Decoding the Signaling of a GPCR Heteromeric Complex Reveals a Unifying Mechanism of Action of Antipsychotic Drugs

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SUMMARY

Atypical antipsychotic drugs, such as clozapine and risperidone, have a high affinity for the serotonin 5-HT2A G protein-coupled receptor (GPCR), the 2AR, which signals via a Gq heterotrimeric G protein. The closely related non-antipsychotic drugs, such as ritanserin and methysergide, also block 2AR function, but they lack comparable neuropsychological effects. Why some but not all 2AR inhibitors exhibit antipsychotic properties remains unresolved. We now show that a heteromeric complex between the 2AR and the Gi-linked GPCR, metabotropic glutamate 2 receptor (mGluR2), integrates ligand input, modulating signaling output and behavioral changes. Serotonergic and glutamatergic drugs bind the mGluR2/2AR heterocomplex, which then balances Gi- and Gq-dependent signaling. We find that the mGluR2/2AR-mediated changes in Gi and Gq activity predict the psychoactive behavioral effects of a variety of pharmacological compounds. These observations provide mechanistic insight into antipsychotic action that may advance therapeutic strategies for disorders including schizophrenia and dementia.

INTRODUCTION

G protein-coupled receptors (GPCRs) are the most common cellular targets for drugs used in the clinic (Rosenbaum et al., 2009). Agonist binding is thought to induce distinct conformational changes that enable GPCRs to couple to and activate specific heterotrimeric G proteins (Oldham and Hamm, 2008). For example, 2AR is a Gq-coupled GPCR that responds to the neurotransmitter serotonin (5-HT) (González-Maeso and Sealfon, 2009), and mGluR2 is a Gi-coupled, pertussis toxin-sensitive GPCR that responds to the neurotransmitter glutamate (Glu) (Moreno et al., 2009). Although considerable biochemical and biophysical data are consistent with monomeric GPCRs binding and activating G proteins (Ernst et al., 2007; Whorton et al., 2007), several recent studies suggest that G protein coupling in cell membranes involves the formation of homomeric and heteromeric GPCR complexes (Han et al., 2009; Lopez-Gimenez et al., 2007; Carriba et al., 2008; Vilardaga et al., 2008). Oligomeric receptor complexes appear to exhibit distinct signaling properties when compared to monomeric receptors (Urizar et al., 2011; Milligan, 2009). The molecular mechanism(s) responsible for such changes in pharmacology are poorly understood, as is the physiological function of GPCR heteromeric complexes.

Atypical antipsychotic drugs have a high affinity for the 2AR (Meltzer et al., 1989; Meltzer and Huang, 2008) and are widely used in the treatment of schizophrenia and other psychiatric disorders (Ross et al., 2006). Interestingly, it has been recently recognized that most clinically effective antipsychotic drugs are, in fact, 2AR inverse agonists—ligands that preferentially bind and stabilize a GPCR in an inactive conformational state (Kenakin, 2002)—rather than simply neutral antagonists (Aloyo et al., 2009; Egan et al., 1998; Weiner et al., 2001)—ligands that compete for the same orthosteric binding site and prevent the cellular responses induced by agonists and inverse agonists. The mechanism underlying the antipsychotic effects of 2AR inverse agonism has not yet been elucidated.

A new class of potential antipsychotic drugs acting as agonists of mGluR2 recently received attention in preclinical (Woolley
et al., 2008) and clinical studies (Patil et al., 2007, Kinon et al., 2011). Previous work demonstrated that mGluR2 and 2AR form a specific heterocomplex in mammalian brain tissue (González-Maeso et al., 2008; Rives et al., 2009). However, the signaling properties of this receptor heterocomplex and its role in transducing antipsychotic drug activity remain unclear. Here, we compared G protein signaling coupled to the heteromeric mGluR2/2AR complex with homomeric signaling through either mGluR2 or 2AR. Our results provide insight into how Gi and Gq signaling are integrated by this GPCR heteromer and uncover a unifying mechanism of action of two families of antipsychotic drugs that target the mGluR2/2AR heteromeric complex. In addition, we provide a predictive metric for the anti- or propysychotic effects of serotonergic and glutamatergic ligands.

RESULTS

Heteromeric Assembly of mGluR2 and 2AR Enhances Glutamate-Elicited Gi Signaling and Reduces 5-HT-Elicited Gq Signaling

2AR and mGluR2 proteins colocalize in mouse cortical slices and neuronal primary cultures (Figure 1A and Figure S1E available online). In addition, the two receptors can be communoprecipitated from mouse frontal cortex (Figure 1B). To investigate the signaling properties of the mGluR2/2AR heterocomplex, we utilized a Xenopus oocyte heterologous expression system (Barela et al., 2006). We expressed each of these GPCRs alone or together and used inhibition of the IRK3 (Kir2.3) current to monitor 2AR-elicted Gq activity (Figure 1C, left) (Du et al., 2004) and activation of the GIRK4* (or Kir3.4*) current to monitor mGluR2-elicted Gi activity (Figure 1C, right) (He et al., 1999, 2002) (see Experimental Procedures).

