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Review

Potential role of G protein-coupled receptor (GPCR) heterodimerization in neuropsychiatric disorders: A focus on depression

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Abstract:
G protein-coupled receptors (GPCRs) represent the largest family of membrane proteins in the human genome and are the target of approximately half of all therapeutic drugs. For many years, GPCRs were thought to exist and function as monomeric units. However, during the past two decades, substantial biochemical, structural and functional evidence have indicated that GPCRs can associate and form heteromers that exhibit functional properties distinct from the corresponding monomers. The understanding of the unique pharmacological and functional properties of such heteromers is a major challenge for neuroscience, particularly given the abundant evidence suggesting that GPCR heteromers may play a crucial role in neuropsychiatric disorders. Herein, we present current data on the role of GPCR heterodimerization in neuropsychiatric disorders, with a focus on its potential implications in depression. The presented examples of pairs of receptors, with their specific pharmacological and functional properties, are likely to lead to novel effective strategies in antidepressant drug development. The currently available techniques for studying GPCR heterodimerization, both *in vitro* as well as *in situ* in native tissue, are also described.

Key words:
heterodimerization, G protein-coupled receptors, neuropsychiatric disorders, depression

Introduction

The neurobiology of mood disorders has been traditionally focused on the monoamine neurotransmitters serotonin and noradrenaline. Based on the analysis of the primary effect of antidepressant action, which is the inhibition of serotonin and/or noradrenaline reuptake, it has been thought that the reduction of synaptic levels of these aminergic neurotransmitters might be a main cause of depression. Unfortunately, the antidepressant drugs currently used in clinical practice are ineffective in many patients. One-third of patients are treatment resistant and do not respond to available antidepressant therapies. Since the discovery of the current serotonin and noradrenergic antidepressant medications, there has been limited progress in the development of new antidepressant treatments.

The new concept that the heterodimerization of receptors is important for the action of aminergic neurotransmitters, i.e., serotonin, noradrenaline and dopamine, among others, opens up a new approach to un-

derstanding the mechanisms of neurotransmission, as well as new strategies in drug development because G protein-coupled receptors (GPCRs) heterodimerization is regarded as a widespread phenomenon that regulates receptors functions. Heteromers can form only when the given receptors are expressed in the same cell, which is not always the case; therefore, heteromers can display unique pharmacological and functional properties. Heteromers of GPCRs may become a new target in drug development strategies.

Heterodimerization of G protein-coupled receptors

G protein-coupled receptors (GPCRs) represent the largest family of cell membrane proteins. They participate in the regulation of major physiological processes and, therefore, represent a key target for current therapeutic drugs on the market. For many years, GPCRs were thought to exist and function as monomeric units. However, recently, substantial biochemical, structural and functional evidence has indicated that GPCRs can associate and form dimers or even higher order oligomers. This interaction appears to be a fundamental component of the regulation and function of GPCRs. The fact that this process has been reported for such a variety of receptors suggests that dimerization is a general phenomenon for this receptor superfamily. Dimerization can occur between the same receptor types (homodimerization) or between different receptors (heterodimerization) from the same or distinct GPCR classes. Heterodimerization is a specific phenomenon that is restricted to certain subtypes of GPCRs; some isoforms of GPCRs do not possess the ability to interact. For example, the somatostatin receptor SST_5 is able to heterodimerize with $SSTR_1$ but not $SSTR_4$ [24].

GPCRs heterodimerization has several important implications. It appears to be essential for the correct action of GPCRs but also results in the diversification of GPCR functioning in terms of ligand binding, signaling and trafficking. Therefore, heteromers may have functional properties that are distinct from those of the corresponding monomers.

The direct evidence that GPCR heterodimerization is critical for receptor activation and normal functionality came from studies with the metabotropic γ-aminobutyric acid $(GABA)_B$ receptor. Each partner in the heterodimer, made up of $GABA_{B1}$ and $GABA_{B2}$ receptors, is non-functional when expressed alone. It has been shown that only the heterodimerization of both – the $GABA_{B1}$ and $GABA_{B2}$ – receptors results in the expression of functional $GABA_B$ receptors at the cell surface [8].

