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Contribution of heteromerization to G protein-coupled receptor function

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Abstract

G protein-coupled receptors (GPCRs) are a remarkably multifaceted family of transmembrane proteins that exert a variety of physiological effects. Although family A GPCRs are able to operate as monomers, there is increasing evidence that heteromerization represents a fundamental aspect of receptor function, trafficking and pharmacology. Most recently, it has been suggested that GPCR heteromers may play a crucial role as new molecular targets of heteromer-selective and bivalent ligands. The current review summarizes key recent developments in these topics.

Introduction

G protein-coupled receptors (GPCRs) are ubiquitous plasma membrane proteins that respond to a variety of extracellular stimuli, including neurotransmitters, hormones, chemokines, proteinases, inflammatory mediators, and peptides, as well as photons, odorant and taste molecules [1–3]. More than 900 GPCRs are encoded by the human genome, and are responsible for a multitude of physiological functions and disease states [4,5].

It is now well accepted that family C GPCRs, such as $GABA_B$ receptor and metabotropic glutamate (mGlu) receptors, require oligomeric assembly for receptor trafficking and receptor-dependent function [6]. Family A GPCRs, such as the β_2-adrenergic receptor [7], rhodopsin [8], and the μ-opioid receptor [9], on the contrary, are known to function efficiently as monomers at least when reconstituted into a phospholipid bilayer. Similarly, numerous mechanisms of intracellular crosstalk exist and several of those are independent of a direct protein-protein interaction between GPCRs [10–12]. Nevertheless, although this concept still remains a matter of intense speculation and debate [13,14], it is particularly intriguing the plausible ability of family A GPCRs to form and function as heterodimers/heteromers that exhibit distinct pharmacological, trafficking and functional properties as compared to their parent monomeric or homodimeric/homomeric GPCRs. Efforts have focused over the past two decades on the identification of GPCR complexes as well as on their signaling properties and stability—these findings were achieved mostly based on

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experimental approaches that included radioligand binding, co-immunoprecipitation, resonance energy transfer, and atomic force microscopy, along with functional testing in heterologous expression systems [15–18]. Similarly, more recent findings have provided evidence for the existence of GPCR heteromerization in native tissues and animal models [15], a concept that had long been considered heretic. This phenomenon not only brings forth a plethora of drug target combinations, but also gives an opportunity to carefully tweak the structure and function of one or more GPCRs involved in the complex, with the final goal of improving our therapeutic strategies.

Family A GPCR homomers as a functional unit in mammalian cells

Dimerization is essential for the plasma membrane expression of the family C receptor $GABA_B$, with γ-aminobutyric acid (GABA) as the major inhibitory neurotransmitter in the CNS [6,19]. This receptor is known to exist as a heterodimer of the $GABA_B$-R1 and the $GABA_B$-R2 subunits—association of which masks the endoplasmic reticulum-retention motif present at the C-terminus of $GABA_B$-R1 [20]. Another family C GPCRs, the metabotropic glutamate (mGlu) receptors, exist as covalently-linked obligatory and strict homodimers [6,19]. Thus, mGlu receptors possess a large N-terminus with a Venus flytrap domain that contains the orthosteric binding site; closure of which is necessary for agonist-mediated receptor activation. Homodimers of mGlu receptors are stabilized by a disulfide bridge at the level of the Venus flytrap domains. Studies have revealed that, for full activity, the closure of the Venus flytrap domains of both protomers in the homodimer is essential for mGlu receptor function [21]. In fact, the purified full-length monomeric mGlu2 receptor (with a mutation [C121A] to prevent potential covalent interaction between two protomers), when reconstituted into nanodiscs (self-assembled discoidal fragments of lipid bilayers stabilized in solution with two helical scaffold proteins) was unable to mediate glutamate-induced G protein activation [22]. This suggests that homodimerization forms the necessary core to induce mGlu receptor-dependent signaling events.