How do the signaling properties of the mGluR2/2AR heteromeric complex differ from those of the homomeric receptors? We first quantified the Gi and Gq activities evoked by Glu and 5-HT, the endogenous ligands of the mGluR2 and 2AR receptors, respectively, and compared these values to Gi and Gq activities in the absence or presence of the heteromeric receptor partner (Figures 1C–1E). Coexpression of mGluR2 with the 2AR reduced 5-HT-elicted Gq activity by approximately 50% (Figures 1C, left panel and 1D). In contrast, coexpression of mGluR2 with the 2AR increased the Glu-elicted Gi activity by nearly 200% (Figure 1C, right panel and 1E).

The metabolotropic glutamate receptor 3 (mGluR3), which shares a high degree of homology with mGluR2, does not form a receptor heterocomplex with the 2AR. Exchanging the transmembrane (TM) domains of mGluR2 and mGluR3 either disrupts (mGluR2ΔSα) or rescues (mGluR3α) mGluR2/2AR receptor complex formation and 2AR-mediated signal-sensing (i.e., Gi signaling) (González-Maeso et al., 2008). mGluR2ΔSα activation does not evoke the Gi and Gq signaling outputs associated with the mGluR2/2AR heteromer, whereas mGluR3α activation induces an increase in Gi- and decrease in Gq-dependent signaling (Figures S1A–S1D). Each of the mGluR chimeras, when expressed as homomers, showed intact Gi signaling (Figures S2A–S2C) and cell-surface localization (Figure S2D). Together these findings suggest that the heteromeric receptor couples Gi and Gq outputs to influence downstream signaling events.

To summarize the difference between Gi and Gq signaling evoked by the mGluR2/2AR heteromers, we developed a metric called the balance index (BI). The BI combines the change in Gi activity (ΔGi) and the change in Gq activity (ΔGq), such that BI = ΔGi − ΔGq (Figure 1F). We used homomeric Gi and Gq signaling levels to normalize the data and found that without stimulation, the mGluR2/2AR complex yielded a BI of 1.45, which we use as the reference BI level (BIr). We obtained the largest BI, when expressing mGluR2/2AR mRNAs in a 1:2 ratio (Figure S3D). As shown in Figure S3C, this ratio of mRNAs yielded a cell-surface localization of receptor protein levels that suggested a higher-order oligomeric complex between mGluR2 and 2AR (see Figures S3A and S3B).

Drugs that Bind 2AR Alter the Balance between Gi and Gq Signaling

We next asked whether drugs bound to one receptor of the heteromer could affect the other receptor’s signaling response to its endogenous ligand. We first investigated the effects of 2AR ligands (a neutral antagonist, a strong agonist, and an inverse agonist) on Glu-elicted Gi signaling by mGluR2. We define DOI as a strong agonist because it evokes greater Gq signaling through 2AR than the endogenous ligand, 5-HT (Figures 2A, 2B, and S4A). In control experiments, the strong agonist (DOI), the neutral antagonist (methysergide), and the inverse agonist (clozapine) (Weiner et al., 2001) worked as expected to stimulate or reduce 5-HT-induced Gq signaling, respectively (Figure 2A).

Occupancy of the 2AR by either methysergide, DOI, or clozapine had different effects on Glu-elicted signaling through mGluR2 (Figure 2B, blue bars). Although Glu-elicted Gi signaling was not altered by 5-HT or methysergide, it was decreased back to baseline by DOI and increased by approximately 40% (240% greater than homomeric levels) by clozapine (Figure 2B).

Using the results obtained for Gi (Figure 2B, blue bars) and Gq signaling (Figures 2A and 2B, red bars), we calculated the BI values for the three ligands in the presence of the endogenous ligands (Figure 2C). The changes evoked by these three drugs were abrogated by mGluR2ΔSα or mimicked by mGluR3α (see Table S1), and they were present when the Gq pathway was blocked by the regulator of G protein signaling subunit 2 (RGS2) (Figures S5D and S5E). The 2AR ligand with the largest overall BI was the inverse agonist clozapine (BI = 2.30; 140% increase in Gi and 100% decrease in Gq).

Could these ligands exert their effects by stabilizing different conformations of the receptor complex? To address this question, we investigated the conformational changes induced by the three 2AR ligands in molecular models of 2AR alone or complexed with mGluR2. To observe large conformational changes in relatively short timescales, we used a combination of adiabatic-biased molecular dynamics (ABMD) and metadynamics simulations (see “Computational Methods” in Experimental Procedures). This approach was recently validated on a prototypic GPCR (Provasi et al., 2011). First, we studied the effects of methysergide, DOI, and clozapine on the activation free-energy profile of a protomeric 2AR (see Figure 2D, top) and identified the most energetically favorable 2AR state for each ligand.
In agreement with known efficacies of these ligands, the clozapine-bound 2AR conformation is inactive (i.e., 2RH1-like), the DOI-bound 2AR conformation is active (i.e., 3P0G-like), and the methysergide-bound conformation adopts an inactive state that is structurally different from the inactive state stabilized by clozapine.

