A critical pocket close to the glutamate binding site of mGlu receptors opens new possibilities for agonist design

Francine C. Acher[a]* , Chelliah Selvam,[a,e] Jean-Philippe Pin,[b,c] Cyril Goudet,[b,c] and Hugues-Olivier Bertrand,[d]

[a] Laboratoire de Chimie et de Biochimie Pharmacologiques et Toxicologiques, CNRS UMR8601, Université Paris Descartes, 45 rue des Saints-Pères, 75270 Paris cedex 06, France

[b] Institut de Génomique Fonctionnelle, Université de Montpellier, CNRS UMR5203, Montpellier, F-34094 France

[c] INSERM, U661, Montpellier, F-34094 France

[d] Accelrys Inc., Parc-Club Orsay Université, 20 rue Jean Rostand, 91898 Orsay cedex, France

[e] Current address: Department of Chemistry, State University of New York at Binghamton, Binghamton, NY 13902.

*Author to whom correspondence should be addressed: Phone +33 (0)1 42 86 33 21. Fax +33 (0)1 42 86 83 87. E-mail francine.acher@parisdescartes.fr .

Abstract.

A recent publication from Ogawa et al. suggested a possible allosteric chloride binding site in the extracellular domain of metabotropic glutamate receptors (mGluRs) by comparison with a similar site found in atrial natriuretic peptide receptor. We simultaneously reported about (*S*)-PCEP an agonist of subtype 4 mGluR that would bind to a similar pocket, adjacent to the glutamate binding site. Here we disclose LSP1-2093, a new derivative of (*S*)-PCEP that holds a nitrophenyl substituent. Docking experiments predict that the nitro group binds to the receptor at the putative chloride ion site. It is thus possible to take advantage of this putative chloride binding site to develop new types of mGluR agonists. This pocket is present in the structural family of Leucine Isoleucine Valine Binding Protein that includes class C GPCRs, suggesting that extended agonists may be identified at receptors bearing such a structural domain.

Abbreviations: ACPD, 1-aminocyclopentane 1,3,4-dicarboxylic acid; ACPT, 1-aminocyclopentane 1,3,4-tricarboxylic acid; ANP, atrial natriuretic peptide; AP4, 2-amino-4-phosphonobutyric acid; ATD, amino terminal domain; GPCR, G-protein coupled receptor; DCG-IV, (2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine; HTS, high throughput screening; LIVBP, Leucine Isoleucine Valine Binding Protein; LSP1-2093, 3-amino-3-carboxypropyl(hydroxy(3-nitrophenyl)methyl)phosphinic acid; mGluR, metabotropic glutamate receptor; NMDA, N-methyl D-aspartate; OlfC, olfactory class C GPCRs; PCEP, 3-amino-3-carboxypropyl-2'-carboxyethylphosphinic acid; PDB, Protein Data Bank; VFT, Venus FlyTrap.

1. Introduction

In 1993 P. O'Hara and colleagues were the first to demonstrate that both metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors hold an amino terminal domain (ATD) that adopts the fold of LIVBP a bacterial periplasmic binding protein and that was thus named the LIVBP domain (O'Hara et al., 1993). They showed that this domain constitutes the glutamate binding site of mGluRs. Later it was found that in NMDA receptors, this domain is the binding domain for zinc and that it modulates the glutamate activation of these receptors. Since that early prediction, much progress has been made and crystal structures of both mGluR and NMDA ATDs have been disclosed (Karakas et al., 2009; Kunishima et al., 2000; Muto et al., 2007).

Periplasmic binding proteins are involved in high affinity active transport of various nutrients such as sugars, inorganic anions and amino acids (Leucine Isoleucine and Valine for LIVBP). A large number have been crystallized and display the same 3D structure with two globular domains linked by a hinge region allowing open and closed conformations (Quiocho and Ledvina, 1996). In the open form, the ligand binds to a first lobe and then is trapped upon closing of the domain in a similar manner as the venus flytrap, a carnivorous plant, catches its prey. By analogy, the LIVBP domain of mGluRs and NMDA receptors has been often named the venus flytrap (VFT) domain.