The heterodimerization of two fully functional receptors was shown for the first time for the opioid receptors κ and δ [9]. The interaction of these two receptors results in the formation of a novel κ-δ heteromer that exhibits ligand binding and functional properties that are distinct from those of either receptor alone. The heterodimers have greatly reduced affinities for their selective ligands and synergistically potentiate signal transduction.

In contrast, heterodimerization has also been shown to inhibit or inactivate receptor function. The interaction between the somatostatin SST_{2A} and SST_3 receptors causes $SSTR_{2A}$-mediated inactivation of the fully functional SST_3 receptor [20].

Two receptors can also form a completely novel signaling complex. It has been shown that the D_1-D_2 heteromer is distinct from its constituent receptors. Agonist stimulation of co-expressed D_1 and D_2 receptors results in an increase in the intracellular calcium levels *via* a G_q-signaling pathway, which is not activated by either receptor alone or when only one of the co-expressed receptors is activated by a selective agonist [11].

Furthermore, heterodimerization occurs between more distantly related GPCRs. The first clear example of this phenomenon was the report that the somatostatin SST_5 receptor can heterodimerize with the dopamine D_2 receptor upon ligand stimulation [23]. The SST_5-D_2 heteromer is functionally and pharmacologically different from the SST_5 and D_2 receptors. The binding affinity of somatostatin is increased by binding of the D_2R agonist and decreased by binding of the D_2R antagonist, which also reflects in the alterations of signaling. As the physiological interaction of dopamine and somatostatin systems is well documented, heterodimerization of these receptors remains of particular interest and will be described below.

Although the concept of receptor dimerization for GPCRs is now well established, the formation, regulation and role of heteromers in receptor signaling and trafficking is still not well understood. Understanding this phenomenon is crucial because heterodimer formation considerably expands the repertoire of pharmacological units that are a potential new drug target with improved selectivity.

GPCR heterodimers in central nervous system disorders

There is evidence that GPCR heteromers may play a crucial role in psychiatric disorders. Recent data suggest that serotonin 5-HT_{2A} and glutamate $mGlu_2$ receptor heteromers might be a promising new target for the treatment of psychoses. Gonzales-Maeso and coworkers [6] have shown that the 5-HT_{2A}-$mGlu_2$ heteromer might be involved in altered cortical function in patients with schizophrenia. Their studies showed that the 5-HT_{2A}-$mGlu_2$ complex may integrate serotonin and glutamate signaling and modulate G protein coupling. Radioligand binding assays revealed that drugs that activate $mGlu_2$ receptors increase the affinity of hallucinogenic drugs for the 5-HT_{2A} receptor and selective 5-HT_{2A} receptor agonists decrease the affinity of the $mGlu_2$ receptor agonist. Moreover, the 5-HT_{2A}-$mGlu_2$ heteromer triggers unique cellular responses when targeted by hallucinogenic drugs. The formation of the 5-HT_{2A}-$mGlu_2$ complex enhances the G_i-dependent signaling pathway, which is necessary for the effects of hallucinogens. Furthermore, the activation of the $mGlu_2$ receptor abolishes hallucinogen-specific signaling and behavioral responses. Interestingly, the receptor densities in cortical membranes from untreated schizophrenic subjects were significantly altered, showing an increased expression level of 5-HT_{2A} receptors and a decreased level of $mGlu_2$ receptors. It was proposed that the dysregulation in expression levels of these receptors could predispose an individual to schizophrenia [6].

Another example of the significance of GPCR heterodimerization in psychiatric disorders is the upregulation of the D_1–D_2 heteromer sensitivity and functional activity, which has been reported in the globus pallidus of schizophrenic patients and in the striatum of rats treated chronically with amphetamine. These findings indicate that the dopamine D_1–D_2 heteromer may contribute to schizophrenia [19]. Abnormal regulation of calcium signaling mediated by a D_1–D_2 heteromer may constitute the central dysfunction that is responsible for generating the psychopathology of schizophrenia.