To provide a structural context for the crosstalk between 2AR and mGluR2, we studied the effects of the three 2AR

Figure 1. Heteromeric Assembly of 2AR and mGluR2 Enhances Glu-Induced Gi Signaling and Reduces 5-HT-Induced Gq Signaling
(A) Representative micrographs showing coexpression of endogenous 2AR (red) and mGluR2 (green) in mouse frontal cortex (left panels) and mouse cortical primary neurons (right panels). Scale bar, 25 μm. See also Figure S1E. 2AR and mGlu2 colocalize and form a receptor complex in mouse frontal cortex.
(B) Mouse frontal cortex membrane preparations were immunoprecipitated (IP) with anti-2AR antibody. Immunoprecipitates were analyzed by western blot (WB) with anti-mGluR2 antibody (lower blot). Mouse frontal cortex membrane preparations were also directly analyzed by WB with anti-2AR antibody (upper blot) or anti-mGluR2 antibody (middle blot). 2AR-KO and mGluR2-KO mouse frontal cortex tissue samples were processed identically and used as negative controls. Frontal cortex tissue samples from 2AR-KO and mGluR2-KO mice were also homogenized together (mixed) and processed identically for immunoprecipitation and WB.
(C) Representative barium-sensitive traces of IRK3 currents obtained in response to 1 μM 5-HT in oocytes expressing 2AR alone, mGluR2 and 2AR together, or mGluR2 alone (left). Representative barium-sensitive traces of GIRK4* currents obtained in response to 1 μM Glu in oocytes expressing mGluR2 alone, mGluR2 and 2AR together, or 2AR alone (right). Barium (Ba) inhibited IRK3 and GIRK4* currents and allowed for subtraction of IRK3- and GIRK4*-independent currents. For illustrative purposes, traces with similar basal currents were chosen.
Summary bar graphs of (D) Gq activity measured as IRK3 current inhibition (mean ± standard error of the mean [SEM]) following stimulation with 5-HT and (E) Gi activity measured as GIRK4* current activation (mean ± SEM) following stimulation with Glu. IRK3 current inhibition was measured relative to basal currents and was normalized relative to that obtained by stimulating 2AR alone with 5-HT (100% or 1). GIRK4* current activation was measured relative to the basal currents and was normalized relative to that obtained by stimulating mGluR2 alone with Glu (100% or 1).
(F) Calculation of the BI as the difference of the increase in Gi signaling in response to Glu from the mGluR2 homomeric level (ΔG_i) and the decrease of Gq signaling in response to 5-HT from the 2AR homomeric level (ΔG_q). A reference BI (BI_ref = 1.45) was calculated for the mGluR2/2AR complex in response to 1 μM Glu and 1 μM 5-HT using mean values (**p < 0.01, ***p < 0.001). Error bars depict standard error of the mean (SEM). See also Figures S1, S2, and S3.
ligands on mGluR2 conformations in the dimeric complex. Figure 2D (bottom) shows that when clozapine is bound to 2AR, the mGluR2 equilibrium shifts toward an activated conformation (i.e., 3DBQ-like), consistent with functional upmodulation of Gi signaling. In contrast, when methysergide and DOI are bound to 2AR, mGluR2 is stabilized in inactive states (i.e., 1U19-like). Although no significant energetic and structural differences were noted between the TM regions of these inactive states, the functional downmodulation of Gi signaling induced by DOI, but not by methysergide, may be ascribed to different interactions between the receptor loop regions and the G protein, which are not taken into account in our simulations. The functional predictions from this computational approach can be used to guide structure-based rational discovery of novel “biased” drugs that are capable of selectively activating specific signaling pathways.

Together, these results indicate that formation of the heteromer enables modulation of the mGluR2-Gi response by 2AR ligands. Whereas drugs such as the strong 2AR agonist DOI can greatly stimulate Gq signaling and decrease Gi signaling (henceforth referred to as dominant agonists), inverse agonists, such as clozapine, have the opposite effect, abolishing Gq and increasing Gi signaling.
Drugs that Bind mGluR2 Alter the Balance between Gi and Gq Signaling

How do drugs bound to the mGluR2 component of the heteromer affect the 5-HT-elicited Gq signaling of the 2AR? Neutral antagonist ethylglutamic acid (eGlu), the strong agonist (LY37) (Figure S4B), and the inverse agonist (LY34) (Figure S4C) worked as expected to reduce, stimulate, or abolish Glu-elicited Gi signaling through mGluR2, respectively (Figure 3A). Consistent with its inverse-agonist properties, LY34 not only completely abolished Gi signaling but also reduced the basal Gi activity of the mGluR2 receptor even in the absence of Glu (Figure S4C).

Occupancy of the mGluR2 receptor by each of the three ligands influenced the 2AR signaling. Relative to the homomeric 2AR levels, formation of the complex reduced the extent of Gq signaling by 50% (Figures 3B, 1D, and 1F). Although 5-HT-elicited Gq signaling was unaffected by Glu or eGlu, it was further decreased by LY37 and restored to near-homomeric levels by LY34 (Figure 3B).

