On the Role of the Balance of GPCR Homo/ Heteroreceptor Complexes in the Brain

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Abstract: The early work on neuropeptide-monoamine receptor-receptor interactions in the Central Nervous System gave the first indications of the existence of G protein-coupled receptors (GPCRs) heteroreceptor complexes and the GPCR field began to expand from monomers into heteromers and higher order heteromers, including also GPCR-ion channel, Receptor Tyrosine Kinases (RTK)-GPCR and Receptor activity-modifying proteins-GPCR heteroreceptor complexes. The existence of heteroreceptor complexes with allosteric receptor-receptor interactions increases the diversity of receptor function including recognition, trafficking and signalling. We have proposed the molecular phenomenon of receptor-receptor interactions as a good way to understand of how brain function can increase through molecular integration of signals. An alteration in specific receptor-receptor interactions or their balance/equilibrium (with the corresponding monomers-homomers) are indeed considered to have a role in the pathogenic mechanisms that lead to various diseases, including drug addiction, depression, Parkinson's disease and schizophrenia. Therefore, targeting protomer-protomer interactions in heteroreceptor complexes or the balance with their corresponding homoreceptor complexes in discrete brain regions may become an important field for developing novel drugs, including heterobivalent drugs and optimal types of combined treatments. Increasing our understanding of molecular integration of signals via allosteric receptor-receptor interactions in the heteroreceptor complexes will have a major impact on the molecular medicine, leading to novel strategies for drug discovery and treatment of diseases.

Keywords: G protein-coupled receptors, Dimerization, Oligomerization, Homdimer, Heterodimer, Homoreceptor complexes, Heteroreceptor complexes, Receptor-receptor interaction, Networks, Dopamine receptor, Serotonin receptor.

INTRODUCTION

The modulation of the binding characteristics, especially the affinity, of the monoamine receptors in a receptor subtype specific way by neuropeptides was tested in 1980-1981 and it opened the door to hypothesized the existence of an intramembrane receptor-receptor interactions phenomenon [1-10]. Thus, it was postulated that direct interactions may exist in addition to indirect actions via receptor phosphorylation, changes in the membrane potential or the involvement of adapter proteins [11-15]. Later on in 1998-1999, it was demonstrated that two non-functional GPCR receptors, GABAB1 and GABAB2 protomers, can assemble in a signalling heterodimer at the cell surface, confirming definitely the phenomenon of GPCR heteromerization [16, 17]. In addition, biophysical approaches (*i.e*. Fluorescence and Bioluminescence Resonance Energy Transfer methods, FRET and BRET respectively) provided the evidence needed to demonstrate the existence of GPCR heteroreceptor complexes in living cells [18-23]. In recent decades, a series of relevant contributions have demonstrated the importance of heterodimerization processes within the GPCR superfamily [18-20, 24-41].

We have suggested that allosteric mechanisms, which make possible the integrative intermolecular activity, take place via the interface interaction in the homoreceptor or heteroreceptor complexes. As to the protomers interface we have observed a high energy strength double arginine-phosphate electrostatic interaction in the adenosine A2AR-dopamine D2R heteroreceptor complexes representing a hot spot in the protomer interface [41, 42]. The allosteric

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E-ISSN: 2409-3564/15

mechanisms in the heteroreceptor complexes open up the door for a marked rise of the diversity of the GPCRs, *e.g*, their recognition, trafficking and signalling [20, 21, 39, 40, 43-48]. This is possible through modulation of the orthosteric/allosteric binding sites of the adjacent protomer, its G protein activation, its selectivity for scaffold protein recruitment and among others switching from G proteins to β-arrestin 2 [43, 46, 49-55]. The concept of moonlighting protein is employed to describe "multifunctional proteins in which several functions can be found in a single strand of amino acids unrelated to splicing, posttranslational changes etc" [56-58]. Moonlighting of GPCR heteroreceptor complexes may be brought about by the allosteric receptor-receptor interactions changing the function of the protomers of the heteroreceptor complexes [55, 59, 60]. The current state of the art in the brain dopamine D2 like, adenosine A2AR and serotonin 5-HT1AR heteroreceptor complexes will be described as well as some relevant methods/-techniques that could allow also the understanding of the balance between corresponding homo-and heteroreceptor complexes [61-63].