Another GPCR interaction that has implications for the treatment of brain disease is the adenosine A_{2A}-dopamine D_2 receptor heteromer. Adenosine A_{2A} and dopamine D_2 receptors are colocalized in GABAergic striatopallidal neurons and form heteromeric complexes. Numerous studies have suggested that the A_{2A}–D_2 heteromer in the central nervous system may provide a new therapeutic target for treating Parkinson's disease [5]. Antagonism of A_{2A} receptors increases the affinity of the D_2 receptor for agonists, including dopamine. Therefore, heterodimerization of adenosine A_{2A} and dopamine D_2 receptors has been proposed as a potential target for improving the side effects induced by L-DOPA treatment in Parkinson's disease. Behavioral studies have shown that selective A_{2A} antagonists inhibit the motor activating effects induced by D_2 agonists, while A_{2A} antagonists enhance the same effects and promote motor inhibition. This enhancement of dopamine D_2R transmission by adenosine $A_{2A}R$ antagonists helps to reverse the motor impairment observed in Parkinson's disease. These observations confirm a close and functional association between A_{2A} and D_2 receptors and clearly show that A_{2A} antagonists have antiparkinsonian activity. Selective adenosine A_{2A} receptor antagonists are currently under investigation in clinical trials for the treatment of Parkinson's disease.

Heterodimerization in depression

Currently, there is no good model to explain the molecular basis of depression or the mechanism by which the diversity of antidepressants work. Current antidepressant drugs, which act by enhancing monoaminergic transmission, have limited therapeutic efficacy in approximately 30% of patients and often cause unwanted side effects. A new strategy may be to search for specific pathways involved in disease pathophysiology that are mediated by receptor-receptor interactions rather than the receptors themselves. The development of molecules that will be able to enhance or block specific receptor pairs may potentially improve the treatment of depression.

Herein, we focus on the potential role of GPCR heterodimerization in depression. The presented examples of heteromers, with their unique pharmacological and functional properties, are likely to lead to novel effective strategies in the development of antidepressant drugs.

Gal-5-HT_{1A} receptor heteromers

Galanin is a neuropeptide that is coexpressed with serotonin and is implicated in the modulation of its release. The coexistence of galanin and serotonin has

been found in the dorsal raphe cell bodies. Intraventricular injections of galanin reduced serotonin metabolism in the ventral limbic cortex, the hippocampal formation and the fronto-parietal cortex, potentially *via* the direct inhibitory actions on dorsal raphe serotonin cells that reduces their firing in the ascending pathways. Galanin has been shown to decrease the affinity of postsynaptic 5-HT_{1A} agonist binding in the limbic system; this indicates that galanin can also contribute to the development of depression because it has been shown that postsynaptic 5-HT_{1A} receptor activation elevates mood. The results concerning GalR modulation of postsynaptic 5-HT_{1A} recognition and signaling provided the first indication that brain Gal-5-HT_{1A} receptor heteromers may exist. The antagonistic Gal-5-HT_{1A} receptor interactions in putative receptor heteromers represent a novel integrative mechanism in serotonergic neurotransmission. The role of galanin in the modulation of serotonergic neurotransmission became more complex, however, with the observations that galanin can also increase potassium-induced serotonin release. This was the first indication that there may be galanin receptors that increase serotonin neurotransmission and thus may be beneficial for the treatment of depression. Other studies have shown that the stimulation of galanin $GalR_1$ and $GalR_3$ results in depression-like behavior, whereas the activation of $GalR_2$ results in antidepressant-like effects. In view of these findings, Gal-5-HT_{1A} receptor heteromers may represent a novel target for antidepressant drugs, as $GalR_1$ and $GalR_3$ antagonists may act as antidepressants, especially in combination with serotonin uptake inhibitors [10].

D_1-D_2receptor heteromers

Although serotonin and noradrenaline are thought to be the key neurotransmitters involved in the pathophysiology of depression, the disturbances in the dopaminergic system can also play an important role in the etiology of mood disorders. Patients suffering from depression often exhibit reduced motivation, anhedonia and lowered motor activity, all of which are linked to dopamine. Thus, targeting the dopamine system is of importance when considering potential mechanisms and novel treatments for depressive disorders.