The metabotropic glutamate receptors belong to the class C of the G-protein coupled receptor family (Pin et al., 2003). All members of that family possess an LIVBP-like ATD connected to a hepta helix transmembrane domain. These receptors function as dimers with a contact surface between the lobes 1 of the ATDs (Kunishima et al., 2000). Agonists stabilize the closed LIVBP domains that result in a relative rotation of two domains then triggering G-protein activation. Eight subtypes of mGluRs have been identified and classified in three groups according to their sequence identity, transduction pathways and pharmacological

profile. Group-I receptors (mGlu1 and mGlu5) are linked to PLC activation while group-II (mGlu2 and mGlu3) and group-III (mGlu4, mGlu6, mGlu7 and mGlu8) inhibit adenylate cyclase (Niswender and Conn, 2010).

Other proteins adopt the LIVBP-like fold. In the SCOP classification (L-arabinose binding protein-like family) 16 different types are listed (http://scop.mrc-lmb.cam.ac.uk/scop/data/scop.b.d.bea.b.b.html) . Many of these proteins function as monomers but several others need to form a dimer in order to be active as seen with class C GPCRs. In this latter group are found the hormone binding domain of the atrial natriuretic peptide receptor (ANPR) and DNA-binding protein purine-, lac- and trehalose repressors. It was known that ANPRs require chloride for their activity. Recently Ogawa et al. showed that it may play an allosteric role in the binding of ANP (Ogawa et al., 2010). Moreover when comparing the crystal structures of ANPR and mGlu1R ATDs, these authors noted that the ANPR chloride binding site is conserved in mGlu1R. Thus they suggested that chloride may also allosterically regulate mGluR activation. In this article, we show that indeed a binding site similar to that of chloride in ANPR is present in several LIVBP-like proteins and that it may be a site for developing a new type of ligands. In fact, we have recently described an adjacent binding pocket to glutamate in mGlu4R that coincides with the ANPR chloride site and where new orthosteric agonists may be binding (Selvam et al., 2010). Here we disclose LSP1-2093, a new molecule of this type.

2. Materials and Methods

2.1 Pharmacology

HEK293 cells (Human Embryonic Kidney cells) were transiently transfected by electroporation with rat clones of mGlu4, mGlu6, mGlu7 and mGlu8 receptors as described elsewhere (Brabet et al., 1998). A high affinity glutamate transporter EAAC1 was co-

transfected with the receptor to avoid the influence of extracellular glutamate. Receptors were co-transfected with a chimeric Gq/Gi-protein. This modified Gq-protein allows the monitoring of naturally Gi-coupled receptor activity by measurements of inositol phospholipid hydrolysis (Gomeza et al., 1996).

[3H]Inositol phosphate ([3H]-InsP) accumulation experiments were performed in 96-well microplates, as described previously (Goudet et al., 2004). Briefly, transfected cells were incubated overnight with [3H]-myoinositol (16 Ci/mmole, Amersham, Buckinghamshire, UK). The day after, ambient glutamate was degraded by incubation with glutamate pyruvate transferase in the presence of pyruvic acid then cells were stimulated with agonists for 30 minutes in the presence of 10 mM LiCl. [3H]-InsP accumulated during receptor stimulation was recovered by anion exchange chromatography and radioactivity was counted using a Wallac 1450 Microbeta stintillation and luminescence counter (Perkin Elmer, Courtaboeuf, France). Results are expressed as the ratio between [3H]-InsP and total radioactivity in each sample. All points are from triplicates. LSP1-2093 was synthesized in the laboratory of F. Acher (Acher et al., 2007).