Using the data shown for Gi (Figures 3A and 3B, blue bars) and Gq signaling (Figure 3B, red bars), we calculated the BI values for the three ligands (Figure 3C). As with 2AR drugs, the neutral antagonist eGlu only affected the mGluR2 side of signaling. The strong agonist LY37, however, affected both types of signaling through the complex and showed a dominant-agonist behavior as defined previously. Furthermore, LY37, like DOI, cross-signaled and elicited Gq signaling in the absence of Glu and 5-HT, respectively (Figures S4F and S4G). The inverse agonist LY34 had the opposite two effects: it blocked Gi but also potentiated Gq signaling, achieving almost 2AR homomeric levels (83%). All effects were disrupted when replacing mGluR2 by mGluR2D or rescued by mGluR3D (see Table S1), and they were present when the Gi pathway was blocked by pertussis toxin (PTX) (Figures S5A–S5C). The largest overall signaling difference between Gi and Gq was obtained by the dominant agonist LY37 (BI = 2.10).

In summary, the formation of the heteromeric complex favors Gi over Gq signaling by endogenous ligands. Dominant agonists...
enhance signaling through the receptor they target as part of the complex but inhibit signaling of the heteromeric receptor partner. Inverse agonists inhibit signaling through the receptor they target as part of the complex but enhance signaling of the heteromeric receptor partner.

**The BI Predicts the Anti- or Propropsychotic Activity of Drugs Targeting mGluR2 or 2AR**

Our results in Figures 2 and 3 indicate that although clozapine and LY37 act on different receptors, both drugs act through the mGluR2/2AR complex to achieve a similar effect—an increase in Gi activity with a concomitant decrease in Gq activity. To test whether the psychoactive effects of specific drugs correlated with differences in the levels of Gi and Gq activity that they induced, we calculated BI values for multiple drugs. For mGluR2, in addition to the dominant agonist LY37, we tested the neutral antagonist eGlu and the inverse agonist LY34, a drug that has been shown to increase locomotor activity and exploratory behavior in mice and might be propropsychotic (Bespalov et al., 2007). For 2AR, besides the inverse agonist clozapine, we tested the following: risperidone, another widely used atypical antipsychotic like clozapine; ritanserin, an antidepressant also used as an adjuvant therapeutic for schizophrenia; methysergide, a neutral antagonist mainly used for migraines; and DOI, a proppsychotic drug with lysergic acid diethylamide (LSD)-like effects.

In the presence of endogenous ligands and absence of drugs, the difference between Gi and Gq signaling is naturally kept in a balance that favors Gi over Gq (BIr). We set the reference level of the BI scale to BIr = 1.45 and compared the relative effect of different drugs on the BI, assuming a 50% occupancy of the receptor by the drug (see Gi-Gq recovery/loss calculation in Experimental Procedures). Results from drugs that target mGluR2 (blue icons), versus those that target 2AR (red icons), are plotted in Figure 4.

Drugs with the most effective antipsychotic properties, regardless of the receptor they target (2AR: clozapine, risperidone; mGluR2: LY37), show the highest BI values. In contrast, drugs with the most effective proppsychotic properties (2AR: DOI; mGluR2: LY34) show the lowest BI values (Figure 4 and Table S2). All of these drugs are either dominant agonists (antipsychotic for mGluR2 and proppsychotic for 2AR) or inverse agonists (antipsychotic for 2AR and proppsychotic for mGluR2).

**Inverse-Agonist Upmodulation Occurs in Mouse Frontal Cortex**

To study the relevance of mGluR2/2AR heteromer signaling in vivo, we examined the pattern of G protein coupling in mouse frontal cortex, a region that plays an important role in schizophrenia and antipsychotic action (González-Maeso and Sealfon, 2009). We first measured the mGluR2/2AR complex-dependent upmodulation of Gi signaling by a 2AR inverse agonist. Membrane preparations from mouse frontal cortex were incubated with the inverse agonist clozapine or the neutral antagonist methysergide (Figures S4D and S4E), together with DCG-IV, a selective mGluR2/3 agonist. Clozapine increased the DCG-IV-mediated Gi signaling (Figure 5A), whereas methysergide did not significantly affect Gi signaling (Figure 5B). Furthermore, clozapine failed to increase the DCG-IV-mediated Gi signaling in frontal cortex membrane preparations from 2AR knockout (KO, Htr2a−/−) mice (Figure S6C; see also Figures S6A and S6B for LY37-dependent activation of Gq in wild-type but not in 2AR-KO mouse frontal cortex).