THE OVERALL ARCHITECTURE OF THE GPCR-GPCR HETERORECEPTOR COMPLEXES

The entire number of demonstrated GPCR heteroreceptor complexes was linked together and represented as a GPCR heterodimer network (GPCR-HetNet: *http: //www.gpcr-hetnet.com/* [64]. In this study static/non-dynamical human GPCR data were manually collected with a total of 187 different nodes/protomers (until August 2014) derived from 537 GPCR-GPCR edges/interactions studies in databases and literature. These data were then integrated into a scale graph, which was named the GPCR heterodimer network (GPCR-HetNet), where the nodes are the heteroreceptor complexes protomers and the edges are the relationships or interactions between them. The analysis of the GPCR-HetNet indicate a scale free model in which only a few protomers dominate the connectivity and sustain the network together. Two hub criteria show that the growth hormone secretagogue receptor type 1, dopamine D2 receptor, the mu-type opioid receptor, the beta-2 adrenergic receptor, the secretin receptor, Cannabinoid receptor 1 and the delta-type opioid receptor are the hubs in the network. Other very well connected protomers are also observed and identified in this work, as well as the potential of existence of novel receptor-receptor interactions or the formation of higher order heteroreceptor complexes. In this study the overall architecture of the GPCR-GPCR heteroreceptor complexes is for the first time presented, which provides important insight into receptor-receptor interaction connectivity, topology and organization. And may contribute to a better understanding of the complexity of GPCR heteroreceptor systems [64].

Although in this work the homomer information was excluded, we have emphasized the facts that a high percent of the total protomers described exists as homomers. [64]. In lines with this evidence, it must be point out that by means of fluorescence correlation spectroscopy it has been demonstrated that biogenic amine receptors, freely diffusing within the plasma membrane, are predominantly homodimers and not monomers [65].

The study of receptor-receptor interaction connectivity (intrafamily and interfamily) has also shown a significant prevalence of intrafamily versus interfamily connections [64]. We have claimed that one mechanism which could explain the marked dominance of intrafamily versus interfamily connections could be a favourable coevolution of the receptor-receptor interface interaction inside each subfamily. One characteristic of GPCRs subfamilies is the high sequence homologies/similarities of its member receptors and their reduced sequence homologies with other GPCR subfamilies [66]. For instance, Class B superfamily (Secretin-like), is suggested to have emerged from a single ancestral gene via gene duplication [67, 68]. As a result it shows a high sequence homology between its members [68]. Furthermore, it was demonstrated that homologues receptors belonging to the same subfamily can share similar interaction interfaces where their exposed residues can be either intermixed or run in parallel to one another [69]. Also, domain swapping phenomena can occur via their conserved domains/motifs, as have been shown for some GPCR heteroreceptor complexes [29, 30, 70]. Another reason could be the particular cell and tissue expression pattern of some GPCR [43, 47, 71-75]. Some receptor classes, for instance TAS2R bitter taste receptors, are expressed in more restricted types of cells, tissues or organ than other receptors that are more widely expressed. Therefore, their members have a lower chance of encounters and interactions. Furthermore, a need for more experimental data who focus on the study of GPCR interfamily heteroreceptor complexes should be considered. So far, only a few groups have focused their efforts on the study of the specificity of GPCR

heteromer interactions, and we expect in a near future a more wide-spread existence of cross-family heterodimerization or a definite experimental confirmation of a lower number of inter family interactions, as shown in the analysis of GPCR-HetNet (*http: //www.gpcr-hetnet.com/*).

It must be highlighted that the GPCR HetNet is so far the first and the only work that took into consideration not only a topological and architectural perspective of GPCR receptor-receptor interactions, but also the relevance of the specificities of the GPCR heteroreceptor complexes. In this database is presented in addition to the GPCR-GPCR interactions, that have been demonstrated experimentally by means of biophysical (BRET, FRET, FCCS) and biochemical (coimmunoprecipitation, *in situ* Proximity Ligation Assay (*in situ* PLA), and radioligand binding) methods, also their specificities and promiscuity. We have developed two networks, one represents the so called "positive receptor-receptor interaction" or GPCR HetNet and another one the GPCR Non-HetNet (see as well, *http: //www.gpcr-hetnet.com/*), which represents the experimentally demonstrated non-interacting GPCR protomers. Relevant facts could be extrapolated from the comparison between both networks, like for example, the interaction pattern of the alpha-1D-adrenergic receptor, which shows a low level of connectivity in the HetNet but a high level of non-interactions in the Non-HetNet.

