Supplemental Information

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. The Increase in Gi and Decrease in Gq Signaling Depend on Heterocomplex Formation

Bar graph summary of IRK3 current inhibition (left) and GIRK4* current activation (right) (mean ± SEM) in oocytes expressing 2AR alone or 2AR together with mGluR2 (A) mGluR2Δ (B) mGluR3 (C) or mGluR3Δ (D). IRK3 current inhibition and GIRK4* current activation were measured and normalized as in Figure 1, D and E respectively. Currents were obtained in response to 1 μM of the endogenous ligand serotonin (5-HT) and 1 μM of endogenous ligand glutamate (Glu). Coexpression of mGluR2Δ with 2AR did not result in a significant decrease in Gq signaling in response to 5-HT or a significant increase in Gi signaling in response to Glu (C) when compared to the group co-expressing mGluR2 and 2AR (A). Similarly, both effects were absent for mGluR3 (B), but present for mGluR3Δ (D) (**p<0.01; ***p< 0.001; n.s., not significant). The Nissl staining of the coronal cortex was taken from the mouse brain atlas with the author's permission (Hof et al., 2000).

(E) Representative micrographs showing co-expression of endogenous 2AR (red) and mGluR2 (green) in mousefrontal cortex sections. Scale bar 100 μm.

Figure S2. Expression Levels of mGluR2, mGluR2Δ, mGluR3 and mGluR3Δ

(A) Representative GIRK4* barium-sensitive traces obtained in response to 1 μM glutamate. mGluR2, mGluR2Δ, mGluR3Δ and mGluR3Δ were functional as Gi-coupled GPCRs.

(B) Statistical summary of basal current (mean ± S.E.M.) (***p<0.001).

(C) Statistical summary of glutamate agonist-induced current (mean ± S.E.M.). GIRK4*

current activation was measured and normalized as in Figure 1D. Current activation for mGluR2 (1), mGluR3 (2), mGluR2Δ (3) and mGluR3Δ (4) were similar compared with non-injected (5) (***p<0.001).

(D) Data summary (mean ± S.E.M.) of measured receptor membrane expression levels. Expression was measured with surface labelled receptors and quantitated with a chemiluminescence assay. Chemiluminescence intensity is expressed in logarithmic units and normalized to the background non-injected intensity level. Expression levels were similar for all constructs (1,2,3 and 4) compared to non-injected (5) (***p<0.001; n.s, not significant).

Figure S3. Changes in 2AR mRNA Injected Alter Expression Levels of the Complex in the Membrane and the Difference in Gi to Gq Signaling

(A) Western Blot analysis of the membrane fraction of oocytes, injected with Injected with 1ng of HA-tagged mGluR2 RNA (mGluR2), or 2 ng of myc-tagged 2AR RNA (2AR), or 1 ng of HA-tagged mGluR2 RNA plus various amounts of 2AR RNA (mGluR2/2AR RNA ratios are shown on top of each lane).

(B) Summary of densitometry data for mGluR2 (left) and 2AR (right) from experiments such as the one shown in (A), normalized relative to mGluR2 (left) or 2AR (right) protein levels in oocytes injected with 1 ng of mGluR2 + 2 ng of 2AR mRNAs. Data are from 1-4 independent experiment (see numbers on top of each bar). “mGluR2” indicates homomeric expression of 1 ng mGluR2 mRNA and “2AR” indicates homomeric expression of 2 ng of 2AR mRNA.

(C) Expression levels of mGluR2 (blue filled bars) and 2AR (red filled bars) in the membrane. Data summary (mean ± S.E.M.) of measured receptor membrane expression levels (as were shown in Figure S2 D) for oocytes injected with 1 ng of mGluR2 mRNA and 1 ng, 2 ng, and 3 ng of 2AR mRNA respectively. Expression was measured with surface labelled receptors and quantitated with a chemiluminescence assay. Chemiluminescence was normalized to the

signal obtained in oocytes injected with 1 ng of mGluR2 alone (empty blue bars). For reference, the levels corresponding to oocytes injected with 1 ng, 2ng, and 3 ng of 2AR alone are also depicted (empty red bars). All signal levels were at least 500 times higher than background.

