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Constitutive activity modulation of human metabotropic glutamate 5a receptors in HEK293 cells: a role for key amino-terminal cysteine residues

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1 Several combinations of cysteine to serine mutations at positions 57, 93, 99 and 129 in the extracellular N-terminal domain of human metabotropic 5a (hmGlu5a) receptors were produced and expressed in HEK293 cells. Quisqualic acid-induced intracellular calcium ($[Ca^{2+}]_i$) mobilization and inositol phosphates (IP) accumulation revealed an apparent increased efficacy and decreased potency for hmGlu5a mutants C57S, C99S and C57/99S, as well as a total loss of function for the mutant C57/93/99/129S.

2 [^{3}H]Quisqualate saturation analysis revealed mutants C57S, C99S, C57/99S and the tetramutant C57/93/99/129S to have unchanged K_D but reduced B_{max} values. [^{3}H]MPEP saturation analysis on the same membrane preparations revealed no difference in K_D for any mutant, but a decrease in B_{max} value for the C57/93/99/129S receptor.

3 Inverse agonism of MPEP at hmGlu5a receptors was partially reduced by mutation C57S, significantly reduced by C99S and C57/99S mutations and totally abolished in the tetramutant.

4 We confirmed the surface expression of all the mutated receptors using [^{3}H]MPEP binding analysis on whole cells. However, B_{max} values were increased for mutant C57S, C99S, and C57/99S but decreased in the C57/93/99/129S receptor.

5 The 24 h preincubation of cells expressing hmGlu5a receptors with 1 μM MPEP followed by extensive washing dramatically increased the wild-type receptor efficacy to quisqualate, to the same levels seen with C57/99S receptors. MPEP preincubation did not affect C57/99S function.

6 We conclude that cysteines 57 and 99 are key residues necessary for modulating hmGlu5a receptor function.

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Abbreviations: GPCR, G-protein-coupled receptor; HEK, human embryonic kidney; hmGlu5a, human metabotropic glutamate receptor 5a; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; MTEP, 5-[(2-Methyl-1,3-thiazol-4-yl)ethynyl]-2,3′-bipyridine; rEAAC1, rat excitatory amino-acid carrier 1; 7TMD, seven-transmembrane domain; VFT, venus fly-trap; WT, wild type

Introduction

Glutamate is the major excitatory neurotransmitter in the central nervous system and is responsible for many neurophysiological roles, ranging from sensory pathway transmission to higher cognitive functions such as mood regulation and memory. To date, two classes of glutamate receptors are characterized in the nervous system: the ionotropic glutamate receptors and the metabotropic glutamate (mGlu) receptors. mGlu receptors modulate synaptic transmission through interactions with heterotrimeric G proteins and subsequent downstream effector pathways.

mGlu receptors belong to the family 3 of G-protein-coupled receptors (GPCRs) that include $GABA_B$-, Ca^{2+}-sensing (CaS), vomeronasal and putative taste receptors (Bockaert & Pin, 1999). Eight mGlu receptors have been identified and classified according to their sequence homology, main signalling pathway and pharmacological properties. mGlu1 and mGlu5 receptors belong to group I, and are coupled to $G_{\alpha q}$ and its associated effector phospholipase C.

In heterologous expression systems as well as *in vivo*, mGlu receptors are believed to exist as dimers held together by both noncovalent and covalent interactions such as intermolecular disulphide bonds (De Blasi *et al*., 2001). Cysteine residues mapped to the proximal part of the N-terminus region of the receptor were identified in mGlu1 (C140), mGlu5 (C129) and CaS (C129 and C131) receptors (Ray *et al*., 1999; Ray & Hauschild, 2000; Romano *et al*., 2001), which are responsible for the disulphide link between two monomers. Despite the fact that the single cysteine residues in mGlu1 and mGlu5 receptors are required for covalent interactions, it appears that mutating this cysteine does not abolish dimer formation by other, noncovalent type of interactions. In contrast, mutant

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CaS receptor C129/131S disrupted dimer formation and also increased the affinity for Ca^{2+} (Ray *et al*., 1999). It is not however clear as to what are the consequences of disrupting this covalent interaction of GPCR dimerization on the function of mGlu receptors in particular. Romano *et al*. (2001) suggested that other cysteines (C57 and C99) located in the extracellular amino-terminal region of hmGlu5a receptors, which are also highly conserved among group III GPCRs, are not implicated in mGlu5 receptor covalent dimerization and speculated that they may play a role in ligand binding (Romano *et al*., 2001). Here, we have further characterized the role that these key cysteines play in hmGlu5a receptor regulation and function.

Methods

Plasmids and mutagenesis, cell culture and transfection

cDNA encoding the hmGlu5a receptors in pcDNA3.1(−) was prepared as described previously (Malherbe *et al*., 2003). All point mutations were constructed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) as described in Romano *et al*. (2001). The coding sequences of all point mutants were confirmed by sequencing in both directions using an automated cycle sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

HEK293 cells were maintained in culture in DMEM supplemented with 10% FCS and 100 U ml^{-1} penicillin/streptomycin at 37°C and 5% CO_2. To obtain expression of hmGlu5a receptors, cells at 70% confluence were washed with MEM and transfected overnight with 20 μg pcDNA3.1(−) encoding cDNAs of interest using Lipofectamine transfection reagents according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, U.S.A.).

Membrane preparation

HEK293 EBNA cells were transfected as previously described in Malherbe *et al*. (2003). Cell pellets were resuspended in buffer A containing 50 mM Tris-HCl and 10 mM EDTA at pH 7.4 and homogenized for 30 s using a Polytron PT 3100 mixer (Kinematica, Littau, Switzerland). Cell homogenates were ultracentrifuged at 50,000 × *g* for 30 min at 4°C. Membrane pellets were then resuspended and homogenized as described above in ice-cold buffer B containing 10 mM Tris-HCl and 0.1 mM EDTA at pH 7.4 and submitted again to ultracentrifugation as before. Finally, the membrane pellet was homogenized again in an appropriate volume of buffer B, aliquoted and stored at −80°C. Before use, the membrane preparations were briefly Polytron-homogenized. Protein concentration of the membrane preparations was determined by the Bradford protein assay (Bio-Rad, Hercules, CA, U.S.A.) using γ-globulin as a standard.