DOPAMINE D2-LIKE AND ADENOSINE A2A HETERORECEPTOR COMPLEXES

There exists evidence for the presence of A2AR-D2R heteroreceptor complexes not only in cell lines [41, 42, 63] but also in the rat striatum using the *in situ* PLA [71, 76] as well as indications for the existence of striatal A2AR-D3R and A2AR-D4R heteroreceptor complexes. Antagonistic A2AR-D2R interactions exist in the striatal A2AR-D2R heteroreceptor complexes reducing D2R recognition and signalling which has led to a novel understanding of the molecular bases behind the pathophysiology of drug addiction, Parkinson's diseases (PD) and L-DOPA-induced dyskinesias of high relevance for their treatments [52, 55, 63, 77-79]. Based on this, A2AR antagonists are being proposed in the symptomatic treatment of PD [52]. In line with these results A2AR agonists have been shown to have atypical antipsychotic actions and to possess anti-cocaine actions in development and reinstatement of cocaine self-administration [77]. Also antagonistic CB1R-D2R interactions likely exist in striatal CB1R-D2R heteromers leading to a reduced D2R signalling and counteraction of D2R-induced hyperlocomotion [80, 81]. Higher order heteromers may also be found since there are indications for the presence of striatal A2AR-CB1R-D2R trimers and A2AR-D2R-mGlu5R trimers with integrative receptor-receptor interactions modulating the activity of the striato-pallidal GABA neurons [82, 83]. In the latter trimer the A2AR and mGlu5R synergize to counteract the D2R signalling to allow firing of the striato-pallidal GABA neurons without being restrained by the inhibitory D2R signalling. In this way these receptors can potentially override a pathologically increased D2R signalling present in schizophrenia. In the former trimer the antagonistic CB1R-D2R interaction activated by endocannabinoids removes the D2 brake on the A2AR signalling to the adenylyl cyclase contributing to a markedly increased activity of the striato-pallidal GABA neurons [81]. The A2AR-CB1R-D2R trimer may function mainly as an inhibitory feedback mechanism to reduce an exaggerated and prolonged activation of D2R removing an excessive silencing of the striato-pallidal GABA neurons. Thus, these two types of GPCR trimers are also potential novel drug targets for treatment of PD, schizophrenia and cocaine addiction.

SEROTONIN 5-HT1A HETERORECEPTOR COMPLEXES

As to the 5-HT1AR heteroreceptor complexes evidence exists that Galanin receptor1 (GalR1) modulates 5-HT1AR signalling via heterodimerization in cellular models [21]. These results amplify the indications that there exist brain GalR1-5-HT1AR heteromers in which the previously observed antagonistic galaninR-5-HT1A receptor-receptor interactions in the limbic system take place [84]. Targeting different types GalR-5-HT1AR heteromers may be a novel therapeutic strategy for treatment of depression [53, 72, 84]. Targeting the recently discovered FGFR1-5-HT1AR heteroreceptor complexes of the hippocampus may be another therapeutic approach in depression in view of their enhancement of hippocampal plasticity [75, 85]. Combined acute and repeated intracerebrocentricular treatment with FGF2 and 8-OH-DPAT have shown evidence as significant antidepressant in the forced swim test [75]. These heteroreceptor complexes were also observed in midbrain 5-HT neurons of the rat [73]. The 5-HT1A autoreceptor protomer upon agonist activation produced an allosteric enhancement of the FGFR1 signaling in the midbrain raphe 5-HT nerve cells leading to increases in their neuroplasticity and

trophism. Thus, the 5-HT1A autoreceptor in this heteroreceptor complex can also have a trophic role of importance for depression and its treatment in addition to its key role in reducing the firing of the ascending midbrain 5-HT neurons.