With regards to family A GPCRs, although in 1975 it had been shown a process of negatively cooperative interactions among the β_2-adrenergic receptor in frog erythrocyte membrane preparations [23], the demonstration of β_2-adrenergic receptor dimerization using bioluminescence resonance energy transfer (BRET) as a biophysical approach in living cells did not occur until 2000 [24]. Since then, an increasing amount of experimental evidence suggests that certain family A GPCRs exist and function as homomers in living cells (for review, see [15]). Among these, findings based on the use of mutant α_{1B}-adrenergic receptor constructs suggested that effective homomerization is required for receptor function in HEK293 cells [25]. Similarly, using an engineered experimental system to test the quaternary structure and function of the dopamine D_2 receptor in Flp-In T-REx 293 cells led to the demonstration that the minimal signaling unit is formed by two receptors and a single G protein [26]. Testing total internal reflection fluorescence imaging of single molecules in living CHO-K1 cells led to the suggestion that approximately one-third of the muscarinic M_1 receptor molecules exist as strict dimers [27]. Using a fluorescence recovery after photobleaching approach, it was proposed that β_1-adrenergic receptors show transient molecular interactions, whereas β_2-adrenergic receptors are able to form stable homomers [28]. This approach was validated using CD86 and CD28 as monomeric and dimeric internal

controls, respectively [28]. More recently, the use of real-time visualization with confocal fluorescence resonance energy transfer (FRET) approaches suggested that homodimerization of the 5-HT_{2C} receptor is required for processes of biogenesis in the endoplasmic reticulum in living HEK293 cells [29].

Along with evidence provided by a variety of crystal structures of family A GPCRs, such as β_1-adrenergic, [30], κ-opioid, and CXCR4 [31] receptors, together, these findings suggest that homodimers/homomers of family A GPCRs play a fundamental role in processes related to protein maturation, G protein coupling and receptor trafficking. This is further supported by recent findings suggesting that three mutations associated with retinitis pigmentosa (a blinding disease) disrupt the ability of rhodopsin to form GPCR homomers via TM1 and TM5 [32].

Nevertheless, this information obtained in heterologous expression systems must be interpreted with caution due to the likelihood of artifacts and false positives as well as false negatives arising from differences in the absolute and relative expression ratios as compared to those observed in native tissue [33–38]. Further work is also needed to integrate our knowledge related to the role of intra-membrane receptor-receptor interactions in GPCR-dependent signaling with recent advances focused on receptor-G protein coupling [39–41], as well as the biophysical rules that dictate the stoichiometry of the protein complexes (i.e., strict dimers versus higher order oligomers). Among these, membrane lipids such as cholesterol have been recently implicated in the modulation of GPCR oligomerization [42].

Family A GPCR heteromers and their role in receptor function

Since the evidence in 1991 showing that the pharmacological properties of the $G_{i/o}$ protein-coupled dopamine D_2 receptor antagonist [^{3}H]raclopride were affected by the G_s protein-coupled adenosine A_{2A} receptor agonist CGS-21680 in rat striatal membranes [43], and the demonstration in 1993 that co-expression of two functionally inactive chimeric constructs of the $G_{i/o}$ protein-coupled α_2-adrenergic receptor and the $G_{q/11}$ protein-coupled muscarinic M_3 receptor rescues regulation of phosphatidylinositol (PI) hydrolysis by the muscarinic receptor agonist carbachol in COS-7 cells [44], there has been a large number of studies suggesting putative family A GPCR heteromers—most of these studies were conducted in heterologous expression systems (for review, see [15–17,44]). Only a few, however, have been able to put forward the idea that GPCR heteromeric assemblies present a functional relevance in vivo in whole animal models [45,46] (Table 1). Here we summarize some of the advances made in understanding the physiological roles of family A GPCR heteromerization.

Heterocomplexes with the dopamine D_2 receptor

Generally, GPCRs in a complex seem to be influenced by their heteromeric partner in terms of the signaling pathway chosen. As an example, it has been suggested that the apo-ghrelin GHSR1a receptor and the dopamine D_2 receptor form heteromers; with the GHSR1a receptor modulating allosterically the signaling properties of the dopamine D_2 receptor, such that it switches its signaling pathway from activating $G_{i/o}$ proteins and inhibiting the production of cAMP to the intracellular mobilization of Ca^{2+} [58]. Immunofluorescence

studies performed on mouse brain slices showed that the two receptors co-localized the most in the hypothalamus—close proximity ex vivo was demonstrated by time-resolved fluorescence energy transfer assays in membrane preparations from rat hypothalamus. This particular brain region is involved in the regulation of feeding behavior, and D_2 receptor agonists such as cabergoline are known to induce anorexia as a secondary effect, a phenotype that was not observed in D_2 receptor knockout mice [58]. Antagonists selective for the GSHR1a receptor blocked the anorexia-related behaviors elicited by cabergoline, thus indicating therapeutic advantage of selective GHSR1a ligands that can specifically target D_2 receptors within the GSHR1a-D_2 heteromer in the hypothalamus for the treatment of eating disorders, such as anorexia or obesity.