We also tested the mGluR2 inverse-agonist upmodulation of Gq signaling in cortical primary cultures. Stimulation of Gq signaling in neurons is known to elicit a transient increase of intracellular calcium via an IP3-mediated Ca2+ release from the endoplasmic reticulum (ER) that can be recorded using fluorescent calcium-sensitive dyes (Pichon et al., 2010). As predicted,
LY34 was able to boost the 5-HT response nearly 5-fold (Figures 5C and 5D) but showed no response alone. In contrast, coapplication of the mGluR2 neutral antagonist eGlu with 5-HT did not elicit a significant increase in intracellular calcium (Figures 5C and 5D).

These data provide evidence that the effects of inverse agonists that bind 2AR or mGluR2 and boost their heteromeric partner receptor’s signaling occur in cortical neurons in vivo (Figures 5A–5D).

2AR and mGluR2 Are Both Necessary for Antipsychotic-like Behavior in Mice

How do the antipsychotic drugs LY37 and clozapine influence behaviors? We determined the effects of the mGluR2/3 agonist LY37 on locomotor behavior precipitated by treatment with MK801 (Figure 5E). Noncompetitive NMDA receptor antagonists, such as phencyclidine (PCP) and ketamine, are used to model schizophrenia in rodents because of their capacity to evoke human behaviors similar to those observed in patients (Morris et al., 2005; Mouri et al., 2007; Patil et al., 2007). The potent and selective noncompetitive NMDA receptor antagonist MK801 (dizocilpine) can also elicit similar symptoms (Reimherr et al., 1986). Activation of mGluR2, but not mGluR3, by LY37 has been shown to reduce hyperlocomotion induced by noncompetitive NMDA antagonists in mouse models of schizophrenia (Woolley et al., 2008). MK801-stimulated locomotor activity in wild-type and 2AR-KO mice was indistinguishable. MK801-stimulated activity was significantly attenuated by LY37 in wild-type mice, but not in 2AR-KO (Htr2a−/−) or mGluR2-KO (Grm2−/−) mice (Figures 5E and S7B).

We next tested the role of mGluR2 in the antipsychotic-like effect induced by the atypical antipsychotic clozapine. Because clozapine binds with high affinity to 2ARs, and with lower affinity to dopamine D2 receptors (Meltzer et al., 1989), we first established the lowest dose of clozapine that induced an antipsychotic-like effect in mice (Figure S7A). The locomotor activity...
induced by MK801 was similar in wild-type and mGluR2-KO mice. Notably, pretreatment with 1.5 mg/kg clozapine significantly decreased the MK801-stimulated locomotion in wild-type mice but not mGluR2-KO mice (Figure 5F), and this treatment had no effect on 2AR-KO mice (Figure S7C; see also Figures S7A–S7C). Although our results are consistent with the absence of antipsychotic-like behavioral effects of methysergide (compare Figure 5F for clozapine with Figures S7D and S7E for methysergide), they do not exclude the possibility that the absence of antipsychotic-like effects of LY37 in 2AR-KO may be affected by the lower expression of mGluR2 in 2AR-KO mice (González-Maeso et al., 2008; Moreno et al., 2011). Coinjection of LY37 and clozapine, followed by MK801 (0.5 mg/kg), or both LY37 and clozapine, did not affect the MK801-induced locomotion in mGluR2 or 2AR heterozygote mice (Figure 6B). However, coadministration of both antipsychotics robust therapeutic-like behavioral effects and reduced unwanted side effects (Uslaner et al., 2009). We postulated that alterations in 2AR and mGluR2 expression levels in our system (Figures 1F and S3D) may model the alterations in 2AR and mGluR2 ratios observed in postmortem brain samples from untreated schizophrenic patients (González-Maeso et al., 2008). We asked whether coadministration of clozapine (a 2AR inverse agonist) with LY37 (a mGluR2 dominant agonist) compensates for alterations in Gi-Gq balance caused by suboptimal expression ratios of the two receptors. To this end, we injected clozapine with LY37 and determined the BI. Figure 6A shows the ΔGI and ΔGq values obtained in response to clozapine, LY37, and both clozapine and LY37 (5 mg/kg). Data are mean ± SEM. (B) Summary bar graphs (mean ± SEM) of the total MK801-induced locomotion as a summation of horizontal activity from t = 30 min to t = 120 min. Injection time was at t = 0 min. mGluR2 heterozygotes (mGluR2+/−) (left) and 2AR heterozygotes (right) are shown. Mice were administered vehicle, clozapine (1.5 mg/kg), LY37 (5 mg/kg), or both LY37 and clozapine, followed by MK801 (0.5 mg/kg) (N = 5–6). (*p < 0.05, n.s.: not significant).