Saturation analysis and whole-cell radioligand binding

[^{3}H]MPEP binding was performed as described in Malherbe *et al*. (2003). Briefly, the membrane preparations were diluted in binding buffer M consisting of 120 mM NaCl, 100 mM KCl, 25 mM $MgCl_2$, 25 mM $CaCl_2$, 15 mM Tris-HCl at pH 7.4 to an assay concentration of 60 μg protein ml^{-1}. Saturation isotherms were determined by the addition of different radioligand concentrations to the membranes in a volume of 200 μl for 1 h at 4°C.

[^{3}H]Quisqualic acid binding was performed in the same manner but in binding buffer Q containing 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 20 mM HEPES, pH 7.4, at RT.

[^{3}H]MPEP binding on living, transiently transfected HEK293 cells was performed as described above for total membrane preparations with the following modifications. At 48 h post-transfection, cells were suspended and washed in Hank's balanced saline solution (HBSS) supplemented with 20 mM HEPES, counted and plated into 96-well plates with different radioligand concentration in a total volume of 200 μl at a final cell density of 25×10^4 cells per well. After 1 h incubation on ice, cells were washed on GF/C filter plates with ice-cold 50 mM Tris-HCl at pH 7.5 as described in Malherbe *et al*. (2003).

Saturation experiments were analysed by Prism 3.0 (GraphPad software, San Diego, CA, U.S.A.) using the rectangular hyperbolic equation derived from the equation of a bimolecular reaction and the law of mass action, $B = (B_{max} \times [F])/K_D + [F])$, where B is the amount of bound ligand at equilibrium, B_{max} is the maximum number of binding sites, $[F]$ is the concentration of free ligand and K_D is the ligand dissociation constant.

Intracellular Ca^{2+} release measurement (fluorometric imaging plate reader, FLIPR)

At 24 h post-transfection, transfected HEK293 cells were harvested and seeded at 5.5×10^4 cells per well in poly-D-lysine-treated, black-, clear-bottomed 96-well plates (BD Biosciences, Palo Alto, CA, U.S.A.). At 48 h post-transfection, the cells were loaded with 4 μM Fluo-4AM (Molecular Probes, Eugene, OR, U.S.A.) in assay buffer corresponding to 1 × HBSS supplemented with 20 mM HEPES, and kept at 37°C for 1 h. Cells were washed five times with assay buffer and intracellular calcium ($[Ca^{2+}]_i$) release was measured using FLIPR (Molecular Devices, Menlo Park, CA, U.S.A.) as described in Porter *et al*. (1999). Responses were measured as peak increase in fluorescence minus basal levels, normalized to the maximal stimulatory effect induced by the agonist. Activation curves were fitted according to the Hill equation: $y = 100/(1+x/EC_{50})^{n_H})$, where n_H is the slope factor, using Prism 3.0 (GraphPad software).

Phosphatidylinositol (PI) hydrolysis measurement: scintillation proximity assay

PI hydrolysis turnover was measured as described in Brandish *et al*. (2003) with the following adaptations. At 24 h post-transfection, HEK293 cells were washed twice in inositol-free DMEM with reduced L-glutamine (1 mM final) and 10% dialysed FCS. Cells were seeded at 7.5×10^4 cells per well in poly-D-lysine-treated 96-well plates in the same medium supplemented with 5 μCi ml^{-1} of *myo*-[2-^{3}H]inositol (specific activity: 15.0 Ci mmol^{-1}). Radiolabelled inositol was incubated with the cells overnight. At 48 h post-transfection, cells were washed three times in assay buffer (1 × HBSS, 20 mM HEPES). To remove any trace of endogenously produced L-glutamate, cells were incubated for 1 h at 37°C in assay buffer containing 1 U ml^{-1} glutamate-pyruvate transaminase (Roche Diagnostics,

Switzerland) and 2 mM sodium pyruvate. The appropriate volume of assay buffer was applied to the cells 5 min before the application of drugs in the presence of LiCl (8 mM final). Stimulation was allowed to proceed for 45 min at 37°C. The assay was terminated by replacing the media from the cells with 100 μl per well of 20 mM formic acid. After 20 min at RT, 20 μl of the resulting cell extract was transferred to 80 μl of yttrium silicate beads (12.5 mg ml^{-1}), and shaken for 30 min at RT. YSi beads were allowed to settle for 30 min before counting on a Packard Top-count microplate scintillation counter with quenching correction (Canberra Packard SA, Zürich, Switzerland). Activation and inhibition curves were fitted according to the Hill equation: $y = 100/(1+x/EC_{50})^{n_H}$), where n_H is the slope factor, using Prism 3.0 (GraphPad software).

Materials

2-Methyl-6-phenylethynyl-pyridine (MPEP) and L-glutamate mono-Na$^+$ salt were obtained form Sigma Chemicals (Buchs, Switzerland). 5-[(2-Methyl-1,3-thiazol-4-yl)ethynyl]-2,3′-bipyridine (MTEP) was synthesized as described (Cosford *et al*., 2002) at F. Hoffmann-La Roche Ltd Laboratories, Basel, Switzerland. L-Quisqualic acid and [^{3}H]MPEP was purchased form Tocris Cookson Ltd (Bristol, U.K.). [^{3}H]Quisqualate, YSi beads and *myo*-[2-^{3}H]inositol were purchased from Amersham Biosciences (Basel, Switzerland). Inositol-free DMEM was purchased from ICN (Irvine, U.S.A.); all other media and cell culture product were from Gibco Invitrogen Corporation (Grand Island, NY, U.S.A.).

Results

Functional effect of cysteine to serine mutations on activation of hmGlu5a receptor

To test whether the mutated receptors C57S, C93S, C99S, C129S, C57/99S and the tetramutant C57/93/99/129S (4C-S) could have a direct effect on hmGlu5a receptor function, HEK293 cells were transiently transfected with the wild-type (WT) or mutated hmGlu5a receptors. All mutants, except the tetramutant 4C-S, elicited a quisqualate and glutamate concentration-dependent intracellular-free calcium increase $[Ca^{2+}]_i$, when monitored using an FLIPR in the presence of the Ca^{2+}-sensitive dye Fluo-4 (Figure 1a and b). The EC_{50} and relative E_{max} values calculated from concentration–response curves are shown in Table 1.

The relative E_{max} of C93S and C129S appeared similar to the E_{max} of the WT, whereas the E_{max} of C57S, C99S and C57/99S yielded approximately two-fold higher values relative to the WT (Figure 1a and Table 1). Furthermore, the mutants C57S, C99S and C57/99S displayed clear right shifts in EC_{50} values of two to three orders of magnitude. Both the observed decrease in potency and increase in efficacy for the mutants C57S, C99S and C57/99S were confirmed when measured by inositol phosphate (IP) accumulation after quisqualic acid stimulation in the same conditions (Figure 1c and Table 2).