(D) Changes in 2AR mRNA injected alter the Gi and Gq balance. ΔGi and ΔGq measured in oocytes injected with 1 ng mGluR2 mRNA and 1 ng, 2 ng, and 3 ng of 2AR mRNA respectively. ΔGi is referenced to the homomeric mGluR2 (1 ng or mRNA) response to 1 μM glutamate. (Left) ΔGq is referenced to multiple homomeric 2AR responses to 1 μM serotonin corresponding to 1ng, 2ng and 3ng of 2AR mRNA injected. (Right) ΔGq is referenced to one single homomeric response to 1 μM serotonin corresponding to 2ng 2AR mRNA injected.

Figure S4. Homomeric and Heteromeric Dose-response Curves for DOI, LY37 and LY34.

(A) Dose-response curve showing normalized Gq activation elicited by DOI in oocytes expressing 2AR. Gq-mediated IRK3 current inhibition was measured and normalized as in Figure 1D (EC_{50} = 3.6 x 10^{-7} ± 1.05 x 10^{-7}).

(B) Dose-response curve showing normalized Gi activation elicited by LY37 in oocytes expressing mGluR2. Gi-mediated GIRK4* current stimulation was measured and normalized as in Figure 1E (EC_{50} = 1.3 x 10^{-7} ± 6.4 x 10^{-9}).

(C) LY34 behaves as an inverse agonist. Dose-response curve showing normalized Gi inhibition elicited by LY34 in oocytes expressing mGluR2. Gi-mediated GIRK4* current inhibition was measured and normalized as in Figure 1E. Gi inhibition is shown indicating LY34 inverse agonism (IC_{50} = 8.2 X 10^{-5} ± 1.7 X 10^{-6}).

(D) (E) Clozapine, and not methysergide, behaves as an inverse agonist in mouse frontal cortex membrane preparations. [^{3}H]Ketanserin binding displacement curves by clozapine (D)

or methysergide (E) in the absence (black) and in the presence (red) of 10 μM GTPγS. Clozapine displacing [^{3}H]ketanserin binding: $pK_{i\text{-}high}$ control, -8.01 ± 0.1; $pK_{i\text{-}low}$ control, -5.64 ± 0.4; fraction high, 0.77 ± 0.06; pK_i GTPγS, -8.07 ± 0.05 (*F* [3,78] = 4.99, p < 0.01). Methysergide displacing [^{3}H]ketanserin binding: pK_i control, -7.16 ± 0.08; pK_i GTPγS, -7.15 ± 0.07 (*F* [3,88] = 0.05, p = 0.98).

THERE IS NO KEY FOR THE SYMBOLS USED IN (F) AND (G)

(F) Dose-response curves showing cross signaling with LY37 in the absence of serotonin (5) (EC_{50} = 9.34 X 10^{-7} ± 1.13 X 10^{-7}), dominant-agonist effect with LY37 (4) (EC_{50} = 8.35 X 10^{-7} ± 3.37 X 10^{-8}), and inverse-agonist effect with LY34 (2) (EC_{50} = 8.2 X 10^{-5} ± 1.6 X 10^{-6}) and serotonin signaling. The Gq-response by heteromer formation is indicated by the dashed lines [from (1) to (3)] (see also Figure S6 A and B for LY37 effects in 2AR-KO mice).

(G) Dose-response curves showing cross signaling with DOI in the absence of glutamate (5) (EC_{50} = 9.49 X 10^{-7} ± 4.3 X 10^{-7}), dominant-agonist effect with DOI (4) (EC_{50} = 3.72 X 10^{-7} ± 8.45 X 10^{-8}), and inverse-agonist effect with clozapine (1) (EC_{50} = 2.02 X 10^{-5} ± 7.10 X 10^{-6}) of Glu signaling. The Gi-response by heteromer formation is indicated by the dashed lines [from (3) to (2)].