2.2 Structure analysis and docking of LSP1-2093 in mGlu4R AminoTerminal Domain.

All calculations were performed in Discovery Studio 2.5.5 (Accelrys Software Inc., San Diego, 92121 CA). Due to the lack of crystal structure for mGlu4 subtype amino terminal domain, LSP1-2093 was docked in a homology model that had been previously generated (Bertrand et al., 2002; Selvam et al., 2010). Our homology model was built upon 2 templates 1ewk (mGlu1R ATD) and 2e4u (mGlu3R ATD) (Selvam et al., 2010). The ligand was initially positioned in the binding site using CDOCKER. CDOCKER uses a CHARMm-based molecular dynamics scheme to dock ligands into a receptor binding site (Wu et al., 2003). Random ligand conformations are generated using high-temperature molecular dynamics. The

conformations are then translated into the binding site. Candidate poses are then created using random rigid-body rotations followed by simulated annealing. A final energy minimization is then used to refine the ligand poses. As CDOCKER takes into account only ligand flexibility, protein-ligand interactions were further optimized by one nano second molecular dynamics using CHARMm where only side chains were flexible while the backbone was harmonically restrained. Once the trajectory was equilibrated, snapshots of the trajectory were analyzed in terms of protein-ligand contacts and the selected ones were submitted to energy minimization leading to the figures presented in this article (Fig. 5).

3. Results

3.1 LIVBP-like domains and homologous ANPR chloride binding site

The LIVBP domain is composed of two similar sub-domains (lobe1 and lobe2) each consisting of a central cluster of beta-sheets sandwiched between alpha-helices and lining a cleft in between. The chloride site of ANPR is found in lobe 1 between β1, β2, β3 sheets and α1, α2, α3 helices (Fig. 1) (He et al., 2006; Ogawa et al., 2010). In the orientation of Figure 1, the ion is situated above the cleft, below two hydrophobic clusters and holds together the lower part of β2 and β3 sheets by making three hydrogen bonds with main chain NH of G85 and C86 and with hydroxyl of S53 (Fig. 1 and SI-1). Ogawa et al. demonstrated that chloride may be replaced by bromide as found in ANPR PDB structure 3ak3 and displayed in resulting figures 1 and SI-1 (Ogawa et al., 2010). When checking the analogous site in structures of the LIVBP-like family members that have been deposited at the PDB, we found several structures with the adequate distance between β2 and β3 sheets. However in some of them, the link between those sheets is provided by direct interaction between residues of the two β-sheets. For example, in Leucine Binding Protein (PDB identifier 1usk) D51 and H76 side chains take the place of the ion (Fig. SI-2) (Magnusson et al., 2004) and similarly T76 and T103 in the

NR2B ATD domain (PDB identifier 3jpy) hold the structure by a hydrophobic contact (Fig. SI-3) (Karakas et al., 2009). Nevertheless, in the mGlu1R, mGlu5R and mGlu3R ATD structures one or two water molecules are found in place of the chloride/bromide ion of the ANPR as observed by the Misono group (Fig. 2 and 3, PDB identifiers 1ewk, 3ks9, 3lmk, 2e4u, 2e4v, 2e4w) (Kunishima et al., 2000; Muto et al., 2007; Ogawa et al., 2010). A hydrophobic cluster lies over this site likewise observed in ANPR. Interestingly the same disposition of the water molecules is found in mGlu1 and 5 receptors with an agonist (glutamate) or an antagonist (LY311495) and for the three different mGlu3R agonists, glutamate, DCG-IV and ACPD. In none of the mGluR X-ray structures was a chloride ion located as in ANPR, although Hampson's investigations proved that chloride may facilitate the binding of DCG-IV to mGlu3R (Kuang and Hampson, 2006). Yet the chloride effect was most effective in L-AP4 binding and activation of mGlu4R, but detailed structural information about the Cl binding site in mGlu4R is lacking due to the absence of crystal structure for the VFT of this specific mGluR.