Although the relative E_{max} of C129S observed in the fluorometric measurement was slightly but not significantly higher than the E_{max} of the WT (difference +12.9%), IP production measurement yielded a lower relative E_{max} for C129S as compared to the WT (difference −6.3%). This relatively small inconsistent difference is thought to be explained by intrinsic variability in functional activities from transiently transfected cells.

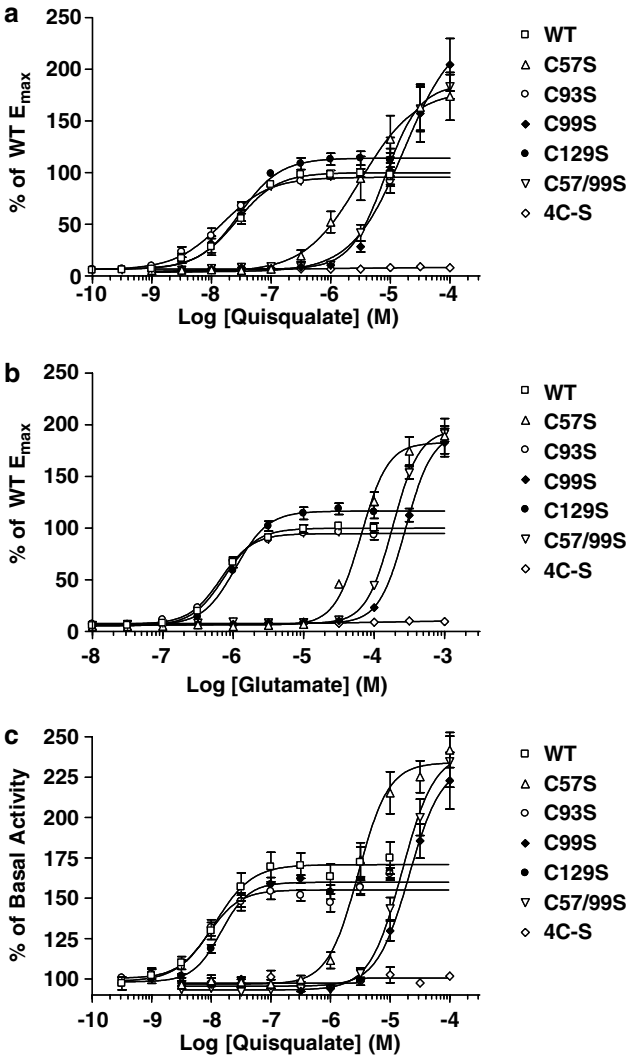

Figure 1 Increased efficacy but decreased potency in C57S, C99S and C57/99S mutants of hmGlu5a receptor upon quisqualate- or glutamate-induced activation. HEK293 cells were transiently transfected with cDNAs coding for hmGlu5a WT or the indicated mutated receptors. (a) Quisqualate- or (b) glutamate-induced $[Ca^{2+}]_i$ mobilization was assessed using the Ca^{2+}-sensitive dye Fluo-4 and FLIPR. In all, 100% correspond to 13 688 fluorescence units. (c) IP production concentration–response curve of quisqualate applied on HEK293 expressing the indicated cysteine to serine mutants together with rat glutamate transporter rEAAC1 and the G protein α_q subunit. Data represent the mean ± s.e.m. of three (a and b) or four (c) measurements performed in quadruplicates from three or four independent transfections, respectively. Note that the tetramutant 4C-S displays total loss of function even at high concentration of the agonist.

Effect of cysteine mutations on [^{3}H]quisqualic acid binding

Saturation binding analyses were performed on membranes isolated from HEK293 cells transfected with either the WT hmGlu5a or mutated receptors using [^{3}H]quisqualic acid.

Table 1 Effect of the cysteine to serine mutations in hmGlu5a receptor on quisqualate- or glutamate-induced $[Ca^{2+}]_i$ response

	Quisqualate		*Glutamate*	
	EC_{50} (nM)	E_{max} (% of WT E_{max})	EC_{50} (μM)	E_{max} (% of WT E_{max})
WT	29.3±12.8	100.0±7.7	0.7±0.2	100.0±1.6
C57S	4914.7±2702.7*	168.5±53.8	68.2±5.6**	189.7±6.9**
C93S	19.7±8.7	94.5±7.2	0.7±0.1	94.8±1.3
C99S	21 228.7±12 089.0*	240.0±25.5*	280.8±14.2 **	192.4±7.3**
C129S	32.1±13.5	112.9±14.1	1.1±0.2	116.6±2.2
C57/99S	15 100.0±9548.3*	186.2±27.1*	183.3±2.5**	194.9±3.7**
4C-S	ND	ND	ND	ND

Average±s.e.m. of $n=3$ independent experiments; ND, not determined; two-tailed *t*-test.
*$P<0.05$.
**$P<0.001$.

Table 2 Effect of the cysteine to serine mutations in hmGlu5a receptor on quisqualate-induced IP accumulation

	EC_{50} (nM)	E_{max} (% of basal activity)
WT	12.1±3.5	167.3±14.1
C57S	3586.3±793.7*	230.2±18.0*
C93S	13.0±5.7	155.9±9.4
C99S	20 046.7±1158.2**	220.0±37.8*
C129S	21.0±8.0	160.6±1.9
C57/99S	16 490.0±425.7**	230.6±32.3*
4C-S	ND	ND

Average±s.e.m. of $n=3$ independent experiments; ND, not determined; two-tailed *t*-test.
*$P<0.05$.
**$P<0.001$.

Table 3 Saturation analysis of [^{3}H]quisqualic acid binding to hmGlu5a and mutants in HEK293 cell membranes

	K_D (nM)	B_{max} (fmol mg^{-1} prot.)
WT	80.9±19.1	1593±284
C57S	97.3±14.8	658±236*
C93S	60.4±7.1	1380±139
C99S	81.6±12.0	515±84*
C129S	69.5±12.4	1556±234
C57/99S	105.9±28.1	661±161*
4C-S	117.0±40.7	673±314*

Average±s.e.m. of $n=4$ independent experiments; two-tailed *t*-test.
*$P<0.05$.