Figure S5. LY37 and LY34 Effects on Gq Activity are PTX Insensitive but RGS-Sensitive.

(A) Representative GIRK4* barium-sensitive current traces in oocytes expressing mGluR2 alone or mGluR2 together with PTX.

(B) Bar graph summary of normalized Gi activity (mean ± SEM) elicited by glutamate (1 μM) in the presence and absence of PTX. GIRK4* current activation was measured and normalized as in Figure 1E.

(C) (D) Bar graph summary of Gq activity (mean ± SEM) in response to 5-HT (1 μM) alone, or

together with LY37 (10 μM) or LY34 (10 μM), measured in oocytes expressing mGluR2, 2AR and PTX (C) or RGS2 (D). IRK3 current inhibition was measured and normalized as in Figure 1D.

(E) Bar graph summary of Gi activity (mean ± SEM) measured in oocytes expressing mGluR2, 2AR and RGS2. GIRK4* current activation was measured and normalized as in Figure 1E.

Figure S6. Biochemical Studies in 2AR-KO Mouse Frontal Cortex: LY37-Mediated Gi Activation Is Unaffected, while Gq Activation and Up-Modulation of mGluR2-Dependent Gi Signaling by Clozapine are Abolished

LY37-stimulated [^{35}S]GTPγS binding in wild-type (WT) and 2AR-KO mouse frontal cortex membrane preparations followed by immunoprecipitation with anti-Gαi (A) or anti-Gαq (B) antibodies after treatment with vehicle or 10 μM LY37. Data are mean ± SEM. (*p < 0.05; ***p < 0.001; n.s., not significant).

(C) DCG IV-stimulated [^{35}S]GTPγS binding in 2A-KO mouse frontal cortex membranes followed by immunoprecipitation with anti-Gαi antibody in the presence or clozapine or vehicle. Data are mean ± SEM. (*p < 0.05; n.s., not significant). (Compare with Figure 5A in wild-type mice).

Figure S7. Dose-Response Effect of Clozapine in the Locomotor Response Induced by MK801

(A) Left panel depicts representative time courses of MK801-induced locomotion measured in 5 min block. Mice were administered clozapine (1.5 or 10 mg/kg), or vehicle followed by MK801 (0.5 mg/kg). Time of injection is indicated by arrow. The right panel shows bar graph

summaries of the total of MK801-induced locomotion as a summation of horizontal activity from t = 30 min to t = 120 min. Mice were administered clozapine (at the indicated dose) or vehicle followed by MK801 (0.5 mg/kg). Data are mean ± SEM (n = 5-20); (*p<0.05; **p<0.01; n.s., not significant).

(B) Data summary of the total MK801-induced locomotion as a summation of horizontal activity from t = 30 min to t = 120 min. mGluR2-KO (B), and 2AR-KO mice (C) were administered clozapine at 10 mg/kg (cyan) and compared the response to that of LY37 (purple), or clozapine at 1.5 mg/kg (purple), or vehicle (red), followed by MK801. Data are mean ± SEM (n = 4-10); (*p<0.05; **p<0.01; n.s., not significant).

(D)(E) Methysergide does not modulate the locomotor response induced by MK801, but abolishes the head-twitch response induced by DOI. (D) Mice were administered methysergide (3 mg/kg) or vehicle, followed by MK801 (0.5 mg/kg) or vehicle. Data are mean ± SEM (n = 4-6) (*p<0.05; n.s., not significant). (E) Head-twitch response was determined in mice injected with DOI (2 mg/kg) 15 min after being injected with methysergide (3 mg/kg). Data are mean ± SEM (n = 5 per group). (***p<0.001). These data suggest that although methysergide binds as a neutral antagonist to 2AR and blocks the behavioral responses induced by 2AR agonists, it does not induce antipsychotic-like effects.