3.2 (*S*)-PCEP binds to the analogous "ANP chloride" site

We recently disclosed a new series of group-III mGluR agonists that derive from the optimization of (*S*)-PCEP that was discovered in a virtual HTS campaign (Selvam 2010). (*S*)-PCEP is composed of an L-AP4 part and a distal acidic chain. A docking of (*S*)-PCEP in our homology model of mGlu4R ATD revealed that the L-AP4 part binds in the cleft of the bilobate structure in the same way as L-AP4 does. The distal acidic chain reached an adjacent pocket located in lobe1 and interacted with K74, S110, S157 and G158 (Fig. 4). A sequence comparison with the residues that bind the chloride/bromide ion in ANPR reveals that these two sites are overlapping. Indeed residues C86 of ANPR and G158 of mGlu4R are aligned and the main chain NH of each is involved in binding chloride and (*S*)-PCEP respectively

(Fig. 1 and 4). Moreover the distal carboxylate of (*S*)-PCEP and the chloride ion both link the two loops between β2 sheet - α2 helix and β3 sheet - α3 helix of the two proteins providing a stabilization of lobe 1 and maintaining α2 and α3 helices. These two helices play a key role in the dimer contact and consequently in the activation of the systems. As a result, it was suggested that the ANP chloride binding site and the analogous one in mGluRs could be a site for allosteric regulation. The discovery of (*S*)-PCEP confirms this hypothesis and demonstrate that new molecules that bind to this site may be discovered.

Since residues binding the water molecule at the "chloride site" of mGlu3R are homologous to those of mGlu4R, we postulated that in mGlu4 a water molecule may also be found at the same position (Fig. 4). Such a model suggests the design of new derivatives of (*S*)-PCEP that would contain a distal substituent that merges both interactions of (*S*)-PCEP and of the water molecule.

3.3 LSP1-2093, a new Group-III mGluR agonist, binds to the "ANP chloride" site

As part of the chemical optimization program of (*S*)-PCEP, we synthesized a series of aromatic derivatives, among which LSP1-2093 (Acher et al., 2007). This compound was tested at cloned mGlu receptors and proved to be a potent agonist of group-III receptors except at mGlu7R. Values of EC_{50}'s are 0.55 +/- 0.06 µM (n=19) for mGlu4R, 8.81 +/ 0.72 µM (n=5) for mGlu6R, > 1000 µM for mGlu7R and 1.53 +/- 0.51 µM (n=6) for mGlu8R (Table 1).

The docking model of LSP1-2093 reveals that the nitro group of the nitrophenyl substituent may replace the putative water molecule and makes hydrogen bonds with T108 side chain and backbone NH of S157 and G158 (Fig. 5). The lack of subtype selectivity among group-III mGluRs may be explained by the absence of contact with the side chains of S157 and G158 that differ in mGlu6 and 8 as previously proposed for (*S*)-PCEP (Selvam et al., 2010). The

pocket is limited by a hydrophobic cluster situated above the nitro group in the orientation of figure 5, however it should be possible to add substituents to the phenyl group that would provide selective interactions with the receptors.

4. Discussion

A simultaneous publication of a chloride binding site in the ANP receptor, and of a new group-III mGluR agonist (*S*)-PCEP revealed the presence of a similar "ANP chloride" site in mGlu4R. Substituents that bind to this pocket provide an increased potency to the ligand emphasizing the druggability of mGluRs' orthosteric binding site. Although the size of the pocket is limited, new derivatives may be developed with improved pharmacological properties. It is interesting to note that this site is found between two highly conserved short loops that connect two β-sheets to two α-helices (β2 to α2 and β3 to α3) in the LIVBP-like family. Moreover this site seems to be only present in members of the structural family that form dimers and for which the dimer interface is found between helix α2 and helix α3 (Fig.6). Such a situation is indeed found with ANP and mGlu receptors (He et al., 2006; Kunishima et al., 2000). The role of the chloride ion or another bridging moiety would be to get closer the two helices and stabilize the VFT conformation that allows dimerization and activation. Thus, as proposed by Misono and colleagues, the entities that bind to this site could be allosteric modulators. This explains why extending phosphinothricine, the methyl phosphinate analog of L-AP4 (Selvam et al., 2007), with a substituent that binds to this site as in (*S*)-PCEP and LSP1-2093, an increased potency is observed. Since all receptors of class C GPCRs are activated via the same dimerization mechanism, the same allosteric site may be found in lobe 1 of their VFT domains. As a matter of fact, the "chloride" binding motif is made up of two backbone NH's (from loop β3 to α3), and a hydrogen bond donor serine or threonine (from loop β2 to α2). This latter residue is found in ANPR sequences but also in a