Another example is the extensively studied dopamine D_2-adenosine A_{2A} receptor heteromer, which has been implicated in Parkinson's disease, schizophrenia and drug abuse [65]. This GPCR heteromer was first described based on allosteric interactions between dopamine D_2 and adenosine A_{2A} receptors in rat striatal membranes (see above). Expression of dopamine D_2 and adenosine A_{2A} as a GPCR heteromer in the main type of neurons in the striatum (GABAergic striato-pallidal neuron) was demonstrated using experimental approaches such as proximity ligation assay (PLA) [48,49]. It has been suggested that the allosteric crosstalk between the components of the A_{2A}-D_2 receptor heteromer is involved in the depression-related behavioral effects induced by adenosine A_{2A} receptor agonists as well as the psychostimulant effects of adenosine A_{2A} receptor antagonists. Structurally, A_{2A} and D_2 receptors are assembled as GPCR heterotetramers: heteromers of homodimers. Using the ability of peptides with the amino acid sequence of transmembrane domains to disrupt A_{2A}-D_2 receptor heteromerization in tissue sections from sheep striatum, it was demonstrated that either the TM5 peptide from the D_2 receptor or the TM5 peptide from the A_{2A} receptor are able to reduce close molecular proximity between A_{2A} and D_2 receptors [48]. The combination of mass spectrometry and pull-down techniques demonstrated that heteromerization between A_{2A} and D_2 receptors also depends on an electrostatic interaction between an arginine-rich epitope on the N-terminal segment ($RRRKR_{217\text{-}222}$) of the D_2 receptor intracellular loop 3 and two adjacent Asp residues ($DD_{401–402}$) or a phosphorylated Ser (S_{374}) residue of the C-terminus of the A_{2A} receptor [66]. These findings related to the structure and function of the A_{2A}-D_2 receptor heteromer are expected to open new ways towards effective treatment for devastating brain disorders.

Heterocomplexes with opioid receptors

A major limitation of morphine and associated opioid analgesics is the development of tolerance, and proinflammatory cytokines and glutamate could be contributing factors [67,68]. The μ-opioid receptor and the mGlu5 receptors are widely distributed in the CNS, and have been shown to co-localize in the spinal cord [69]. There is also an allosteric crosstalk between μ-opioid and mGlu5 receptors that suggests GPCR heteromerization [70]. Co-administration of morphine and the mGlu5 receptor antagonist m-methoxy-2-methyl-6-(phenylethynyl) pyridine (MPEP) leads to enhanced antinociception and fewer morphine-related side effects [56]. Notably, recent findings based on the development bivalent ligands having pharmacophoric elements of the μ-opioid receptor agonist oxymorphone and MPEP, joined by spacers of varying lengths, suggest that the heteromer could be a target for the

treatment of chronic pain. One of their bivalent compounds MMG-22 (22 atom spacer) has proven to be a prime candidate for the treatment of chronic pain [56].

Within this context, selective ligands that have enhanced affinity at, or specifically bind to, GPCR heteromers instead of homomers/monomers can provide tremendous information about function of the receptor complex, as well as a pharmacological tool to target specifically GPCR heteromers [71]. Subtypes of the δ and κ opioid receptors form GPCR heteromers [72] and co-localize in CNS regions such as spinal cord neurons [73]. Competition binding studies in HEK293 cells demonstrate that KDN21, a bivalent ligand having pharmacophores of the δ and κ receptors, possessed almost 50-fold higher affinity in cells co-expressing δ and κreceptors, as compared to cells singly expressing either δ or κreceptors and then mixed [74]. Additionally, the opioid agonist ligand 6'-guanidinonaltrindole (6'-GNTI) specifically activates the δ and κreceptor heteromer, but not homomers, in the spinal cord [55]. This not only provides strong evidence in favor of the formation of the receptor heteromer, but also shows that this could be a novel target for analgesic drugs, circumventing many of the side effects of morphine and associated drugs [75].