Dissociation constants (K_D) and maximum binding sites (B_{max}) derived from saturation isotherms are given in Table 3. Each value represents an average of four independent experiments performed in triplicate. There were no significant alterations in K_D values of the mutated receptors. However, relative to the WT receptor, C57S, C99S, C57/99S and the C57/93/99/129S mutant receptors displayed a significant decrease in binding site densities (Table 3).

Decreased constitutive activity in hmGlu5a receptor cysteine mutants

Along with other GPCRs and mGlu receptors (Prezeau *et al*., 1996; de Ligt *et al*., 2000; Carroll *et al*., 2001), hmGlu5a receptors have been shown to display a high level of constitutive activity (Joly *et al*., 1995; Gasparini *et al*., 1999).

To assess the impact on the constitutive activity of the cysteine to serine mutations in hmGlu5a receptor, we measured IP accumulation in HEK293 cells transiently transfected with the WT or mutated receptors after exposure to the inverse agonist MPEP using concentrations from 0.3 nM to 3 μM. In the same range as for the WT receptor, which displayed 21.3±2.6% inhibition (Figure 2a), MPEP also decreased the constitutive IP production of C93S (23.7±2.2%) and C129S (17.9±2.3%). Although not statistically significant, MPEP-induced inhibition was slightly less pronounced for C57S expressing cells (15.5±1.4%) when compared to the WT. However, MPEP did exhibit significantly reduced inverse agonism in cells expressing C99S or C57/99S receptors (11.3±2.0 and 8.6±1.5% inhibition, respectively). In the tetramutant-expressing cells, MPEP had no effect. The potency of inverse agonism remained the same between the WT and mutated receptors, except for the loss of function mutant (Table 4).

Comparable results were observed using another noncompetitive antagonist of hmGlu5a receptors, MTEP (Anderson *et al*., 2002; Roppe *et al*., 2004), where C57S, C99S and C57/99S receptors had a markedly reduced inhibition of constitutive activity (17.1±1.9, 8.6±1.6 and 8.1±1.8% inhibition, respectively) as compared to the WT, C93S and C129S (23.2±1.9, 26.0±3.0 and 22.0±2.6% inhibition, respectively).

Effect of cysteine mutations on [^{3}H]MPEP binding

Extensive mutational analysis of hmGlu5a receptors (Pagano *et al*., 2000; Malherbe *et al*., 2003) localized the MPEP binding site in an allosteric site that resides within the seven-transmembrane domain (7TMD). Since the cysteine mutations studied in this report are located in an area distinct from the MPEP binding site but can still influence MPEP-induced inhibition, we investigated the impact of the mutations on MPEP binding to hmGlu5a receptors. Saturation analysis was performed on membranes obtained from HEK293 cells transiently expressing the WT or mutated hmGlu5a receptors using 0.04 to 100 nM [^{3}H]MPEP. Table 5 lists the K_D and B_{max} calculated from the saturation isotherms. Dissociation constants obtained for the mutants were indistinguishable from the WT and are consistent with published data (Malherbe *et al*.,

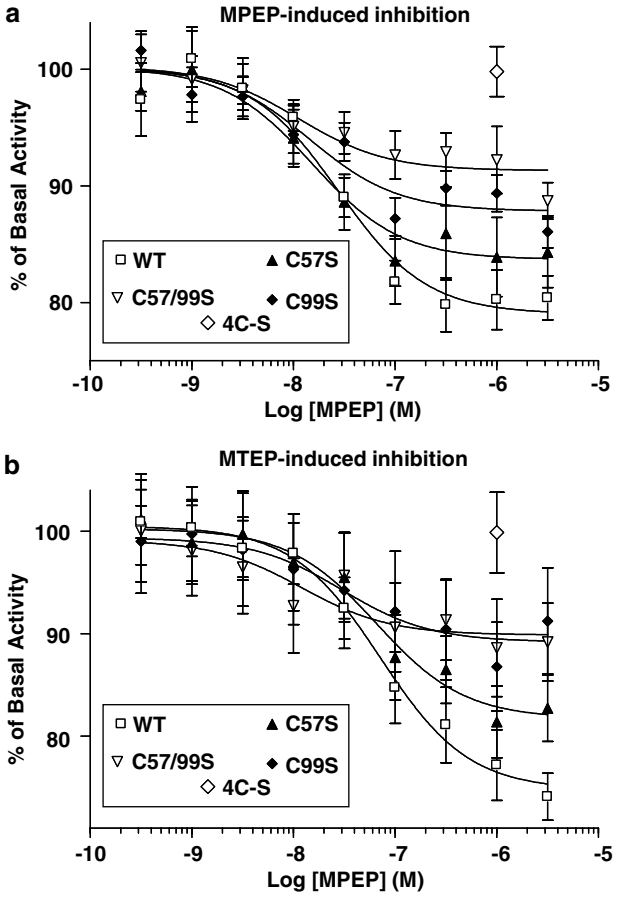

Figure 2 Constitutive activity of hmGlu5a receptor is decreased by N-terminal cysteine to serine mutations. HEK293 cells were transiently transfected with cDNAs coding for hmGlu5a WT or the indicated mutated receptors. IP production concentration–response curve of MPEP (a) or MTEP (b) applied on HEK293 expressing the WT or the indicated mutated receptors together with rEAAC1 and $G\alpha_q$. The signal window between the constitutive activity and the ground state corresponds to 498±44 to 368±15 c.p.m.for the WT and 431±83 to 367±64 c.p.m. for the C57/99S mutant, respectively. For the tetramutant 4C-S, no MPEP- or MTEP-mediated inhibition could be measured. Mutant receptors C93S and C129S behaved as the WT (not shown). The presented data are the mean±s.e.m. of three experiments performed in quadruplicates from three independent transfections and is expressed as the percentage of the WT alone.

Table 4 IC_{50} values obtained from MPEP- and MTEP-induced inhibition of basal values of transiently transfected hmGlu5a WT or mutated receptors by IP accumulation measurement

	MPEP (nM)	*MTEP* (nM)
WT	25.7±4.1	71.4±110
C57S	17.0±8.4	55.9±20
C93S	20.7±6.2	48.5±15
C99S	8.8±6.1	19.4±46
C129S	16.5±6.9	60.4±26
C57/99S	11.3±7.6	ND
4C-S	ND	ND

Average±s.e.m. of $n=3$ independent experiments; ND, not determined.