**Figure 6. Control of BI through a Drug Combination Approach**

(A) BI calculations at 50 μM concentrations of ligands. ΔGI referenced to the homomeric mGluR2 (1 ng of mRNA) response to 1 μM Glu and ΔGq referenced to the homomeric 2AR (2 ng of RNA) response to 1 μM 5-HT and 1 μM Glu. Responses to a concentration of 50 μM clozapine, LY37, or LY37 together with clozapine were measured in oocytes injected with 1 ng mGluR2 mRNA and 1 ng (left), 2 ng (center), and 3 ng (right) 2AR mRNA, respectively. Data are mean ± SEM. (B) Summary bar graphs (mean ± SEM) of the total MK801-induced locomotion as a summation of horizontal activity from t = 30 min to t = 120 min. Injection time was at t = 0 min. mGluR2 heterozygotes (mGluR2+/−) (left) and 2AR heterozygotes (right) are shown. Mice were administered vehicle, clozapine (1.5 mg/kg), LY37 (5 mg/kg), or both LY37 and clozapine, followed by MK801 (0.5 mg/kg) (N = 5–6). (*p < 0.05, n.s.: not significant).
in mGluR2 or 2AR heterozygotes significantly decreased the MK801-stimulated locomotor activity (Figure 6B). These results suggest that a combination of mGluR2 dominant agonists with 2AR inverse agonists is likely to synergize in vivo to achieve an optimal signaling ratio in cases where a suboptimal Gi-Gq signaling balance exists.

**DISCUSSION**

Previous work demonstrated that mGluR2 and 2AR form a functional heteromeric complex through which hallucinogenic drugs cross-signal to the Gi-coupled receptor (González-Maeso et al., 2008). It was not clear how the heteromeric complex signaled in response to ligands binding to either receptor and whether the differential pharmacology of this GPCR heteromer could be considered widely as a tractable therapeutic target for psychotic behavior. Our current study indicates that the mGluR2/2AR heteromer establishes a Gi-Gq balance in response to endogenous ligands (e.g., Glu and 5-HT). We utilized a simple metric, the BI, that quantifies the change in Gi (increase) and Gq (decrease) signaling upon heteromerization relative to the homomeric signaling levels.

The BI can be modulated either by dominant agonists that take control over their counterpart receptors or by inverse agonists that lift this control. This establishes a map between the ligand input to the heteromer (e.g., agonist/agonist, dominant agonist/agonist, neutral antagonist/agonist, inverse agonist/agonist) and its signaling output in terms of how specific ligands affect the BI. Our results are in agreement with results from a GPCR complex of D2 receptor homomers coupled to a single Gi protein subunit (Han et al., 2009). Our study further extends these previous findings and demonstrates a signaling crosstalk between the 2AR and mGluR2, which are individually coupled to two different subtypes of G proteins (Gq and Gi). Our data indicate that signaling crosstalk through the mGluR2/2AR heterocomplex may be a causal mechanism for the induction of cellular and behavioral responses that differ from those of mGluR2 and 2AR homomers.

In our model (Figure 7), psychedelics invert the signaling balance through the complex (Gi signaling decreases, whereas Gq signaling increases, thus decreasing the BI), tipping the balance from being in favor of Gi signaling (normal complex) to being in favor of Gq signaling (propsychotic) (Figure 7A). Similarly, disease states involving psychosis, such as schizophrenia, would be expected to be associated with a variable disruption of the Gi-Gq balance (i.e., decrease in Gi, increase in Gq, and decrease in BI) (Figure 7B), consistent with the mGluR2 downregulation and 2AR upregulation observed in untreated schizophrenic patients (González-Maeso et al., 2008). Such disruption would be reversed by antipsychotics that recover the Gi-Gq balance again in favor of Gi as in the normal complex (i.e., increasing Gi and decreasing Gq, thus increasing the BI) (Figure 7B). Because the Gi-Gq balance is the regulated variable predicting psychotic state, it is not surprising that inverse agonists on the 2AR side and strong agonists on the mGluR2 side are the most effective antipsychotics. The present study also suggests a unifying mechanism of action of atypical antipsychotics and the new glutamate antipsychotics. Our findings suggest inverse agonism as a common feature of 2AR ligands with antipsychotic properties. We show that dysregulation of an optimal ratio of mGluR2 to 2AR expression via injection of different mRNA ratios greatly decreases BI values, and single

![Figure 7. Gi-Gq Balance Model of the Mechanism of Action of Antipsychotic and Psychedelic Drugs through the mGluR2/2AR Complex](image-url). Formation of the receptor complex establishes an optimal Gi-Gq balance in response to Glu and 5-HT (increase in Gi, decrease in Gq). (A) Psychedelics (LY34 and DOI) invert the balance (strong Gi decrease, strong Gq increase). (B) Disruption of the optimal balance in psychotic states (decrease in Gi, increase in Gq) can be compensated for by antipsychotics (LY37, clozapine, and risperidone) that recover the Gi-Gq balance (increasing Gi and decreasing Gq).
application of 2AR inverse agonists or mGluR2 dominant agonists may not push the BI into the therapeutic range (see Figure 4). Yet coadministration of the most effective mGluR2 and 2AR drugs yields BI values in the therapeutic range (Figure 6). These findings in heterologous systems were paralleled in vivo using heterozygous mice for 2AR or mGluR2: coinjection of both clozapine and LY37 was needed to decrease the MK801-dependent locomotor activity. In some schizophrenic patients, atypical antipsychotics produce complete remission of psychotic symptoms. However, two-thirds of schizophrenic patients are considered treatment resistant, with persistent psychotic and other symptoms despite the optimal use of available antipsychotic medications (Lieberman et al., 2005). The absence of antipsychotic-like behavioral effect by injection of either LY37 or clozapine in 2AR or mGluR2 heterozygous mice, but not in the same mice coinjected with LY37 and clozapine, points toward potential beneficial use of combination therapy in treatment-resistant schizophrenia.