large number of class C GPCRs that includes, besides mGluRs, the calcium sensing receptor (CaSR), hGPRC6a and the fish olfactory receptor OR5.24 (Hu and Spiegel, 2007; Luu et al., 2004; Pi et al., 2005). Moreover in the repertoire of class C GPCRs found in the genome of the zebrafish (54 OlfC receptors), a Ser, Thr or Asn is found most of the time in that position, allowing the critical H-bond of the "chloride" site (residue of OlfC receptors aligning with residue 112 of OlfCq6 in alignment displayed in (Alioto and Ngai, 2006). Indeed serine, threonine and asparagine may secure the same distance between helix $\alpha2$ and helix $\alpha3$. Also in that same position, a threonine is present in T1R3 of taste receptors while it is a valine in T1R1 and T1R2 meaning that a putative chloride binding site may only be found in T1R3 and not in T1R1 or T1R2. However, since these receptors function as dimers T1R1/T1R3 (umami taste) and T1R2/T1R3 (sweet taste), the "chloride" site in T1R3 may offer a new opportunity for taste modulation. It will be interesting to support the present observations with a series of mutagenesis experiments.

One major outcome of the present findings is the presence in many class C GPCRs of an allosteric pocket adjacent to the agonist binding pocket and that extended agonists may reach that site. This situation may allow overcoming the lack of selectivity that is inherent to the high conservation of agonist binding site within a group of similar subtypes. As reported above, the motif that would bind a putative chloride ion is highly conserved in class C GPCRs, however this site adjoins a variable loop between $\beta1$ and $\alpha1$, that may bring the required differences. Accordingly it seems now possible to discover selective orthosteric agonists of class C GPCRs, changing the dogma about these ligands. Additionally, the polar structure of these compounds may be an advantage considering their high aqueous solubility and minimal metabolisation.

In conclusion, the current observations support the presence of an allosteric site located in lobe 1 of the VFT domain of LIVBP-like proteins forming dimers that are in contact through

their α2 and α3 helices. In addition we show that this site is druggable since it may be fitted with extended agonists. This opens large possibilities for the development of potent and selective ligands since many of the concerned proteins are important therapeutical targets.

Supplementary Information Available:

Figures with expanded view of ANPR (Fig. SI-1), of Leucine Binding Protein (Fig. SI-2), of NR2B subunit of NMDA receptor (Fig. SI-3). Chemical structures of group-III mGluR agonists (Fig. SI-4).

References

Acher, F., C. Selvam, N. Triballeau, J.P. Pin and H.O. Bertrand, 2007, Hypophosphorous acid derivatives and their therapeutical applications, Patent Application WO2007052169.

Alioto, T.S. and J. Ngai, 2006, The repertoire of olfactory C family G protein-coupled receptors in zebrafish: candidate chemosensory receptors for amino acids, BMC Genomics. 7, 309.

Bertrand, H.-O., A.-S. Bessis, J.-P. Pin and F. Acher, 2002, Common and selective molecular determinants involved in metabotropic glutamate receptor agonist activity, J. Med. Chem. 45, 3171.

Brabet, I., M.-L. Parmentier, C. De Colle, J. Bockaert, F. Acher and J.-P. Pin, 1998, Comparative effect of L-CCG-I, DCG-IV and γ-carboxy-L-glutamate on all cloned metabotropic glutamate receptor subtypes, Neuropharmacology 37, 1043.

Gomeza, J., S. Mary, I. Brabet, M.-L. Parmentier, S. Restituito, J. Bockaert and J.-P. Pin, 1996, Coupling of mGluR2 and mGluR4 to Gα15, Gα16 and chimeric Gαq/i proteins: characterization of new antagonists, Mol. Pharmacol. 50, 923.