2003). The maximum binding sites observed in the mutants were comparable with the WT, with exception to the non-functional mutant 4C-S, which had a nearly two-fold decreased B_{max} when compared to the WT and the other mutants.

Whole-cell binding

The basal activity of GPCRs can be modulated by expression levels (Prezeau *et al*., 1996; Hermans & Challiss, 2001). In order to evaluate a possible effect of the cysteine mutations on cell surface expression levels, we performed saturation binding analysis on intact living cells using 0.3 to 100 nM [^{3}H]MPEP. In agreement with the results obtained from the [^{3}H]MPEP binding on total membrane preparations, none of the mutated receptors had altered affinity for the radioligand (Figure 3 and Table 6). However, the C57S, C99S and C57/99S mutants had statistically significant increased B_{max} values, indicative of higher surface expression. In contrast, the tetramutant 4C-S had a markedly reduced relative B_{max}. It is worth noting that all the mutants were present at the cell surface, including the total loss of function mutant 4C-S (Figure 3, Table 6).

MPEP preincubation

The efficacy of the WT receptors was dramatically increased when incubated for 24 h with 1 μM MPEP followed by

Table 5 Saturation analysis of [^{3}H]MPEP binding to hmGlu5a and mutants in HEK293 cell membranes

	K_D (nM)	B_{max} (fmol mg^{-1} prot.)
WT	2.1±0.7	4983±527
C57S	2.5±0.9	5091±2514
C93S	2.6±0.9	6509±1346
C99S	2.7±0.5	5463±207
C129S	2.6±0.7	4766±294
C57/99S	2.9±0.9	5835±575
4C-S	2.4±0.6	2338±55*

Average±s.e.m. of $n=4$ independent experiments; two-tailed *t*-test.
*$P<0.05$.

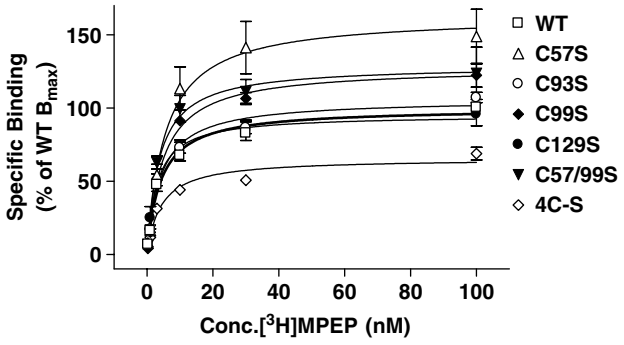

Figure 3 Saturation isotherms of [^{3}H]MPEP binding analysis performed on living HEK293 cells transiently transfected with the hmGlu5a WT or cysteine to serine mutants as indicated. Cells were plated at a density of 25×10^4 cells per well. The average difference in DPM between total binding and nonspecific binding (defined by 10 μM MPEP) was then expressed as a percentage of that obtained for the WT (11 122.3±622.2DPM). Each point represents the mean±s.e.m. of at least three independent transfections and experiment performed in quadruplicates.

extensive washing prior to agonist stimulation. The increased E_{max} values reached the maximal efficacy over basal as the C57/99S mutant receptors (WT $E_{max} = 151.3 \pm 9.4\%$ of basal activity, WT + MPEP $E_{max} = 241.9 \pm 24.7\%$, two-tailed *t*-test, $P < 0.05$; C57/99S $E_{max} = 229.1 \pm 8.1\%$, $P < 0.001$ and C57/99S + MPEP $E_{max} = 239.0 \pm 5.6\%$, $P < 0.001$; see Figure 4, Table 7). The mutated receptor, however, did not significantly change its efficacy when incubated with MPEP before testing. EC_{50} values of the WT receptor (26.2 ± 5.8 nM) did not change upon MPEP treatment (27.7 ± 9.4 nM). As presented above, the double mutant C57/99S, both MPEP-treated and -untreated, displayed drastically diminished affinity for quisqualic acid when compared to the WT (EC_{50} C57/99S: 7.8 ± 0.5 μM and C57/99S + MPEP: 15.9 ± 1.7 μM).

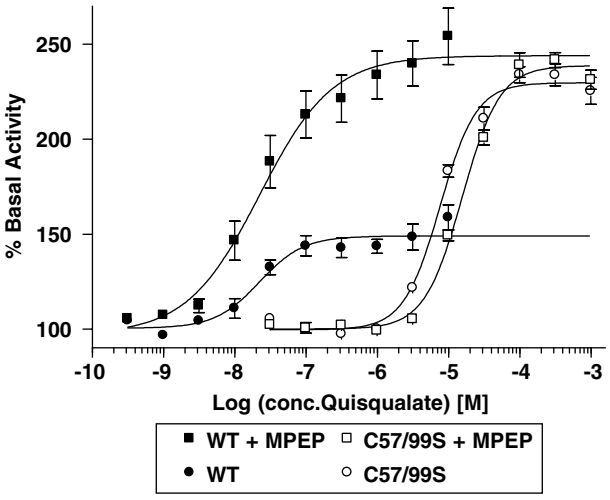

Figure 4 Increased quisqualate-induced IP accumulation after inverse agonist pretreatment of hmGlu5a receptor. At 24 h post-transfection, HEK293 cells expressing the WT hmGlu5a or C57/99S mutant (each cotransfected with rEAAC1 and the $G\alpha_q$ subunit) were split into equal parts: the first parts (WT and C57/99S) received no special treatment and the second parts (WT + MPEP and C57/99S + MPEP) were incubated in the presence of 1 μM MPEP during 24 h before measuring the IP turnover as described in Methods. Extensive washing steps ensured that MPEP traces were removed from the assay medium prior to agonist stimulation. Each curve represents the mean ± s.e.m. of three (WT) or four (C57/99S) dose–response measurements performed in quadruplicates from three or four independent transfections, respectively. MPEP-treated WT cells displayed more than 2.5-fold increase in efficacy as compared to nontreated cells.

Discussion

The structural basis underlying the constitutive activity of GPCRs is beginning to be explored. The present study demonstrates that key cysteine residues in the amino-terminal region of the VFT module of hmGlu5a receptors have regulatory activity in modulating hmGlu5a receptor's constitutive activity when expressed in HEK293 cells. We showed that substituting cysteines at position 57 and 99 for serine residues gives rise to a higher IP production and $[Ca^{2+}]_i$ mobilization relative to the WT receptors, and lowered potency as revealed by the functional assays. C93S and C129S mutant receptors did not appear to affect hmGlu5a receptors function. These results are in general agreement with a previous study (Romano *et al*., 2001) where C57S and C99S mutants were functionally responding to glutamate only at very high doses and where residue C129 was not necessary for receptor functionality but reported to be critical for covalent dimerization of hmGlu5a receptors.