SUPPLEMENTAL TABLE 1

Gi	mGluR2 / 2AR	mGluR2Δ / 2AR	mGluR3 / 2AR	mGluR3Δ / 2AR
5-HT	0.43 ± 0.04	1.02 ± 0.23	0.98 ± 0.14	0.65 ± 0.26
5-HT + LY34	0.83 ± 0.18	1.07 ± 0.17	0.98 ± 0.16	1.00 ± 0.13
5-HT + LY37	0.23 ± 0.03	1.00 ± 0.05	0.87 ± 0.31	0.23 ± 0.14
LY37	0.26 ± 0.14	0.02 ± 0.01	0.021 ± 0.01	0.11 ± 0.06
Gq	**mGluR2 / 2AR**	**mGluR2Δ / 2AR**	**mGluR3 / 2AR**	**mGluR3Δ / 2AR**
Glu	1.98 ± 0.87	0.94 ± 0.27	1.07 ± 0.17	2.30 ± 0.68
Glu + Clozapine	2.80 ± 0.37	1.23 ± 0.32	0.90 ± 0.11	2.97 ± 0.29
Glu + DOI	0.99 ± 0.22	1.23 ± 0.24	1.07 ± 0.32	1.12 ± 0.37
DOI	0.35 ± 0.18	0.01 ± 0.00	0.01 ± 0.00	0.37 ± 0.16

Table S1. Cross Signaling, Down-Modulation, and Up-modulation Depend on Heterocomplex Formation

Summary of GIRK4* current activation (Gi) and IRK3 current inhibition (Gq) (mean ± SD) in oocytes expressing 2AR alone or 2AR together with mGluR2, mGluR2Δ, mGluR3, or mGluR3Δ. IRK3 current inhibition and GIRK4* current activation were measured and normalized as in Figure 1, D and E respectively. Currents were obtained in response to 1 μM of the endogenous ligand serotonin (5-HT) and 1 μM of endogenous ligand glutamate (Glu). For Gq values were obtained in response to 1 μM of the endogenous ligand serotonin (5-HT), 1 μM serotonin together with 50 μM LY34, serotonin 1 μM together with 50 μM LY37, or 50 μM LY37 alone. For Gi values were obtained in response to 1 μM of the endogenous ligand glutamate (Glut), 1 μM glutamate together with 50 μM clozapine, serotonin 1 μM together with 50 μM DOI, or 50 μM DOI alone. Gq: 5-HT, 5-HT + LY34, and 5-HT + LY37 treatments were significantly different for mGluR2/2AR and mGlUR3Δ/2AR ($p<0.05$). Gi: Glu, Glu + Clozapine,

and Glu + DOI treatments were only significantly diffferent for mGluR2/2AR and mGluR3Δ/2AR ($p<0.005$).

SUPPLEMENTAL TABLE 2

	Δ Gi	Δ Gq	BI_{10}	Δ Gi	Δ Gq	BI_{50}
Clozapine	1.32	-0.98	**2.3**	1.68	-0.98	**2.66**
Risperidone	1.36	-0.82	**2.18**	1.69	-0.9	**2.59**
LY37	1.45	-0.65	**2.1**	1.55	-0.77	**2.32**
Ritanserin	1.12	-0.77	**1.89**	1.17	-0.85	**2.02**
Methysergide	0.96	-0.80	**1.76**	0.95	-0.82	**1.77**
eGlu	0.54	-0.44	**0.98**	0.39	-0.45	**0.84**
LY34	-1.02	-0.20	**-0.82**	-1.07	-0.17	**-0.9**
DOI	-0.01	0.95	**-0.96**	-0.01	1.39	**-1.4**

Table S2. Balance Index Calculations

Balance Index (BI) Calculations for clozapine, risperidone, LY37, methysergide, eGlu, LY34 and DOI at 10 μM (BI_{10}) and 50 μM concentration (BI_{50}).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Drugs

5-hydroxytryptamine (serotonin, 5-HT), 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), and (+)-MK801 hydrogen maleate (MK801) were purchased from Sigma-Aldrich.