Recently, Pei and co-workers reported the first direct evidence implicating dopamine D_1 and D_2 receptor heterodimers in depression [18]. Using a co-immunoprecipitation assay in *post-mortem* brains, they have shown elevated levels of D_1–D_2 heteromers in the striatum of patients suffering from major depression. They have also designed a peptide that disrupts the D_1–D_2 interaction but does not perturb the normal functioning of each individual receptor. Administration of this interfering peptide into the prefrontal cortex of rats resulted in a significant reduction of immobility time in the forced swim test. This antidepressant-like behavior was correlated with decreased levels of D_1–D_2 complexes. The potential antidepressant effects of this disrupting peptide in a learned helplessness paradigm have also been tested. Rats that had developed learned helplessness showed increased levels of D_1–D_2 heteromers. Disruption of D_1–D_2 complexes by the interfering peptide resulted in a decreased escape rate without affecting locomotor activity. These results provide interesting evidence not only on the role of receptor heterodimerization in the mechanism of action of antidepressant drugs but also the role of dopaminergic neurotransmission in depression.

D_2-SST receptor heteromers

The interaction between dopaminergic and somatostatinergic systems has been suggested for many years based on anatomical, behavioral and biochemical studies. Dopamine and somatostatin have also been implicated in the pathophysiology of depression considering their potential role in mood regulation. Reduced levels of somatostatin and dopamine metabolites were shown in the cerebrospinal fluid of depressive patients [14]. Intracerebroventricular administration of somatostatin results in antidepressant-like effects in the forced swim test in rats [4]. It also appears that antidepressants interact with the dopamine and somatostatin pathways. Chronic desipramine treatment selectively potentiates somatostatin-induced dopamine release in the nucleus accumbens and the striatum in rats [16]. It has also been shown that the chronic administration of antidepressants influences somatostatin levels and somatostatin and dopamine receptor density in rat brains [3, 17]. Imipramine up-regulates somatostatin release in the mouse hypothalamus and elicits antidepressant-like effects in the tail suspension test [15]. More recent studies demonstrate transmitter switching between dopamine and somatostatin in neurons induced by exposure to short- and long-day photoperiods [2]. The molecular basis of this functional interaction between the somatostatinergic and dopaminergic systems may stem from the

interaction of somatostatin and dopamine receptors, as they were shown to undergo ligand-dependent heterodimerization [23].

Recent studies reported by Szafran and co-workers indicate that antidepressant drugs promote heterodimerization of dopamine D_2 and somatostatin SST_5 receptors despite the lack of affinity of these drugs for the dopamine or somatostatin receptors [25]. These results may be considered as an evidence for a potential molecular mechanism underlying the antidepressant-like effects of somatostatin, which influences dopamine-mediated behavioral responses.

$5HT_{1A}$-$5HT_7$ receptor heteromers

Serotonin 5-HT_{1A} and 5-HT_7 receptors are highly co-expressed in brain regions implicated in depression. It has been reported that heterodimerization decreases the 5-HT_{1A} receptor-mediated activation of G_i proteins without affecting 5-HT_7 receptor mediated G_s protein activation. 5-HT_{1A}-5HT_7 heteromers also show elevated internalization upon ligand stimulation. The 5-HT_{1A} receptor is expressed both as a presynaptic autoreceptor in serotonergic neurons of the raphe nuclei and a postsynaptic receptor in multiple brain regions including the hippocampus and cortex. Analysis of the regional brain distribution of the 5-HT_7 receptor has revealed that this receptor is highly enriched in serotonergic neurons of the dorsal raphe nuclei. Higher levels of 5-HT_{1A}-5-HT_7 heteromers present in presynaptic neurons compared to postsynaptic neurons may represent a mechanism responsible for the differential desensitization observed for the 5-HT_{1A} receptors. It has been reported that chronic receptor stimulation results in functional desensitization of only 5-HT_{1A} autoreceptors without affecting postsynaptic 5-HT_{1A}R. Because 5-HT_{1A} receptors expressed alone are resistant to agonist-mediated internalization, serotonin released in the hippocampus or cortex will not reduce the amount of postsynaptic 5-HT_{1A} receptors at the cell surface. Thus, the balanced ratio of heterodimerization on pre- and postsynaptic neurons may be involved in the response to the treatment of depression [22].