In general, receptor overexpression can result in increased constitutive activity. The observation that mutations at positions 57 and 99 resulted in higher [^{3}H]MPEP whole-cell binding but no change in homogenate binding levels (Figure 3, Table 5) suggests that a higher proportion of the expressed receptors are present on the cell surface. Further, the significantly reduced agonist EC_{50} values for these mutations when coupled with the increased efficacy over baseline relative to the WT receptors suggests a reduced propensity for agonist-independent G-protein coupling. This hypothesis is supported by the [^{3}H]quisqualate binding data demonstrating a reduced density of sites in the high-affinity state (G-protein coupled) but no change in affinity for quisqualate (Table 3). The low binding affinity of [^{3}H]quisqualate, coupled with the relatively low levels of specific binding (approximately 60%), precluded any direct testing of this hypotheses due to high levels of variability. Consequently, attempts to fit the WT saturation plots to two sites were unsuccessful. Unfortunately, an antagonist radioligand for hmGlu5a receptors binding at the glutamate recognition site is not available, but we would hypothesize that it would not discriminate between any of the studied receptors, assuming its binding was not directly affected by the mutations (Sleight *et al*., 1996; Strange,

Table 6 Whole-cell binding: saturation analysis of [^{3}H]MPEP with hmGlu5a and mutants in intact HEK293 cells

	K_D (nM)	*Relative* B_{max} (% of WT)
WT	4.2 ± 0.1	100.0 ± 0.0
C57S	5.5 ± 0.3	163.2 ± 20.5*
C93S	4.4 ± 0.6	106.4 ± 3.3
C99S	4.9 ± 0.6	127.5 ± 3.4**
C129S	3.2 ± 0.6	97.6 ± 6.0
C57/99S	3.6 ± 0.1	127.2 ± 10.7*
4C-S	4.4 ± 0.6	65.8 ± 3.7

Average ± s.e.m. of $n = 4$ independent experiments; two-tailed *t*-test.
*$P < 0.05$.
**$P < 0.001$.

Table 7 EC_{50} and relative E_{max} values of quisqualate-induced IP production by hmGlu5a WT and C57/99S after preincubation with MPEP

Receptor	*Treatment*	*EC*$_{50}$	E_{max} (% basal)
WT		26.2 ± 5.8 (nM)	151.3 ± 9.4
WT	+ MPEP	27.7 ± 9.4 (nM)	241.9 ± 24.7*
C57/99S		7.8 ± 0.5 (μM)**	229.1 ± 8.1**
C57/99S	+ MPEP	15.9 ± 1.7 (μM)**	239.0 ± 5.6**

Average ± s.e.m., $n = 3$ (WT) or 4 (C57/99S) independent experiments; two-tailed *t*-test.
*$P < 0.05$.
**$P < 0.001$.

2002). The discrepancy between the large decrease in functional potency for quisqualate and no change in [^{3}H]quisqualate binding affinity for the C57S and C99S mutant receptors (Tables 1–3) cannot be explained at this time, except to suggest that more than the affinity of agonist binding is necessary to induce a functional response in the current experiments.

It has recently been reported that mGlu5 receptors display constitutive activity even in the absence of its extracellular domain (Goudet *et al*., 2004). However, our data are supportive of an interaction between the extracellular domain and the 7TMD. The ability of the inverse agonists MPEP and MTEP to inhibit the constitutive activity of hmGlu5a receptors appeared to be significantly lowered for C99S, C57/99S, 4C-S and to some extent C57S, suggesting that the N-terminus can influence basal activity of hmGlu5a receptors. An impairment of the MPEP binding site can be ruled out because none of the mutations disturbed the binding affinity of radiolabelled MPEP. If the cysteine mutations induced a conformational change in the receptor, resulting in decreased basal activity, it could explain the higher magnitude of agonist-induced response observed in the calcium mobilization assay and the IP hydrolysis assay. If this is the case, we showed for the first time that these cysteine residues are implicated in the stabilization of an active state of hmGlu5a receptors.

The lowered constitutive activity attributed to C57S, C99S or C57/99S mutations (Figure 2) cannot solely be explained by the small increase in cell surface expression level as observed in the whole-cell binding experiments (Prezeau *et al*., 1996; Hermans & Challiss, 2001). One potential explanation may be that because of the lack in constitutive activity, C57S, C99S and C57/99S mutants are less susceptible to desensitization; therefore, less receptor internalization occurs explaining the slightly increased cell surface expression. In line with this interpretation, preincubation of the WT receptor with MPEP thus artificially decreasing its constitutive activity prior to agonist stimulation resulted in a drastically increased response, reaching amplitudes identical to the one observed for the double mutant C57/99S whose basal activity was already close to its minimal level and could not be lowered further with MPEP preincubation.

A possible peptidic misfolding could underlie the reduced surface expression and loss of function of the tetramutant 4C-S, thereby explaining the reduced B_{max} obtained in the binding experiments with [^{3}H]quisqualate and [^{3}H]MPEP. The fact that the maximum number of 4C-S receptors was $\sim$34% lower than the WT receptors would not however explain the total loss of function of the tetramutant.

Our data are consistent with the hypothesis that dimerization has an important role in GPCR activation. It has recently been shown using fluorescence resonance energy transfer analysis (Tateyama *et al*., 2004) that agonist binding on mGlu1α receptor dimers does not clearly change the conformation of a single subunit but rather changes the dimeric allocation of the cytoplasmic regions, which is likely to influence downstream signalling. It could therefore be that the functional consequences of the cysteine mutation reflect interaction impairment between the two partners of a dimer. This could explain the fact that truncation of the N-terminal domain of mGlu5a receptor does not impair constitutive activity (Goudet *et al*., 2004).