Goudet, C., F. Gaven, J. Kniazeff, C. Vol, J. Liu, M. Cohen-Gonsaud, F. Acher, L. Prézeau and J.-P. Pin, 2004, Heptahelical domain of metabotropic glutamate receptor 5 behaves like rhodopsin-like receptors, Proc Nat Acad. Sci. USA 101, 378.

He, X., A. Dukkipati and K.C. Garcia, 2006, Structural determinants of natriuretic peptide receptor specificity and degeneracy, J Mol Biol. 361, 698.

Hu, J. and A.M. Spiegel, 2007, Structure and function of the human calcium-sensing receptor: insights from natural and engineered mutations and allosteric modulators, J Cell Mol Med. 11, 908.

Karakas, E., N. Simorowski and H. Furukawa, 2009, Structure of the zinc-bound amino-terminal domain of the NMDA receptor NR2B subunit, EMBO J. 28, 3910.

Kuang, D. and D.R. Hampson, 2006, Ion dependence of ligand binding to metabotropic glutamate receptors, Biochem Biophys Res Commun. 345, 1.

Kunishima, N., Y. Shimada, Y. Tsuji, T. Sato, M. Yamamoto, T. Kumasaka, S. Nakanishi, H. Jingami and K. Morikawa, 2000, Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor, Nature 407, 971.

Luu, P., F. Acher, H.-O. Bertrand, J. Fan and J. Ngai, 2004, Molecular determinants of ligand selectivity in a vertebrate odorant receptor, J. Neurosci. 24, 10128.

Magnusson, U., B. Salopek-Sondi, L.A. Luck and S.L. Mowbray, 2004, X-ray structures of the leucine-binding protein illustrate conformational changes and the basis of ligand specificity, J. Biol. Chem. 279, 8747.

Muto, T., D. Tsuchiya, K. Morikawa and H. Jingami, 2007, Structures of the extracellular regions of the group II/III metabotropic glutamate receptors, Proc Natl Acad Sci U.S.A. 104, 3759.

Niswender, C.M. and P.J. Conn, 2010, Metabotropic glutamate receptors: physiology, pharmacology, and disease, Annu Rev Pharmacol Toxicol. 50, 295.

Ogawa, H., Y. Qiu, J.S. Philo, T. Arakawa, C.M. Ogata and K.S. Misono, 2010, Reversibly bound chloride in the atrial natriuretic peptide receptor hormone-binding domain: possible allosteric regulation and a conserved structural motif for the chloride-binding site, Protein Sci. 19, 544.

O'Hara, P.J., P.O. Sheppard, H. Thogersen, D. Venezia, B.A. Haldeman, V. Mc Grane, K.H. Houamed, C. Thomsen, T.L. Gilbert and E.R. Mulvihill, 1993, The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins, Neuron 11, 41.

Pi, M., P. Faber, G. Ekema, P.D. Jackson, A. Ting, N. Wang, F.-P. M., R.W. Mays, K.R. Brunden, J.J. Harrington and L.D. Quarles, 2005, Identification of a novel extracellular cation-sensing G-protein-coupled receptor, J Biol Chem. 280, 40201.

Pin, J.-P., T. Galvez and L. Prézeau, 2003, Evolution, structure and activation mechanism of family 3/C G-protein coupled receptors, Pharmacol. Ther. 98, 325.

Quiocho, F.A. and P.S. Ledvina, 1996, Atomic structure and specificity of bacterial periplasmic receptors for active transport and chemotaxis: variation of common themes, Mol. Microbiol. 20, 17.

Selvam, C., C. Goudet, N. Oueslati, J.-P. Pin and F. Acher, 2007, L(+)-2-amino-4-thiophosphonobutyric acid (L-thioAP4), a new potent agonist of group III metabotropic glutamate receptors: increased distal acidity affords enhanced potency, J.Med.Chem. 50, 4656.

