoinositide-coupled metabotropic glutamate receptor in cortical astrocytes. *J. Neurosci.* 15, 6103–6109

5 Hall, J.M. *et al.* (1993) Receptor subtypes or species homologues: relevance to drug discovery. *Trends Pharmacol. Sci.* 14, 376–383

6 Tian, Y. *et al.* (1997) High throughput 96-well plate assay for receptor-mediated phosphoinositol turnover. *J. Biomol. Screening* 2, 91–97

7 Veliçelebi, G. *et al.* (1998) Fluorescent techniques for measuring ion channel activity. *Methods Enzymol.* 294, 20–47

8 Sullivan, E. *et al.* (1999) Measurement of $[Ca^{2+}]$ using the Fluorometric Imaging Plate Reader (FLIPR). *Methods Mol. Biol.* 114, 125–133

9 Daggett, L.P. *et al.* (1995) Molecular and functional characterization of recombinant human metabotropic glutamate receptor subtype 5. *Neuropharmacology* 34, 871–886

10 Lin, F.F. *et al.* (1997) Cloning and stable expression of the mGluR1b subtype of human metabotropic receptors and pharmacological comparison with the mGluR5a subtype. *Neuropharmacology* 36, 917–931

11 Varney, M.A. *et al.* (1999) SIB-1757 and SIB-1893: selective, noncompetitive antagonists of metabotropic glutamate receptor type 5. *J. Pharmacol. Exp. Ther.* 290, 170–181

12 Gasparini, F. *et al.* (1999) 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. *Neuropharmacology* 38, 1493–1503

13 Pagano, A. *et al.* (1999) 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a novel potent non-competitive mGluR5 antagonist, acts at a site in the transmembrane region. *Neuropharmacology* 10, 107P

14 Litschig, S. *et al.* (1999) CPCCOEt, a noncompetitive metabotropic glutamate receptor 1 antagonist, inhibits receptor signaling without affecting glutamate binding. *Mol. Pharmacol.* 55, 453–461

15 Offermanns, S. and Simon, M.I. (1995) $G\alpha_{15}$ and $G\alpha_{16}$ couple a wide variety of receptors to phospholipase C. *J. Biol. Chem.* 270, 15175–15180

16 Gomeza, J. *et al.* (1996) Coupling of metabotropic glutamate receptors 2 and 4 to $G\alpha_{15}$, $G\alpha_{16}$, and chimeric $G\alpha_{q/i}$ proteins: characterization of new antagonists. *Mol. Pharmacol.* 50, 923–930

17 Blahos, J. *et al.* (1998) Extreme C terminus of G protein α-subunits contains a site that discriminates between G_i-coupled metabotropic glutamate receptors. *J. Biol. Chem.* 273, 25765–25769

18 Corti, C. *et al.* (1998) Cloning and characterization of alternative mRNA forms for the rat metabotropic glutamate receptors mGluR7 and mGluR8. *Eur. J. Neurosci.* 10, 3629–3641

19 Milligan, G. and Rees, S. (1999) Chimaeric G alpha proteins: their potential use in drug discovery. *Trends Pharmacol. Sci.* 20, 118–124

20 Phillips, T. *et al.* (1998) Human metabotropic glutamate receptor 2 couples to the MAP kinase cascade in Chinese hamster ovary cells. *NeuroReport* 9, 2335–2339

21 Kowal, D. *et al.* (1998) A $[^{35}S]$GTPγS binding assessment of metabotropic glutamate receptor standards in Chinese hamster ovary cell lines expressing the human metabotropic receptor subtypes 2 and 4. *Neuropharmacology* 37, 179–187

22 Cartmell, J. *et al.* (1998) Characterization of $[^3H]$-(2S,2′R,3′R)-2-(2′,3′-dicarboxy-cyclopropyl)glycine ($[^3H]$-DCG IV) binding to metabotropic mGlu2 receptor-transfected cell membranes. *Br. J. Pharmacol.* 123, 497–504

23 Akam, E.C. *et al.* (1997) Pharmacological characterization of type 1α metabotropic glutamate receptor-stimulated $[^{35}S]$-GTPγS binding. *Br. J. Pharmacol.* 121, 1203–1209

24 Kolczewski, S. *et al.* (1999) Synthesis of heterocyclic enol ethers and their use as group 2 metabotropic glutamate receptor antagonists. *Bioorg. Med. Chem. Lett.* 9, 2173–2176

25 Wichmann, J. *et al.* (1999) Structure–activity relationships of substituted 5*H*-thiazolo[3,2-a]pyrimidines as group 2 metabotropic glutamate receptor antagonists. *Bioorg. Med. Chem. Lett.* 9, 1573–1576

26 Stratowa, C. *et al.* (1995) Use of a luciferase reporter system for characterizing G-protein-linked receptors. *Curr. Opin. Biotechnol.* 6, 574–581