Another possible consequence of the cysteine to serine mutations is that they alter the interaction of hmGlu5a receptors with Homer proteins through indirect intramolecular conformational changes. Homer 1b and 1c proteins have been shown to be constitutively expressed in HEK293 cells (Ciruela *et al*., 1999; Soloviev *et al*., 2000) and to interact directly with hmGlu5a receptors (Tu *et al*., 1998). It has been suggested that a dynamic interplay between Homer 1a and Homer 1b/1c is responsible for the regulation of the constitutive activity of hmGlu5a either by controlling the targeting of receptor to the membrane surface or by modulating the spontaneous activation of the G protein through physical constrains on the C-terminus of hmGlu5a receptors (Hermans & Challiss, 2001; Fagni *et al*., 2002). The lowered constitutive activity observed with the mutant receptors C57S, C99S and C57/99S could therefore be explained by a strengthened interaction with Homer proteins 1b/1c, which is thought to stabilize hmGlu5a receptors in their inactive state (Ango *et al*., 2001). Furthermore, Homer 1c has been shown to enhance the clustering of hmGlu1α on the cell surface and to increase agonist-induced IP3 production (Ciruela *et al*., 2000). This could explain our findings that mutant receptors C57S, C99S and C57/99S are relatively more abundant at the cell surface. Further experimentation involving Homer proteins are necessary to test this hypothesis.

Interestingly, it has recently been shown that mGlu1α activation by extracellular divalent cations such as calcium (Ca^{2+}) (Kubo *et al*., 1998) is sensitive to DTT treatment, indicating that preservation of disulphide bonds in the extracellular domain of mGlu receptors is essential for stimulation by Ca^{2+} (Francesconi & Duvoisin, 2004). An increase in extracellular [Ca^{2+}] has also been reported to trigger activation of mGlu5a receptors (Hermans & Challiss, 2001). Despite the fact that the DTT treatment is likely to disrupt disulphide bridges in the cysteine-rich region of the receptor and not only in the N-terminal part of the extracellular domain, it is tempting to hypothesize that the cysteines at position 57 and 99 of hmGlu5a receptors could be involved in the maintenance of the integrity of the Ca^{2+}-sensing function of the receptor. Indeed, the Ca^{2+} sensitivity of mGlu1α has been attributed to a residue that is immediately adjacent to the agonist binding site (O'Hara *et al*., 1993; Kubo *et al*., 1998). Since [^{3}H]quisqualate binding is affected in mutants C57S, C99S, C57/99S and 4C-S, it remains to be tested whether these conserved cysteines confer structural integrity not only to the glutamate binding pocket but also to the calcium binding site. However, these would explain in part the decreased potency of these mutants but not the increased efficacy.

To conclude, we have demonstrated that cysteines at position 57 and 99 play a key role for the function of hmGlu5a receptors because they are involved in several aspects of the receptors fine regulation including agonist binding kinetics as well as allosteric transduction mechanism from the extracellular part of the receptor to the intracellular domains.

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References

ANDERSON, J.J., RAO, S.P., ROWE, B., GIRACELLO, D.R., HOLTZ, G., CHAPMAN, D.F., TEHRANI, L., BRADBURY, M.J., COSFORD, N.D.P. & VARNEY, M.A. (2002). [^{3}H]Methoxymethyl-3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine binding to metabotropic glutamate receptor subtype 5 in rodent brain: *in vitro* and *in vivo* characterization. *J. Pharmacol. Exp. Ther.,* **303,** 1044–1051.

ANGO, F., PREZEAU, L., MULLER, T., TU, J.C., XIAO, B., WORLEY, P.F., PIN, J.P., BOCKAERT, J. & FAGNI, L. (2001). Agonist-independent activation of metabotropic glutamate receptors by the intracellular protein Homer. *Nature,* **411,** 962–965.

BOCKAERT, J. & PIN, J.P. (1999). Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J.,* **18,** 1723–1729.

BRANDISH, P.E., HILL, L.A., ZHENG, W. & SCOLNICK, E.M. (2003). Scintillation proximity assay of inositol phosphates in cell extracts: high-throughput measurement of G-protein-coupled receptor activation. *Anal. Biochem.,* **313,** 311–318.

CARROLL, F.Y., STOLLE, A., BEART, P.M., VOERSTE, A., BRABET, I., MAULER, F., JOLY, C., ANTONICEK, H., BOCKAERT, J., MULLER, T., PIN, J.P. & PREZEAU, L. (2001). BAY36-7620: a potent non-competitive mGlu1 receptor antagonist with inverse agonist activity. *Mol. Pharmacol.,* **59,** 965–973.

CIRUELA, F., SOLOVIEV, M.M., CHAN, W.Y. & MCILHINNEY, R.A. (2000). Homer-1c/Vesl-1L modulates the cell surface targeting of metabotropic glutamate receptor type 1alpha: evidence for an anchoring function. *Mol. Cell. Neurosci.,* **15,** 36–50.

CIRUELA, F., SOLOVIEV, M.M. & MCILHINNEY, R.A.J. (1999). Co-expression of metabotropic glutamate receptor type 1.alpha with Homer-1a/Vesl-1S increases the cell surface expression of the receptor. *Biochem. J.,* **341,** 795–803.

COSFORD, N.D.P., TEHRANI, L., ARRUDA, J., KING, C., MCDONALD, I.A., MUNOZ, B., ROPPE, J., SCHWEIGER, E., SMITH, N., WANG, B. & CO Merck-USA. LO San Diego, Cal., USA (2002). 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine (MTEP): design and synthesis of a potent and highly selective metabotropic glutamate subtype 5 (Mglu5) receptor antagonist with anxiolytic activity. *Neuropharmacology,* **43,** 282–283 (Coden: Nephbw Issn: 0028-3908).

DE BLASI, A., CONN, P.J., PIN, J.P. & NICOLETTI, F. (2001). Molecular determinants of metabotropic glutamate receptor signaling. *Trends Pharmacol. Sci.,* **22,** 114–120.

DE LIGT, R.A., KOUROUNAKIS, A.P. & AP, I.J. (2000). Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery. *Br. J. Pharmacol.,* **130,** 1–12.

FAGNI, L., WORLEY, P.F. & ANGO, F. (2002). Homer as both a scaffold and transduction molecule. *Sci. STKE,* **2002,** RE8.

FRANCESCONI, A. & DUVOISIN, R.M. (2004). Divalent cations modulate the activity of metabotropic glutamate receptors. *J. Neurosci. Res.,* **75,** 472–479.

GASPARINI, F., LINGENHOHL, K., STOEHR, N., FLOR, P.J., HEINRICH, M., VRANESIC, I., BIOLLAZ, M., ALLGEIER, H., HECKENDORN, R., URWYLER, S., VARNEY, M.A., JOHNSON, E.C., HESS, S.D., RAO, S.P., SACAAN, A.I., SANTORI, E.M., VELICELEBI, G. & KUHN, R. (1999). 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. *Neuropharmacology,* **38,** 1493–1503.