Another step in this direction was the generation of antibodies selective for the μ-δ opioid receptor heteromer [53]. The same group identified a biased agonist CYYM51010, which selectively targeted the heteromer and had antinociceptive properties but with a lower tendency to cause tolerance and side effects compared to morphine [54]. These studies characterized the heteromer and suggested up-regulation of the receptor complex in response to chronic morphine treatment, which might be instrumental in developing tolerance to morphine in the first place [53,54].

The use of opioid analgesics is compelling in chronic pain, especially in terminally ill patients. However, this is more often than not accompanied with the activation of the itch response [76]. Interestingly, a splice variant of the μ-opioid receptor (MOR1D) forms GPCR heteromers with the gastrin releasing peptide (GRP) receptor (GRPR) in the dorsal horn. Morphine leads to internalization of both GRPR and MOR1D, whereas GRP induces only internalization of GRPR and morphine-independent scratching. In addition, using peptides corresponding to the C-terminal of MOR1D, it was demonstrated that blocking heteromeric formation between GRPR and MOR1D attenuates morphine-induced scratching but not morphine-induced analgesia [64].

Mechanisms of crosstalk between 5-HT_{2A} and mGlu2 receptors

Serotonin and glutamate are neurotransmitter systems involved in neuropsychiatric disorders such as schizophrenia and depression. The serotonin 5-HT_{2A} receptor is a target of most of the second generation (or atypical) antipsychotic drugs, such as clozapine and risperidone [77]. Activation of the 5-HT_{2A} receptor by psychedelic drugs, such as lysergic acid diethylamide (LSD) or psilocybin, induces in healthy volunteers behavioral changes that present certain similarities with those observed in schizophrenia patients [78]. Comparably, activation of the mGlu2 receptor by either orthosteric mGlu2/3 receptor agonists or positive allosteric modulators of the mGlu2 receptor induces antipsychotic-related phenotypes in preclinical models of psychosis.

A significant amount of work has shown that the 5-HT_{2A} receptor and the metabotropic glutamate 2 (mGlu2) receptor crosstalk in brain regions involved in cognition and sensorimotor gating, such as the frontal cortex. One of the first studies that raised interest in the possibility that 5-HT_{2A} and mGlu2 receptors could crosstalk was the demonstration using rat brain slices that activation of the 5-HT_{2A} receptor elicited excitatory post-synaptic currents (EPSCs) in layer V pyramidal neurons, and that this post-synaptic electrophysiological response was suppressed by addition of the mGlu2/3 receptor agonist LY354740 to the recording chamber [79]. One of the potential mechanisms used to explain this functional crosstalk was based on the modulation of glutamate release via activation of the mGlu2 receptor located pre-synaptically in the frontal cortex [79] (Figure 1A). An alternative, although not mutually exclusive, mechanism of crosstalk between 5-HT_{2A} and mGlu2 receptors is their assembly as a post-synaptic GPCR heteromer in cortical pyramidal neurons.

Accordingly, it was suggested that the mGlu2 receptor was expressed both pre- and post-synaptically in the frontal cortex [80,81], an hypothesis that has been validated recently [82]. Using neuroanatomical approaches such as fluorescent in situ hybridization (FISH) [36] and immunohistochemistry [83], it was demonstrated that 5-HT_{2A} and mGlu2 co-localized in layer V mouse cortical pyramidal neurons. These data, however, do not provide evidence as to whether these two particular receptor subtypes interact at the sub-cellular level. This was approached with the use of electron microscopy to explore the ultrastructural localization of 5-HT_{2A} and mGlu2 receptors in the frontal cortex [59]. These assays showed that labeling for both 5-HT_{2A} and mGlu2 receptors was observed in close sub-cellular proximity, particularly at or near synaptic junctions [59]. Using a sub-cellular fractionation approach to purify fractions enriched in presynaptic active zone (PAZ) and postsynaptic density (PSD) proteins, it has recently been confirmed that the 5-HT_{2A} receptor is detected mostly in the PSD, whereas the mGlu2 receptor is detected in both the PSD and the PAZ fractions [60]. In addition, 5-HT_{2A} and mGlu2 receptors can be co-immunoprecipitated from mouse [83] and human [36] frontal cortex plasma membrane preparations. Although these data do not exclude the possibility of 5-HT_{2A} receptor-dependent phenotypes that require expression of either mGlu2 [79] or 5-HT_{2A} receptors [84] in cortical presynaptic terminals, together, they suggest that 5-HT_{2A} and mGlu2 form part of a protein complex at the PSD in mouse frontal cortex.