27 George, S.E. *et al.* (1997) Functional coupling of endogenous serotonin ($5\text{-}HT_{1B}$) and calcitonin (C1a) receptors in CHO cells to a cyclic AMP-responsive luciferase reporter gene. *J. Neurochem.* 69, 1278–1285

28 George, S.E. *et al.* (1998) Functional analysis of the D2L dopamine receptor expressed in a cAMP-responsive luciferase reporter cell line. *Biochem. Pharmacol.* 56, 25–30

29 Weyer, U. *et al.* (1993) Establishment of a cellular assay system for G protein-linked receptors: coupling of human NK2 and $5\text{-}HT_2$ receptors to phospholipase C activates a luciferase reporter gene. *Recep. Channels* 1, 193–200

30 Stables, J. *et al.* (1997) A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. *Anal. Biochem.* 252, 115–126

31 Schoepp, D.D. *et al.* (1994) 3,5-dihydroxyphenylglycine is a highly selective agonist for phosphoinositide-linked metabotropic glutamate receptors in the rat hippocampus. *J. Neurochem.* 63, 769–772

32 Doherty, A.J. *et al.* (1997) (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG) activates mGlu5, but not mGlu1, receptors expressed in CHO cells and potentiates NMDA responses in the hippocampus. *Neuropharmacology* 36, 265–267

33 Littman, L. *et al.* (1999) Cyclobutane quisqualic acid analogues as selective mGluR5a metabotropic glutamic acid receptor ligands. *J. Med. Chem.* 42, 1639–1647

34 Schoepp, D.D. *et al.* (1996) The novel metabotropic glutamate receptor agonist 2*R*,4*R*-APDC potentiates stimulation of phosphoinositide hydrolysis in the rat hippocampus by 3,5-dihydroxyphenylglycine: evidence for a synergistic interaction between group 1 and group 2 receptors. *Neuropharmacology* 35, 1661–1672

35 Monn, J.A. *et al.* (1997) Design, synthesis, and pharmacological characterization of (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740): a potent, selective, and orally active group 2 metabotropic glutamate receptor agonist possessing anticonvulsant and anxiolytic properties. *J. Med. Chem.* 40, 528–537

36 Tanabe, Y. *et al.* (1993) Signal transduction, pharmacological properties, and expression patterns of two rat metabotropic glutamate receptors, mGluR3 and mGluR4. *J. Neurosci.* 13, 1372–1378

37 Gasparini, F. *et al.* (1999) (*R*,*S*)-4-phosphonophenylglycine, a potent and selective group III metabotropic glutamate receptor agonist, is anticonvulsive and neuroprotective *in vivo*. *J. Pharmacol. Exp. Ther.* 289, 1678–1687

38 Hayashi, Y. *et al.* (1994) Analysis of agonist and antagonist activities of phenylglycine derivatives for different cloned metabotropic glutamate receptor subtypes. *J. Neurosci.* 14, 3370–3377

39 Kingston, A.E. *et al.* (1995) Pharmacological analysis of 4-carboxyphenylglycine derivatives: comparison of effects on mGluR1α and mGluR5a subtypes. *Neuropharmacology* 34, 887–894

40 Knopfel, T. *et al.* (1995) Pharmacological characterization of MCCG and MAP4 at the mGluR1b, mGluR2 and mGluR4a human metabotropic glutamate receptor subtypes. *Neuropharmacology* 34, 1099–1102

41 Jane, D.E. *et al.* (1996) Potent antagonists at the L-AP4- and (1*S*,3*S*)-ACPD-sensitive presynaptic metabotropic glutamate receptors in the neonatal rat spinal cord. *Neuropharmacology* 35, 1029–1035

42 Toms, N.J. *et al.* (1996) The effects of (*RS*)-α-cyclopropyl-4-phosphonophenylglycine ((*RS*)-CPPG), a potent and selective metabotropic glutamate receptor antagonist. *Br. J. Pharmacol.* 119, 851–854

43 Thomas, N.K. *et al.* (1996) α-Methyl derivatives of serine-O-phosphate as novel, selective competitive metabotropic glutamate receptor antagonists. *Neuropharmacology* 35, 637–642

44 Johnson, B.G. *et al.* (1999) [^{3}H]-LY341495 as a novel antagonist radioligand for group II metabotropic glutamate (mGlu) receptors: characterization of binding to membranes of mGlu receptor subtype expressing cells. *Neuropharmacology* 38, 1519–1529

45 Schaffhauser, H. *et al.* (1998) *In vitro* binding characteristics of a new selective group II metabotropic glutamate receptor radioligand, [^{3}H]-LY354740, in rat brain. *Mol. Pharmacol.* 53, 228–233

46 Eriksen, L. and Thomsen, C. (1995) [^{3}H]-L-2-amino-4-phosphonobutyrate labels a metabotropic glutamate receptor, mGluR4a. *Br. J. Pharmacol.* 116, 3279–3287