New findings have shown that combine treatment with 8-OH-DPAT and FGF2 but not treatment with the 5-HT1A agonist alone significantly increases the BRETmax values and markedly reduces the BRET50 values on the 5HT1A homodimerization in cellular models co-expressing 5-HT1A and FGFR1 [86]. FGF2 produces a rapid rise in FGFR1 homodimerization which, to certain extent, declined over a 10 min period. Combine treatment with 8-OH-DPAT blocked this decline in FGFR1 homodimerization. Also, it was observed that FGF2 only produced a small rise in the $BRET^2$ signal from the 5-HT1A-β-arrestin2 complex which was additive to the significant effect of 8-OH-DPAT alone. All together, these results have shown a dynamic modulation of the allosteric receptor-receptor interactions in the FGFR1-5-HT1A heteroreceptor complexes with regard to their impact on 5-HT1AR and FGFR1 homodimerization [86].

METHODS FOR STUDYING GPCR RECEPTOR-RECEPTOR INTERACTIONS

In the last years, interactions involving GPCRs were demonstrated through diverse biophysical (FRET, BRET, BiFC) and biochemical or microscopy-based procedures (*e.g*. coimmunolocalization, coimmuno-precipitation, radioligand binding, co-internalization analysis) including methods that assess receptor-receptor interactions [22, 23, 85, 87, 88]. Each methods or tools employed has gave a precise and valuable information which has been considered with caution to avoid undesirable drawbacks. Some controversy regarding some approaches have also emerged [89, 90]. Nevertheless, when these methods are properly assessed it is possible to demonstrate elegantly the direct interactions between GPCRs and as well as their dynamics. [91-93].

Novel technologies to study receptor heteroreceptor complexes and their receptor-receptor interactions have recently been developed such as real-time FRET experiments in living cells [94], dual-colour fluorescence cross-correlation spectroscopy (FCCS) [65] and mass spectrometry of phosphorylation fingerprints from receptor protomers of receptor heteromer and homomers [95]. A new dimension in the GPCR heteromer field was also introduced in 2010 by the evidence obtained, in cellular models first [48] and in brain tissue later [43, 46, 47, 72-76, 85], of the existence of GPCR heteroreceptor complexes by means of the *in situ* PLA. This method offers the advantages of the analysis of any receptor-receptor interactions for which suitable antibodies are available without using genetic constructs. As with any method there are limitations, for instance, in situ PLA could only be used in *ex vivo* fixed material. The method strongly relies on the quality of the antibodies used, and we must take into account parameters such as antibody concentration, epitopes targeted by the antibodies, fixation, antigen-retrieval, blocking conditions, etc.

ON THE UNDERSTANDING OF THE BALANCE BETWEEN THE HOMO-AND HETERORECEPTOR COMPLEXES IN THE BRAIN

Until now, most experimental work was focused on the study of GPCR receptor-receptor interactions as a phenomenon of binary interactions. In other words, it was only focused on the understanding of face-to-face protomer-protomer interactions and their allosteric receptor-receptor modulation and functional specificities (See www.gpcr-hetnet.com [64] for a complete list of all binary pairs of GPCR-GPCR interactions described so far). However, the phenomenon of GPCR oligomerization is a more complex and dynamic process, which involve many more parameters than the classical analysis of the interactions of the two involved protomers [86]. One emerging concept is that direct physical receptor-receptor interactions in heteroreceptor complexes or altered balance with their homoreceptor complexes population can contribute to brain disease progression inter alias PD, depression, Schizophrenia, Alzheimer and addiction [61, 79]. When discussing the role of GPCR heteroreceptor complexes, it is of substantial interest to understand the balance/equilibrium between the corresponding homo- and heteroreceptor complexes in the plasma membrane of the cell (Figure **1**). It should be important to also consider a disruption or shift of the balance between corresponding receptor homomers and heteromer populations as a mechanism for disease development which therefore also represents a pharmacological target.