(1R,4R,5S,6R)-4-Amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268; LY37), (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495; LY34), methysergide, clozapine, paliperidone (active compound of risperidone), ritanserin, (2S)-a-ethylglutamic acid (eGlu), and (2S,2'R,3'R)-2-(2',3'-Dicarboxycyclopropyl) glycine (DCG-IV) were obtained from Tocris Cookson Inc. [^{35}S]GTPγS and [^{3}H]ketanserin were purchased from PerkinElmer Life and Analytical Sciences, Inc.

Molecular Constructs

PCR amplification and subcloning of 2AR, mGluR2, mGluR3, mGluR2Δ (mGluR2 chimera containing transmembrane helices 4 and 5 of the mGluR3) and mGluR3Δ (mGluR3 chimera containing transmembrane helices 4 and 5 of the mGluR2) into pcDNA3.1 was described previously (González-Maeso et al., 2008). The human 2AR, the human mGluR2, and the mGluR2Δ were digested with BamHI and NotI and sub-cloned into the pXOOM vector. The human mGluR3 and the mGluR3Δ chimeras were digested with EcoRI and NotI and sub-cloned into pXOOM (González-Maeso et al., 2008). The human RGS2 (University of Missouri-Rolla, UMR) was PCR-amplified using the primers RGS2/S (TTTTggatccATGCAAAGTGCTATGTTCTT) and RGS2/A (TTTTgcggccgcTCATGTAGCATGAGGCTCTG) and sub-cloned into the BamHI and NotI sites of pXOOM. Constructs sub-cloned into the pGEMHE vector with the active point mutants of the human Kir3.4, Kir3.4-S143T or Kir3.4*, Kir2.3 (IRK3) and Pertussis Toxin (PTX) subunit B were previously described (Vivaudou et al., 1997).

Surface Labeling of Oocytes

We engineered HA-tagged 2AR, mGluR2, mGluR3, mGluR2ΔTM4,5 and mGluR3ΔTM4,5 constructs as previously described (González-Maeso et al., 2002) and used an ELISA-based chemiluminescence assay adapted for oocytes. Oocytes were blocked for 30-60 min in experimental medium (ND96 with 1% bovine serum albumin (BSA)) at 4°C, labeled with 0.5 µg/ml rat monoclonal anti-HA antibody (3F10; Boehringer Mannheim) (overnight at 4°C), extensively washed at 4°C, and incubated with 2 µg/ml HRP-coupled secondary antibody (goat anti-rat) (in 1% BSA for 60 min). Cells were washed (experimental medium, 4°C) and transferred to ND96 without BSA. Individual oocytes were placed in 100 µl SuperSignal ELISA (Pierce) and incubated at room temperature for 1 min. Chemiluminescence was quantitated in a TD-20E luminometer (Turner Designs, CA). As a control, we used non-injected oocytes.

Computational Methods

Conformational sampling of ligand-free 2AR or mGluR2. We used the crystal structures of the G protein-interacting opsin (PDB 3DQB (Scheerer et al., 2008)) and the nanobody-stabilized activated β2-adrenergic receptor (PDB 3PX0 (Rasmussen et al. 2011) as target active conformations for mGluR2 and 2AR, respectively. The CHARMM27 force-field (MacKerell et al., 1998) was used to describe the receptors (along with the CMAP backbone energy correction (Mackerell et al., 2004)), as well as the lipids. All calculations were performed using NAMD 2.7 (Phillips et al., 2005), enhanced with the Plumed plug-in (Bonomi et al., 2009). Briefly, we carried out multiple 10 ns-long ABMD simulations in an explicit hydrated 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/10% cholesterol membrane bilayer with an elastic constant of 0.1 kJ/nm2 for each system. The ligand-free sampled conformations of either 2AR or mGluR2 were grouped using average linkage agglomerative hierarchical clustering based on the root mean square deviation (RMSD) of the TM domains,

and path collective variables for metadynamics simulations were derived using the maximum number of clusters k that would define mono-dimensional paths for each receptor. To avoid rapid switching between clusters and to ensure all clusters were visited in a sequential fashion by the conformations of a given trajectory, we considered a maximum number of clusters k=8.