Selvam, C., N. Oueslati, I.A. Lemasson, I. Brabet, D. Rigault, T. Courtiol, S. Cesarini, N. Triballeau, H.-O. Bertrand, C. Goudet, J. Pin and F.C. Acher, 2010, A virtual screening hit reveals new possibilities to develop group III metabotropic glutamate receptor agonists, J.Med.Chem. 53, 2797.

Wu, G., D.H. Robertson, C.L. Brooks and M. Vieth, 2003, Detailed analysis of grid-based molecular docking: A case study of CDOCKER - A CHARMm-based MD docking algorithm, J. Comp. Chem. 24, 1549.

Figure captions

Figure 1. Crystal structure of ANP receptor extracellular domain (monomer) bound with bromide (PDB identifier 3a3k). A) Ribbon diagram with β1, β2, β3 sheets and α1, α2, α3 helices highlighted in yellow; residues binding the bromide ion are displayed. B) Expanded view of the bromide binding site. Atom colors: carbon cyan, hydrogen white, oxygen red, nitrogen blue, sulphur yellow, bromine brown. Hydrogens have been removed for clarity except for those involved in hydrogen bonds that are displayed as green dotted lines.

Figure 2. Crystal structure of mGlu1 receptor extracellular domain (monomer A) bound with glutamate (PDB identifier 1ewk). A) Ribbon diagram with β1, β2, β3 sheets and α1, α2, α3 helices highlighted in yellow. The two water molecules located at the ANPR chloride/bromide analogous site are displayed (red spheres) as well as their binding residues. B) Expanded view of this site with a neighbouring hydrophobic cluster. Atom colors: carbon gray, cyan for binding residues, orange for residues of the hydrophobic cluster, hydrogen white, oxygen red, nitrogen blue. Hydrogens have been removed for clarity except for those involved in hydrogen bonds that are displayed as green dotted lines.

Figure 3. Crystal structure of mGlu3 receptor extracellular domain (monomer A) bound with glutamate (PDB identifier 2e4u). A) Ribbon diagram with β1, β2, β3 sheets and α1, α2, α3 helices highlighted in yellow. The water molecule located at the ANPR chloride/bromide analogous site is displayed (red sphere) as well as its binding residues. B) Expanded view of this site with a neighbouring hydrophobic cluster. Atom colors: carbon gray, cyan for binding residues, orange for residues of the hydrophobic cluster, hydrogen white, oxygen red, nitrogen

blue. Hydrogens have been removed for clarity except for those involved in hydrogen bonds that are displayed as green dotted lines.

Figure 4. Homology model of mGlu4 receptor extracellular domain bound with (*S*)-PCEP. A) Ribbon diagram with β1, β2, β3 sheets and α1, α2, α3 helices highlighted in yellow. Residues binding the distal part of (*S*)-PCEP and their shape (magenta) are displayed (Selvam et al., 2010). B) Expanded view of this site, residues for which the shape is shown in panel A are boxed in magenta. A putative water molecule, positioned as in mGlu3 receptor crystal structures (PDP identifiers 2e4u, 2e4v, 2e4w) is displayed (red sphere). Atom colors: carbon gray, cyan for binding residues, hydrogen white, oxygen red, nitrogen blue, phosphorus orange. Hydrogens have been removed for clarity except for (*S*)-PCEP and those involved in hydrogen bonds that are displayed as green dotted lines.

Figure 5. Expanded view of the homology model of mGlu4 receptor extracellular domain bound with LSP1-2093. Residues binding the distal part of LSP1-2093 and the neighbouring hydrophobic cluster are displayed. Atom colors same as in Figure 3 and 4.

Figure 6. Dimers of crystal structure of ANP (panel A) and mGlu1 (panel B) receptors (PDB identifiers 1yk0 and 1ewk). Ligands, ANP (panel A) and two glutamates (panel B) and the chloride ion (green sphere) and water molecules (red spheres) are displayed. Monomer A is colored in gray, monomer B in cyan, α2 and α3 helices that build up the dimer interface are shown in yellow for monomer A and blue for monomer B in both panels. Atom colors as in Figure 1.