The metric (BI) that we provide allows quantification and prediction of anti-/propsychotic effects of new drugs acting through the mGluR2/2AR receptor heterocomplex. Although long-term effects of drugs targeting mGluR2/2AR signaling are not taken into account in the way we have estimated the BI metric, the ability of this scale to predict appropriately the most effective anti- and propsychotic drugs acting through the receptor heterocomplex makes it a promising tool in predicting the efficacy of new drugs. This metric, as well as structural insights from ligand-specific heteromeric conformations, could be used extensively for screening new compounds with potential antipsychotic effects.

Our results pave the way toward a new understanding of the cellular signaling, function, and pharmacology of other heteromeric GPCRs that have been implicated as therapeutic targets for the treatment of disease (Milligan, 2009). Provided that the receptor complex signaling output can classify accurately the behavior of drugs targeting the complex and used to treat disease, the case of the mGluR2/2AR complex can serve as a guiding example of development of therapeutic potency scales that can be used to classify existing drugs and predict the behavior of novel ones. Because the most effective antipsychotic drugs targeting the mGluR2/2AR complex all gave the highest BI values, it is likely that somehow signaling through this complex is uniquely coupled to specific targets. The mechanism of such signaling specificity as well as the detailed actions of Gi versus Gq signaling through the mGluR2/2AR complex, aiming to achieve a homeostatic balance that ensures a normal nonpsychotic state, are likely to become an active pursuit of future studies.

EXPERIMENTAL PROCEDURES

Drugs, Molecular Constructs, Analysis of mGluR2 and 2AR Protein Levels, and Surface Expression Assays
See Extended Experimental Procedures for details on all of these.

Expression of Recombinant Proteins in Xenopus Oocytes
Oocytes were isolated and microinjected with equal volumes (50 nl), as previously described (Lopes et al., 2002). In all two-electrode voltage-clamp experiments (TEVC), oocytes were injected with 1 ng of mGluR2, 2 ng of mGluR2ΔTM4.5, 2 ng of mGluR3, 2 ng of mGluR3ΔTM4.5, 2 ng of 2AR, 2 ng of GIRK4*, 2 ng of IRK3, 1 ng of PTX, or 4 ng of RGS2 and were maintained at 18°C for 1–4 days before recording.

TEVC Recording and Analysis
Whole-cell currents were measured by conventional TEVC with a GeneClamp 500 amplifier (Axon Instruments, Union City, CA, USA), as previously reported. A high-potassium (HK) solution was used to superfuse oocytes (96 mM KCl, 1 mM NaCl, 1 mM MgCl2, 5 mM K2HPO4, pH 7.4) to obtain a reversal potential for potassium (E钾) close to zero.

Inwardly rectifying potassium currents through GIRK4* and IRK3 were obtained by clamping the cells at ~80 mV. In order to isolate Gi, GIRK4* was co-injected with RGS2 in order to eliminate the Gq component in the current. Basal IRK3 and GIRK4* currents were defined as the difference between inward currents obtained at ~80 mV in the presence of 3 mM BaCl2 in HK solution and those in the absence of BaCl2 and measured for each trace. Current inhibition and current activation were measured respectively and normalized to basal current to compensate for size variability in oocytes.

Computational Methods
Molecular modeling: Because there are no available crystal structures of the 2AR or mGluR2 available to date, we generated initial molecular models of these two receptors. Specifically, we built initial inactive conformations of 2AR or mGluR2 using a combination of homology modeling for the TM helices and an ab initio loop prediction approach implemented in the Rosetta 2.2 code (Wang et al., 2007) for the loop regions of the receptors. According to specific structural and functional similarities, the i2-adrenergic (PDB 2RH1 (Cherezov et al., 2007)) or -rhodopsin (PDB 1U19 (Okada et al., 2004)) crystal structures were used as structural templates for the homology modeling of the TM regions of 2AR or mGluR2, respectively. We generated activation pathways for each receptor using the diabatic-biased MD (ABMD) algorithm (Paci and Karplus, 1999) and a recently published simulation protocol (see Provasi et al., 2011 for details). Free-energy values were calculated using a Monte Carlo scheme. (see Extended Experimental Procedures for additional details.)

Experimental Animals
Experiments were performed on adult (8- to 12-week-old) male 129S6/SvEv mice. 2AR-KO mice have been previously described (Gonzalez-Maeso et al., 2007), mGluR2-KO mice were obtained from the RIKEN BioResource Center, Japan (see reference Yokoi et al., 1996 for details) and backcrossed for at least ten generations onto a 129S6/SvEv background. All subjects were offspring of heterozygote breeding. For experiments involving genetically modified mice, 2AR wild-type or mGluR2 wild-type littermates were used as controls. Animals were housed for 12 hr light/dark cycles at 23°C with food and water ad libitum. The Institutional Animal Use and Care Committee approved all experimental procedures at the Mount Sinai School of Medicine and Virginia Commonwealth University.