CRH_1-V_{1b}receptor heteromers

Other GPCR receptor interactions have been recently associated with depression and anxiety disorders. Vasopressin V_{1b} receptor and corticotrophin releasing hormone receptor type 1 ($CRHR_1$) are co-expressed mainly in corticotropes in the pituitary and were shown to form functional heterodimeric complexes. V_{1b}R and $CRHR_1$ heterodimerization mediates the synergistic biological actions of vasopressin and corticotrophin releasing hormone (CRH), which are the key regulators of the hypothalamic-pituitary-adrenal (HPA) axis. Vasopressin, *via* the V_{1b} receptor, stimulates ACTH secretion from the anterior pituitary corticotropes and potentiates the release of ACTH induced by CRH. A dysregulation of the HPA axis is the most common and consistently reported symptom of depression. Therefore, direct molecular interaction between V_{1b}R and $CRHR_1$ may play an important role in the pathogenesis of stress-related psychiatric conditions such as anxiety disorders and depression [27].

Identification of GPCR heterodimers *in vitro* and in native tissue

The development of techniques for studying the phenomenon of heterodimerization in native tissue is significant. Initial studies were performed *in vitro* in cell systems, but recent studies are shifting the focus to the *in vivo* relevance of heterodimerization. Because there is increasing biochemical and functional evidence supporting the existence of GPCR homo- and heterodimers, it is important to identify protein-protein interactions *in vivo* to understand their functional implications. Therefore, in 2007, the International Union of Basic and Clinical Pharmacology (IUPHAR) suggested criteria that should be used to define physiologically relevant GPCR dimmers [21]. At least two of the following criteria should be met:

– both subunits of the receptor heterocomplex must be coimmunolocalized within the same cellular and subcellular compartment;

– physical interaction between both subunits should be documented in native tissue. This can be achieved using a co-immunoprecipitation technique, antibodies selective for a specific receptor dimer, energy transfer technologies using labeled ligands, labeled antibodies or transgenic animals expressing physiological levels of recombinant fluorescent proteins that could be used to demonstrate close receptor proximity in native tissue;

– identification of unique functional properties (pharmacological, signal transduction or trafficking) of GPCR dimers;

– the response mediated by a unique dimeric receptor should be notably modified in the absence of either of the subunits; these results can be achieved by using knockout animals or RNA interference technology.

For the detection and visualization of GPCR dimerization, the use of biophysical and biochemical approaches is the first choice. Biophysical techniques are based on the Förster Resonance Energy Transfer (FRET) phenomenon. FRET is a process that relies on the distance-dependent transfer of energy between an excited donor molecule and an acceptor molecule in a non-radiative way. Due to its sensitivity to distance, FRET has been used to investigate interactions between proteins that are in close proximity (less than 10 nm); thus, FRET is an excellent method for measuring dimerization of receptors. FRET techniques, such as fluorescence and bioluminescence resonance energy transfer (FRET and BRET), are widely used to investigate GPCR heterodimerization in heterologous systems [12]. FRET is a microscopic technique based on the principle that a donor fluorophore (e.g., cyan fluorescent protein, CFP) can transfer energy to another fluorophore, the acceptor (e.g., yellow fluorescent protein, YFP), if the distance between them does not exceed 10 nm. When FRET occurs, the donor's fluorescence intensity reduces while the acceptor's fluorescence intensity increases. The emission spectrum of the donor must overlap the excitation spectrum of the acceptor. BRET (Bioluminescence Resonance Energy Transfer) is another method for studying protein-protein interactions. In the BRET technique, one receptor is fused to *Renilla reniformis* luciferase (RLuc), whereas the other receptor is fused to a fluorescent protein (e.g., YFP). If the two receptors are in close proximity, the energy generated by the enzymatic degradation of RLuc's substrate (e.g., coelenterazine) is transferred to YFP, causing it to emit its characteristic fluorescent wavelength, which can be detected. The advantage of this technique is that it does not require a conventional light source to initiate energy transfer because the luciferase is excited by the addition of its substrate; thus, it is suitable for light-sensitive proteins.

Biophysical techniques provide strong support for the existence of receptor heteromers in artificial cell systems but are difficult to perform in native tissues. Although working in experimental cell lines can generate initial possible models, final proof requires the localization of interacting heterocomplexes in their proper physiological environment. However, through the development of microscopy and high-resolution imaging techniques and by combining them with transgenic animal models, such as mice transfected with genetically expressed fluorophores, it is becoming possible to study GPCR heterodimerization *in situ* in tissue slides or even in living organisms [13].