Ligand parameterization and docking. Clozapine, methysergide, DOI were parameterized based on the General CHARMM force-field optimization protocol (Vanommeslaeghe et al., 2010). These ligands were docked into the initial inactive model of 2AR, using a standard Autodock 4.0 (Morris et al., 2009) protocol. Inferences from published experimental work on clozapine (Kanagarajadurai et al., 2009), methysergide (Roth et al., 1997), or DOI (Braden et al. 2006) binding were considered to select the most accurate initial binding poses of these ligands for further metadynamics simulations.

Metadynamics of ligand-bound 2AR. Metadynamics simulations were performed on each ligand-bound 2AR system within an explicit environment, using as reaction coordinates path variables (Branduardi et al., 2007) describing the position along (s) and the distance from (z) the predetermined activation trajectories of the 2AR. A bias was added to the system using the well-tempered metadynamics algorithm (Barducci et al., 2008), with a deposition rate of 150 ps, an initial energy of the Gaussian bias of w=0.1 kcal/mol, and a bias-factor of 10. Convergence of the reconstructed free-energy was monitored by checking the height of the Gaussian bias contributions as a function of simulation time, and the simulation was stopped after 150 ns.

Exploration of the dimer interfaces. To study which interfaces involving TM4 and/or TM5

helices are stabilized by the ligand-specific 2AR conformations, we carried out all-atom Monte Carlo simulations with an implicit membrane model. Focus on these helices was based on inferences from the literature on several GPCR complexes, as well as both published (González -Maeso et al., 2008) and unpublished data on 2AR and mGluR2. The receptors were described using the same force-field used in the metadynamics simulations (MacKerell et al., 1998; Mackerell et al., 2004), while the effect of the membrane was approximated by the IMM1 implicit solvent model (Lazaridis et al., 2003) Standard parameters were used, modeling the solvent as water, and the interior of the membrane as cyclohexane.

The free-energy of different interfacial arrangements was calculated using a Monte Carlo scheme. A step consisted in the choice of a putative new conformation obtained by rotating each receptor around the axis of its own helical bundle of a random angle (sampled from a uniform distribution $-30° \leq \alpha, \beta \leq +30°$), and increasing or decreasing the distance between the centers of mass of a random distance ($-2Å \leq \delta \leq +2Å$). Proposed moves were rejected if the distance between the centers of mass increased over 35 Å or if the distance between the two TM4 helices increased over 20Å. A simulated annealing protocol was used to optimize the positions of the side chains of the putative conformations obtained after the proposed moves, keeping the TM Cα fixed. The total energy in the new conformation after the simulated annealing relaxation was used to either accept or reject the proposed move. The cycle was repeated 10^5 times, and the free-energy was calculated as a function of the angles around the axis of the helix bundle of each protomer (α and β), integrating the dependence on the distance.

Metadynamics of the 2AR/mGluR2 complexes interacting at symmetric TM4/TM4 or TM4,5/TM4,5 interfaces. Based on the results of the conformational sampling of the ligand-free receptors and of the interface exploration, we built 4 different reference states for the

symmetric TM4/TM4 interface of the clozapine-bound 2AR/mGluR2 complex, and 4 states for the symmetric TM4,5/TM4,5 interface of the methysergide-bound and the DOI-bound 2AR/mGluR2 complex, one for each conformation sampled along the activation pathway of mGluR2. These reference states were used to derive the path variables for three metadynamics simulations, a first one for the TM4/TM4 clozapine-bound 2AR/mGluR2complex, a second one for the TM4,5/TM4,5 methysergide-bound 2AR/mGluR2 complex, and a third one for the TM4,5/TM4,5 DOI- bound 2AR/mGluR2 complex. We then reconstructed the free-energy of activation of mGluR2 in the presence of the three different dimeric arrangements with clozapine-bound, the methysergide-bound and the DOI-bound 2AR. The parameters were the same as those used in the exploration of the 2AR conformational ensemble described above.