Another potential mechanisms of crosstalk between 5-HT_{2A} and mGlu2 receptors include genetic and epigenetic factors. Thus, at a genetic level, it was initially suggested and validated most recently that schizophrenia patients homozygous (T/T) at single nucleotide polymorphism rs7330461 in the 5-HT_{2A} (Htr2a) gene showed a greater improvement during treatment with pomaglumetad (LY2140023; pro-drug of the mGlu2/3 receptor agonist LY404039) as compared to A/A homozygous patients [85,86]. This is the first pharmacogenetic finding that identifies and replicates in multiple and independent studies a genetic marker that predicts efficacy for the treatment of schizophrenia (Figure 1B).

Long-lasting treatment with atypical antipsychotic drugs has also been shown to affect the therapeutic effects induced by pomaglumetad. Using mouse models of chronic antipsychotic drug treatment, it was suggested that atypical antipsychotic drugs, such as clozapine and

risperidone, but not typical antipsychotic dugs, such as haloperidol, induced repressive covalent histone modifications at the promoter region of the mGlu2 (Grm2) gene in the frontal in the frontal cortex [87]. These repressive histone modifications induced by chronic atypical antipsychotic drug treatment included decreased acetylation of histone H3, decreased acetylation of histone H4, and increased methylation of lysine 27 (H3K27me3), and correlated with transcriptional repression of the mGlu2 gene [87]. Although additional work will be necessary to fully characterize the basic signaling mechanisms underlying this epigenetic crosstalk, it was also demonstrated that the repressive epigenetic changes induced at the mGlu2 promoter in mouse frontal cortex required 5-HT_{2A} receptor-signaling, as they were absent in the frontal cortex of 5-HT_{2A} knockout mice [87] (Figure 1C). Notably, the translational significance of these preclinical findings has been validated recently: schizophrenia patients previously treated with haloperidol responded clinically to pomaglumetad, whereas the effects of pomaglumetad in patients previously treated with atypical antipsychotic medications did not differ from placebo [88]. Considering that there were also repressive histone modifications at the promoter region of the mGlu2 gene in the frontal cortex of 5-HT_{2A} knockout mice as compared to wild-type littermates, together, these data suggest that 5-HT_{2A} receptor-dependent signaling epigenetically affects mGlu2 transcription.

Based on all these findings, it can be concluded that 5-HT_{2A} and mGlu2 receptors crosstalk at multiple neurobiological levels that include mGlu2-mediated action on cortical presynaptic sites to decrease glutamate release, the involvement of 5-HT_{2A} genetic variants in the treatment response to mGlu2/3 receptor agonists as antipsychotic medications, 5-HT_{2A} receptor-dependent repressive histone modifications at the mGlu2 promoter in frontal cortex regions, and allosteric crosstalk through the 5-HT_{2A}-mGlu2 receptor heteromer.

The role of heteromerization as an event involved in the mechanisms of crosstalk between the $G_{q/11}$-coupled 5-HT_{2A} receptor and the $G_{i/o}$-coupled mGlu2 receptor has been studied extensively over the past few years [16]. We [36,59,60] and others [12] have confirmed in HEK293 cells that at least a fraction of the populations of mGlu2 and 5-HT_{2A} receptors constitute part of the same protein complex as defined by co-immunoprecipitation assays (Figure 1D). It has also been demonstrated that these two receptors are expressed in close molecular proximity as defined independently by biophysical assays such as BRET [36], microscope-based FRET [36], flow cytometry-based-FRET [59,60], antibody-based time-resolved FRET [12], and combination of time-resolved FRET and the SNAP-tag approach [33]. Using wild-type 5-HT_{2C} and wild-type mGlu3 receptors as well as mGlu2/mGlu3 chimeric constructs that form or do not form the 5-HT_{2A}-mGlu2 heteromeric receptor complex, previous results suggest the existence of a crosstalk mechanism between these two receptors that requires their physical association [36,59,60,83].