Measurement of Intracellular Ca2+
Measurement of intracellular free calcium was performed as described in the literature with minor modifications (Pichon et al., 2010) (see Extended Experimental Procedures for additional details).

Coimmunoprecipitations and [3H]Ketanserin and [35S]GTP Binding Assays
Mouse frontal cortex membrane preparations and binding assays were performed as previously described with minor modifications (Gonzalez-Maeso et al., 2008) (see Extended Experimental Procedures for additional details).

Cortical Primary Cultures and Immunocytochemistry
Mouse cortical primary neurons were cultured as previously reported (Gonzalez-Maeso et al., 2008). (See Extended Experimental Procedures for additional details.)

Behavioral Studies
Locomotor and head-twitch behavioral studies were performed as previously described (Gonzalez-Maeso et al., 2008). Motor function was assessed with
Calculation of Gi-Gq Balance Recovery and Loss

The total Gi-Gq balance achieved by the mGluR2/2AR heteromer in the presence of a drug was calculated with the following equation:

\[
\text{Total balance} = x \times B_{Ir} + B_{I} (1 - x)
\]

where \( x \) is the fraction of heteromer that binds the drug, \( B_{Ir} \) is the balance index of the drug at a fixed concentration, and \( B_{I} \) is the reference balance index of the complex (1.45).

In the absence of drug (\( x = 0 \)), the total balance achieved will be \( B_I \) (1.45). If the drug has a \( B_{Ir} > B_{I} \), the total Gi-Gq balance is also \( B_I \) (1.45) for any fraction \( x \) of drug-bound heteromer.

In a state where the mGluR2/2AR heteromer is signaling at a \( B_I < B_{Ir} \) (disease state), a drug with a \( B_{Ir} > B_{I} \) will be able to compensate for the total Gi-Gq balance loss and reestablish a total balance of 1.45 (\( B_I \)), if \( B_{Ir} \) is sufficiently large.

The Gi-Gq balance recovery (\( R \)) was calculated with the following equation:

\[
R = 100 \cdot \left( \frac{\text{Total balance}}{B_{Ir}} - 1 \right)
\]

This value expressed as a percentage indicates the amount of Gi-Gq balance that could be recovered by a drug with a balance index \( B_{Ir} \). It is determined by the difference between the total balance achieved in the presence of the drug compared to \( B_I \). A positive Gi-Gq balance (total balance > \( B_I \)) indicates that the drug is able to recover Gi-Gq balance (e.g., a drug with a \( B_{Ir} \) of 2.3 with a fractional occupancy \( x = 0.5 \) will have a total balance of 1.875, which will allow recovery up to ~30% loss from the reference level of 1.45). A negative Gi-Gq balance (total balance < \( B_I \)) indicates that the drug induces a loss in balance (e.g., a drug with a \( B_{Ir} \) of 0.315 with a fractional occupancy \( x = 0.5 \) will have a total balance of 0.315, which will result in ~80% loss from the reference level of 1.45).

In order to compare drugs, we established an arbitrary reference level of fractional occupancy of the heteromer by the drug (\( x = 0.5 \)). This allowed us to establish the differences in the ability of the drugs to recover or lose the Gi-Gq balance based on their \( B_I \) at equal conditions. Changes in the fractional occupancy used for comparison (e.g., \( x = 0.25 \)) changed the magnitude but not the relative order in the classification of the drugs shown in Figure 4.

Statistical Methods

Statistical significance of behavioral experiments involving four groups and two treatments was assessed by two-factor ANOVA followed by Bonferroni’s post-hoc test. Statistical significance of behavioral experiments involving one treatment was assessed by Student’s t test.

Each electrophysiological experiment in Xenopus oocytes was performed in two batches. Every group in each experiment was tested in both batches. Data for both batches were compiled (n = 8–16), and one-way ANOVA tests applied followed by a multiple comparison procedure using Tukey’s honestly significant difference test.

Intracellular calcium measurements were performed in three different isolations. Data for all isolations were compiled (n = 7–11), and one-way ANOVA tests applied followed by a multiple comparison procedure using Tukey’s honestly significant difference test.

[3H]Ketanserin binding experiments were performed 3–5 times in duplicate/triplicate. A one-site model versus a two-site model, as a better description of the data, was determined by F test. [32P]GTP i binding experiments were performed three times in triplicate. Two-way ANOVA tests were applied to the compiled data followed by a Bonferroni’s post-hoc test.


Woolley, M.L., Pemberton, D.J., Bate, S., Corti, C., and Jones, D.N. (2008). The mGlu2 but not the mGlu3 receptor mediates the actions of the mGluR2/3 agonist, LY379268, in mouse models predictive of antipsychotic activity. Psychopharmacology (Berl.) 196, 431–440.