Analysis of mGluR2 and 2AR protein levels

Membrane fractions from oocytes expressing HA-tagged mGluR2, myc-tagged 2AR or both, were prepared as described previously (Michailidis et al., 2011). The pellets were re-suspended with lysis buffer (0.5ml per oocyte), mixed with SDS-PAGE loading buffer, boiled for 10min and analyzed by Western Blotting, using anti-HA (rat monoclonal anti-HA antibody 3F10; Boehringer Mannheim) and anti-myc (Myc-Tag 9B11 Mouse mAb, Cell Signaling Technology) antibodies. Protein levels for mGluR2 and 2AR were quantified by densitometric scanning and expressed relative to mGluR2 or 2AR levels in oocytes injected with 1ng of mGluR2 + 2ng of 2AR mRNAs.

Measurement of Intracellular Ca^{2+}

For measurement of intracellular free calcium, mouse cortical primary cultures were plated in coverslips and incubated for 1h with 10 mM fura-2 (Molecular Probes Inc., USA). A

photometry based epifluorescence microscopy system (Ionoptix; Milton, MA; objective, x40; 0.9 numerical aperture) was used to detect the intracellular Ca^{2+} transient. A Hyperswitch (HSW400, Ionoptix; Milton, MA) with a galvanometer driven mirror was used to alternatively excite fura-2 at wavelengths 340 and 380 nm at 2.5 Hz, and the Ca^{2+} transient was estimated as the ratio (f340/f380) of the emissions at 510 nm. Imaging was obtained using Ion Wizard fluorescence analysis software version 4.4. Experiments were performed on days 6, 7, and 8 after isolation. Image pairs were collected integrating a region of interest covering the whole cell at 2.5 Hz and filtered using a low-pass filter with a time constant τ=1s to reduce noise. Intracellular calcium concentration $[Ca^{2+}]_i$ was expressed as a 340/380 nm ratio and its increase was normalized to the basal 340/380 nm ratio level before perfusion of the drug.

[^{3}H]Ketanserin and [^{35}S]GTPγS Binding Assays

Membrane preparations were incubated for 60 min at 37 ºC. Non-specific binding was determined in the presence of 10 μM methysergide. [^{35}S]GTPγS binding experiments were initiated by the addition of membranes containing 35 μg of protein to an assay buffer (20 mM HEPES, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM ascorbic acid and 0.5 nM [^{35}S]GTPγS) supplemented with 0.1 μM or 10 μM GDP for $G\alpha_{q/11}$ and $G\alpha_i$, respectively, and containing the indicated concentration of ligands. Non-specific binding was determined in the presence of 100 μM GTPγS. Reactions were incubated for 30 min at 30 ºC, and were terminated by the addition of 0.5 ml of ice-cold buffer, containing 20 mM HEPES, 3 mM $MgCl_2$, 100 mM NaCl and 0.2mM ascorbic acid. The samples were centrifuged at 16,000 g for 15 min at 4 ºC, and the resulting pellets were re-suspended in solubilization buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, 1.25% Nonidet P40) plus 0.2% SDS. Samples were pre-cleared with Pansorbin (Calbiochem), followed by immunoprecipitation with antibody against $G\alpha_{q/11}$or $G\alpha_{i1,2,3}$ (Santa

Cruz Biotechnology). Finally, the immunocomplexes were washed twice with solubilization buffer, and bound [^{35}S]GTPγS was measured by liquid-scintillation spectrometry.