Further work is needed to define the biophysical and cellular trafficking processes that modulate heteromeric assembly of 5-HT_{2A} and mGlu2 receptors. Thus, according to this concept, it has been shown that the presence or absence of crosstalk between the components of the 5-HT_{2A}-mGlu2 heteromer profoundly depends upon the appropriate ratios of the two protomers [60,89]. Nevertheless, the use of different experimental approaches, such as stable expression together with inducible expression versus transient

transfection of the appropriate expression vectors all within the same groups of assays that included measure of close molecular proximity and functional assays such as [Ca^{2+}] mobilization, may provide an explanation for the absence of functional and pharmacological differences previously observed between cells expressing 5-HT_{2A} alone, mGlu2 alone, or 5-HT_{2A} and mGlu2 together [33].

Conclusions

GPCRs in a heteromer are thought to moonlight, which in the context of the concept introduced by Constance Jeffery refers to one protein having multiple unrelated functions [90]. GPCRs as part of a complex can switch between different G proteins affecting an array of downstream effectors, each having a multitude of effects of their own. In terms of the CNS, this has fundamental implications as receptors defining the very basis of our existence interact with each other with different permutations and combinations. This phenomenon has already shown the potential for developing selective pharmacological interventions and preventing several limiting side effects. As more GPCR crystal structures are identified, we glean crucial physiological information, and although we have made exceptional progress in the identification and characterization of GPCR complexes, we may have only grazed the surface of what might be an unimaginable reserve of protein-protein interactions that can be tapped to our therapeutic advantage.

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Highlights

- Family A GPCRs can function as monomers but they also form higher order complexes
- These complexes are involved in almost every step of the GPCR life cycle
- They are also involved numerous neuropsychiatric conditions
- GPCR heteromers represent novel targets for selective pharmacological treatments