GOUDET, C., GAVEN, F., KNIAZEFF, J., VOL, C., LIU, J., COHEN-GONSAUD, M., ACHER, F., PREZEAU, L. & PIN, J.P. (2004). Heptahelical domain of metabotropic glutamate receptor 5 behaves like rhodopsin-like receptors. *Proc. Natl. Acad. Sci. U.S.A.,* **101,** 378–383.

HERMANS, E. & CHALLISS, R.A. (2001). Structural, signalling and regulatory properties of the group I metabotropic glutamate receptors: prototypic family C G-protein-coupled receptors. *Biochem. J.,* **359,** 465–484.

JOLY, C., GOMEZA, J., BRABET, I., CURRY, K., BOCKAERT, J. & PIN, J.P. (1995). Molecular, functional, and pharmacological characterization of the metabotropic glutamate receptor type 5 splice variants: comparison with mGluR1. *J. Neurosci.,* **15,** 3970–3981.

KUBO, Y., MIYASHITA, T. & MURATA, Y. (1998). Structural basis for a Ca^{2+}-sensing function of the metabotropic glutamate receptors. *Science,* **279,** 1722–1725.

MALHERBE, P., KRATOCHWIL, N., ZENNER, M.-T., PIUSSI, J., DIENER, C., KRATZEISEN, C., FISCHER, C. & PORTER, R.H.P. (2003). Mutational analysis and molecular modeling of the binding pocket of the metabotropic glutamate 5 receptor negative modulator 2-methyl-6-(phenylethynyl)-pyridine. *Mol. Pharmacol.,* **64,** 823–832.

O'HARA, P.J., SHEPPARD, P.O., THOGERSEN, H., VENEZIA, D., HALDEMAN, B.A., MCGRANE, V., HOUAMED, K.M., THOMSEN, C., GILBERT, T.L. & MULVIHILL, E.R. (1993). The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. *Neuron,* **11,** 41–52.

PAGANO, A., RUEGG, D., LITSCHIG, S., STOEHR, N., STIERLIN, C., HEINRICH, M., FLOERSHEIM, P., PREZEAU, L., CARROLL, F., PIN, J.P., CAMBRIA, A., VRANESIC, I., FLOR, P.J., GASPARINI, F. & KUHN, R. (2000). The non-competitive antagonists 2-methyl-6-(phenylethynyl)pyridine and 7-hydroxyiminocyclopropan[*b*]chromen-1a-carboxylic acid ethyl ester interact with overlapping binding pockets in the transmembrane region of group I metabotropic glutamate receptors. *J. Biol. Chem.,* **275,** 33750–33758.

PORTER, R.H., BENWELL, K.R., LAMB, H., MALCOLM, C.S., ALLEN, N.H., REVELL, D.F., ADAMS, D.R. & SHEARDOWN, M.J. (1999). Functional characterization of agonists at recombinant human 5-HT2A, 5-HT2B and 5-HT2C receptors in CHO-K1 cells. *Br. J. Pharmacol.,* **128,** 13–20.

PREZEAU, L., GOMEZA, J., AHERN, S., MARY, S., GALVEZ, T., BOCKAERT, J. & PIN, J.P. (1996). Changes in the carboxyl-terminal domain of metabotropic glutamate receptor 1 by alternative splicing generate receptors with differing agonist-independent activity. *Mol. Pharmacol.,* **49,** 422–429.

RAY, K. & HAUSCHILD, B.C. (2000). Cys-140 is critical for metabotropic glutamate receptor-1 dimerization. *J. Biol. Chem.,* **275,** 34245–34251.

RAY, K., HAUSCHILD, B.C., STEINBACH, P.J., GOLDSMITH, P.K., HAUACHE, O. & SPIEGEL, A.M. (1999). Identification of the cysteine residues in the amino-terminal extracellular domain of the human Ca(2+) receptor critical for dimerization. Implications for function of monomeric Ca(2+) receptor. *J. Biol. Chem.,* **274,** 27642–27650.

ROMANO, C., MILLER, J.K., HYRC, K., DIKRANIAN, S., MENNERICK, S., TAKEUCHI, Y., GOLDBERG, M.P. & O'MALLEY, K.L. (2001). Covalent and noncovalent interactions mediate metabotropic glutamate receptor mGlu5 dimerization. *Mol. Pharmacol.,* **59,** 46–53.

ROPPE, J.R., WANG, B., HUANG, D., TEHRANI, L., KAMENECKA, T., SCHWEIGER, E.J., ANDERSON, J.J., BRODKIN, J., JIANG, X., CRAMER, M., CHUNG, J., REYES-MANALO, G., MUNOZ, B. & COSFORD, N.D. (2004). 5-[(2-Methyl-1,3-thiazol-4-yl)ethynyl]-2,3′-bipyridine: a highly potent, orally active metabotropic glutamate subtype 5 (mGlu5) receptor antagonist with anxiolytic activity. *Bioorg. Med. Chem. Lett.,* **14,** 3993–3996.

SLEIGHT, A.J., STAM, N.J., MUTEL, V. & VANDERHEYDEN, P.M. (1996). Radiolabelling of the human 5-HT2A receptor with an agonist, a partial agonist and an antagonist: effects on apparent agonist affinities. *Biochem. Pharmacol.,* **51,** 71–76.

SOLOVIEV, M.M., CIRUELA, F., CHAN, W.Y. & MCILHINNEY, R.A. (2000). Molecular characterisation of two structurally distinct groups of human Homers, generated by extensive alternative splicing. *J. Mol. Biol.,* **295,** 1185–1200.

STRANGE, P.G. (2002). Mechanisms of inverse agonism at G-protein-coupled receptors. *Trends Pharmacol. Sci.,* **23,** 89–95.

TATEYAMA, M., ABE, H., NAKATA, H., SAITO, O. & KUBO, Y. (2004). Ligand-induced rearrangement of the dimeric metabotropic glutamate receptor 1(alpha). *Nat. Struct. Mol. Biol.,* **11/7,** 637–642.

TU, J.C., XIAO, B., YUAN, J.P., LANAHAN, A.A., LEOFFERT, K., LI, M., LINDEN, D.J. & WORLEY, P.F. (1998). Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. *Neuron,* **21,** 717–726.

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