Co-Immunoprecipitation in Mouse Frontal Cortex

Tissue samples of mouse frontal cortex were homogenized using a Teflon-glass grinder (10 up-and-down strokes at 1,500 rpm) in 1 ml of homogenization buffer (50 mM Tris-HCl, pH 7.4), supplemented with 0.25 M sucrose. The crude homogenate was centrifuged at 1,000 x g for 5 min at 4°C, and the supernatant was re-centrifuged at 40,000 x g for 15 min at 4°C. The resultant pellet (P_2 fraction) was washed twice in homogenization buffer and re-centrifuged in similar conditions. Aliquots of 1 mg of protein were stored at –80°C until co-immunoprecipitation assay. The protein pellets were re-suspended in 1 ml RIPA buffer (150 mM NaCl, 5 mM EDTA, 0.8 % SDS, 1% Triton X-100, 50 mM HEPES; pH 7.4, supplemented with protease inhibitors), and rotated at 4°C for 60 min. After centrifugation at 14,000 rpm for 15 min, 900 µl of the supernatant were rotated with 40 µl of protein A/G beads (Santa Cruz Biotechnology, Inc.) at 4°C for 60 min. After centrifugation at 14,000 rpm for 1 min, 400 µl of the supernatant were incubated overnight with 4 µl of anti-2AR antibody (Abcam, ab16028) and 40 µl of protein A/G beads at 4°C. Beads were then washed three times with RIPA buffer. Equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis. Detection of proteins by immunoblotting using using anti-2AR and anti-mGluR2 (Abcam, ab15672) antibodies was conducted using the ECL system according to the manufacturer's instructions.

Cortical Primary Cultures and Immunocytochemistry

Cortical primary cultures mice were sacrificed at the E15 stage. The media was removed and the cells were fixed with 3% formaldehyde in PBS for 15 min. The coverslips were washed

with 3 x 2 ml PBS before being treated for 10 min with 0.3 % Triton X-100 to permeabilize the cell membrane to antibodies. Coverslips were further washed with PBS and incubated in PBS containing 3% horse serum and 1% bovine serum albumin (blocking buffer) to reduce non-specific binding. Primary antibodies anti-2AR and anti-mGluR2 (see above) were incubated for 60 min at room temperature. After washing with blocking buffer (3 x 2 ml), the cells were incubated with the secondary antibodies Alexa 488-conjugated goat anti-rabbit (Invitrogen, A11011) and Alexa 568-conjugated goat anti-mouse (Invitrogen, A11001) for 60 min at room temperature. After washing (PBS, 6 x 2 ml), the coverslips were dried and treated with anti-fade before being fixed onto glass slides.

For immunochemistry in brain section, mice were deeply anesthetized with with ketamine/xylazine. Transcardiac perfusion was performed with 10 ml PBS, followed by 30 ml of freshly prepared 4% paraformaldehyde (PFA) in PBS at room temperature. Brains were removed and immersion-fixed in 4% PFA in 1ml at 4°C for 60 minutes and stored at 30% sucrose in PBS at 4°C for an additional 12 hours before to obtain the frontal cortex section. The slides were were further washed with PBS and incubated in PBS containing 0.2% Tx-100 and 5% bovine serum albumin to permeabilize the cells. Primary antibodies anti-2AR and anti-mGluR2 (see above) were incubated for overnight at 4ºC. After washing with PBS the slices were incubated with the secondary antibodies Alexa 488-conjugated goat anti-rabbit (Invitrogen, A11011) and Alexa 568-conjugated goat anti-mouse (Invitrogen, A11001) for 60 min at room temperature. After washing (PBS, 6 x 2 ml), the slices were mounted onto coverslips treated with anti-fade. Sections incubated with secondary antibody alone exhibited background immunofluorescence (data not shown). Tissue samples were examined by upright epifluorescence confocal microscopy (Zeiss Axioplan 2IE).

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