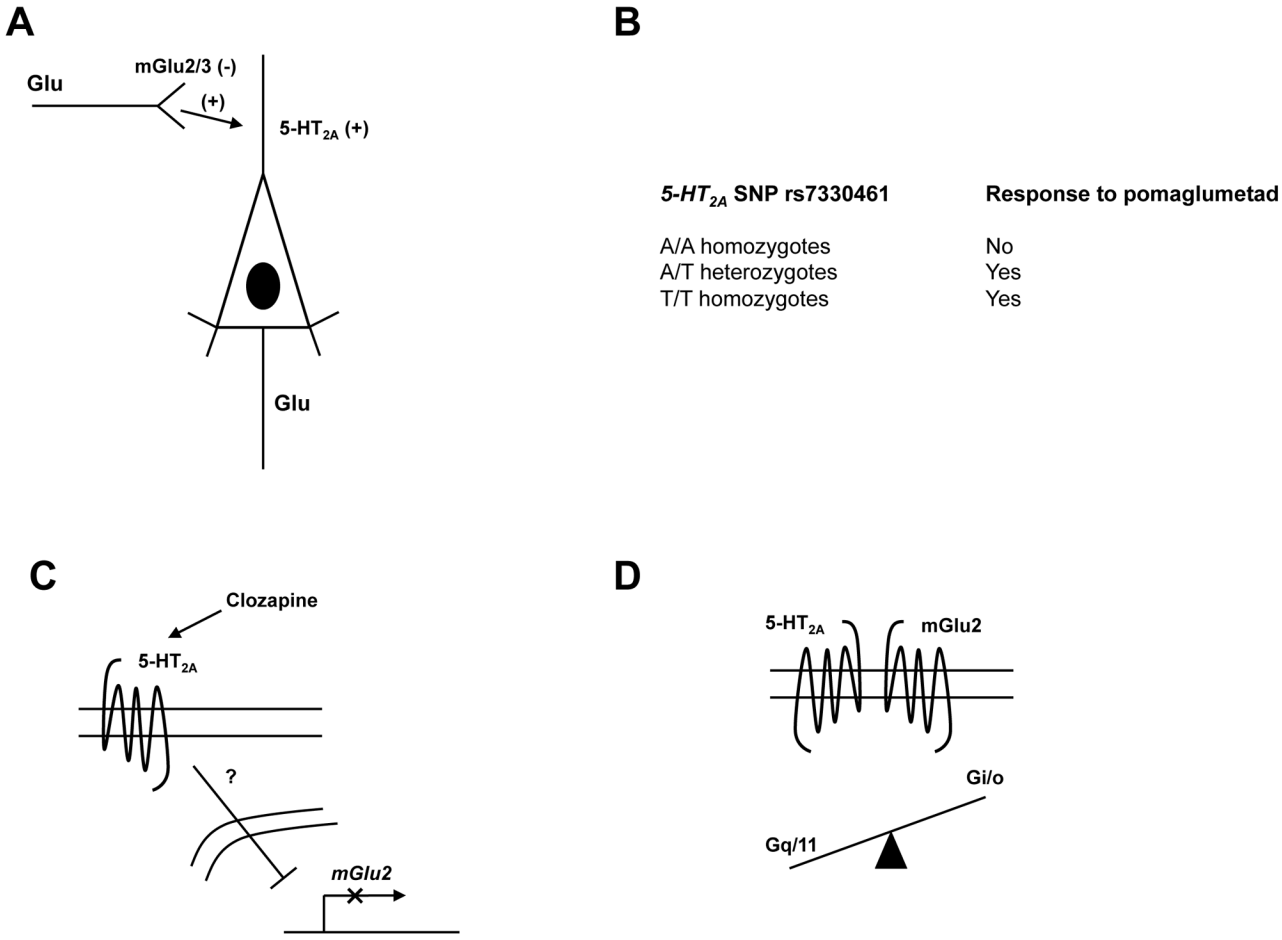

Figure 1.

Schematic diagram of the crosstalk mechanisms between 5-HT_{2A} and mGlu2 receptors. **A,** Activation of the 5-HT_{2A} receptor produces an increase of glutamate release in the frontal cortex. It has been suggested that activation of pre-synaptic mGlu2/3 receptors located at glutamatergic terminals modulates negatively this 5-HT_{2A} receptor-dependent glutamate release. **B,** Schizophrenia patients carrying the T/T genotype at the *5-HT_{2A}* receptor SNP rs7330461 showed significantly greater improvement in positive and negative syndrome scale (PANSS) total scores during treatment with the mGlu2/3 receptor agonist pomaglumetad compared to A/A homozygous patients. **C,** Chronic treatment with atypical antipsychotic drugs, such as clozapine, induces repressive histone modifications at the promoter region of the mGlu2 gene via 5-HT_{2A} receptor-dependent signaling. **D,** Gq/11-coupled 5-HT2A and Gi/o-coupled mGlu2 receptors form a specific GPCR heteromeric complex though which serotonin and glutamate ligands modulate the pattern of G protein coupling in a way that predicts their psychoactive behavioral effects.

Table 1

GPCR complexes in the CNS and their therapeutic implications

Receptor complex	Implications	References
A_1-A_{2A}	drug tolerance	[47]
A_1-D_1	PD	[47]
A_{2A}-D_2	schizophrenia, drug addiction	[48,49]
A_1-$mGlu_1$	schizophrenia	[47]
A_{2A}-$mGlu_5$	PD	[50]
A_{2A}-D_2-$mGlu_5$	schizophrenia, PD	[50]
CB_1-δ	chronic pain	[51]
CB_1-D_2	addiction	[52]
μ-δ	chronic pain	[53,54]
δ-κ	chronic pain	[55]
μ-$mGlu_5$	chronic pain	[56]
D_2-5-HT_{2A}	schizophrenia, hallucination	[57]
Apo-GSHR1a-D_2	anorexia/antiobesity	[58]
5-HT_{2A}-$mGlu_2$	schizophrenia	[36,59,60]
CXCR4-$α_{1A/B}$	blood pressure	[61]
D_1-D_2	depression	[62]
MT_1-MT_2	retinal function	[63]
GRPR-MOR1D	itch	[64]

PD: Parkinson’s disease