We have suggested that the introduction of *in situ* PLA (Figure **1**) in combination with western blot, radioligand binding experiments and co-immunoprocipitation could open a new window to the understanding of the importance of the balance of the corresponding homo- and heteroreceptor complexes [85]. The analysis of animal/human brain material with *in situ* PLA can

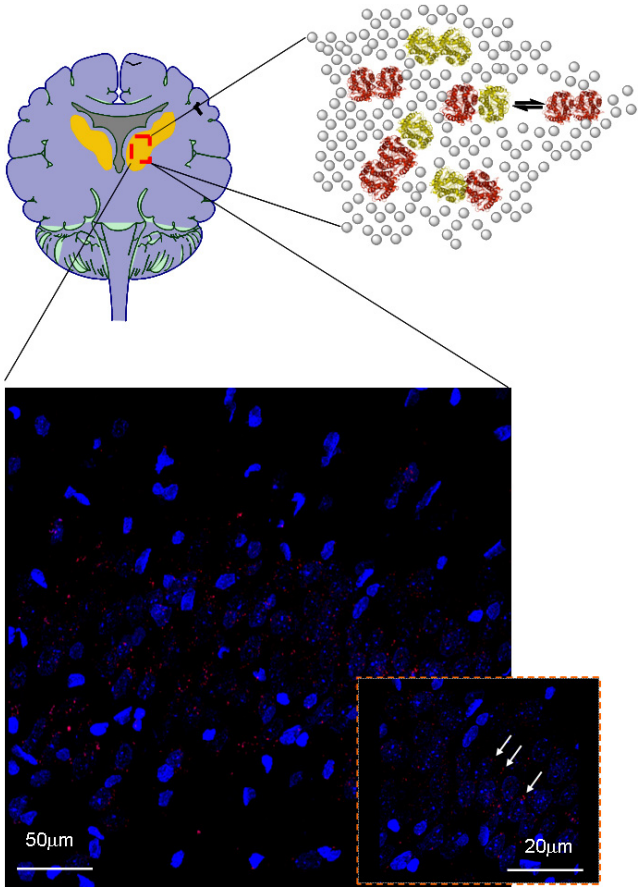

Figure 1: On the understanding of the balance/equilibrium between GPCR homo- and heteroreceptor complexes population in the neuron membrane in the brain. (Top right) Schematic representation of the balance between different populations of homo and hetero-receptor complexes in a portion of the membrane cell. (Bottom) The panel represent an example of *in situ* PLA experiment performed in rat hippocampal free-floating sections using primary antibodies of different species directed to adenosine A2A and A2B receptors. The detected A2A-A2B heteroreceptor complexes are seen as red clusters indicated by arrows in each panel. Specific A2A-A2B clusters are visualized within discrete regions of the pyramidal cell layer of the Ammon´s horn and the molecular cell layer of the dentate gyros (ventral leaflet). The complex was also observed throughout the cortex and in the piriforme layer but were almost absent in the molecular cell layer of the dentate gyros (dorsal leaflet). The analysis of animal/human brain material with *in situ* PLA can reveal if the relative abundance of specific homo- heteroreceptor complexes in discrete brain regions is altered in brain diseases or under certain drug treatments.

reveal if the relative abundance of specific homo-and heteroreceptor complexes in discrete brain regions is altered in brain diseases or under certain drug treatments, for instance, chronic L-dopa treatment in PD [61, 79].

In this analysis, it is important also to determine the ratio between heteromers versus total number of the two participating receptor populations, using in addition to Western blots, receptor autoradiography and biochemical binding methods. The two latter methods show the densities and affinities of the two functional receptor populations. The relationship between these parameters will help to normalize the heteromer values for comparison between groups in addition to evaluating the potential changes in the total number of the two receptor populations [85].

Certainly, we cannot compare or determine directly a balance between the homo- and heteroreceptor complexes populations in the same tissue using the *in situ* PLA approach, because of a technical limitation of the procedure itself. But the method could help us determine each population independently and compare their relative expression levels after an appropriate numerical analysis. Furthermore, it should help understand the effects that could be induced by agonist/antagonist treatments on the regulation of these homo-/heteroreceptor complexes in order to understand their potential roles as drug targets or as markers of brain disease progression.

ACKNOWLEDGMENTS

This work has been supported by the Karolinska Institutets Forskningsstiftelser 2014/2015 to D.O.B-E,

by the Swedish Medical Research Council (62X-00715-50-3) and AFA Försäkring (130328) to KF and D.O.B-E. I.B and D.O.B-E belong to Academia de Biólogos Cubanos.

CONFLICTS OF INTEREST

The authors declare no conflict of interest

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Received on 26-12-2014 Accepted on 30-01-2015 Published on 14-04-2015

http://dx.doi.org/10.15379/2409-3564.2015.02.01.5