Until recently, the most common biochemical method routinely used over the past several decades to study protein-protein interactions in native tissue was co-immunoprecipitation. However, it requires the homogenization of tissue and provides little information on how protein interactions occur at the individual cell level. Thus, it cannot demonstrate the direct interaction between two partners but only suggests that they are part of the same cellular complex.

Therefore, the quantitative analysis of receptor-receptor interactions *in situ* is crucial for understanding its function and regulation and, until recently, remained a major challenge. Promising new techniques have been developed where no modification of cells or tissues is required, allowing endogenous protein interactions to be studied *in situ.*

Fluorescently labeled ligands and antibodies

The presence of endogenous GPCRs can be detected in the tissue by a time-resolved fluorescent resonance energy transfer (TR-FRET) approach based on fluorescently labelled receptor-specific ligands [1]. Labelling the ligand rather than the receptor makes it possible to examine native untagged receptors in real tissues. This approach is based on the use of lanthanides as energy donors and compatible fluorescence acceptors that allow FRET measurements in a delayed manner to avoid naturally occurring cellular autofluorescence, which results in a higher sensitivity and signal-to-noise ratio. Additional information regarding the conformational state of the receptor can be provided because both agonists and antagonists can theoretically be labeled. Another useful technique to examine GPCR heterodimerization in the native environment may be labeling monoclonal antibodies that are highly selective to individual receptors with complementary TR-FRET donor-acceptor pairs.

In situ proximity ligation assay (PLA)

The *in situ* PLA is a novel method of protein-interaction detection and has been recently used to detect the presence of endogenous GPCR heteromers in

tissue. In simple terms, PLA detects protein-interactions through a pair of antibodies that bind to the proteins of interest in close proximity, giving rise to an amplifiable fluorescent detection signal.

In situ PLA has the potential to enable a more complete understanding of GPCR receptor-receptor interactions and could be highly suited to investigate GPCR heteromers in tissue; thus, this technique may provide new insights into basic biological mechanisms, heteroreceptor levels and their locations, e.g., in the brain. *In situ* PLA permits the analysis of interactions among any receptors for which suitable antibodies are available without using genetic constructs; it can be performed in all samples of cells and tissues. This method is suitable to investigate human specimens that may be used to investigate patterns of changes in heteromers in physiological and pathophysiological conditions. It provides an enhanced sensitivity and selectivity compared with other methods. However, one has to be aware that, as with other antibody-based techniques, it shows that two proteins are in close proximity but not necessarily physically interacting with each other [26].

Heterodimer-selective antibodies and drugs

Monoclonal antibodies that selectively recognize a GPCR heteromer can be used to determine the tissue distribution of heteromeric complexes in environmental contexts [7]. In addition, the development of new therapeutics affecting specific receptor heteromers could demonstrate the existence of heterodimers *in vivo.* Moreover, new ligands that are being synthesized to selectively target receptor heterodimers may provide an approach for the design of therapeutics with reduced side effects [18]. GPCR heterodimers show tissue-selective expression and/or activity; thus, therapeutics targeting these unique signaling complexes may be expected to act more specifically.

Conclusions

Heterodimerization of G protein-coupled receptors (GPCRs) is a concept that may lead to the re-evaluation of the actions of neurotransmitters, hormones, pharmacological ligands and other mediators acting on GPCRs. Receptor-receptor interactions may modify neurotransmitter signaling and appear to have clinical and therapeutic significance in a wide range of diseases associated with altered neurotransmitter signaling.

At the physiological level, GPCRs dimerize for several reasons: as a quality control mechanism following their biosynthesis, to diversify receptor conformations and the state of activity, to control the duration and strength of cell signaling, and to diversify their functions and responses.

Because the receptor heteromers show a higher selectivity in drug actions due to the receptor-receptor interactions, a new field for pharmacology should be considered. In addition to drugs that specifically target receptor heterodimers, it is also possible to modulate one receptor through an action on the second receptor that belongs to the same heteromer. As shown by examples provided in the present paper, some clinical trials have already been carried out demonstrating possible therapeutic implications [5]. However, additional studies concerning the issue of GPCR heterodimerization and its functional significance are still necessary.

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