Family a GPCR heteromers in animal models

Javier González-Maeso*

Departments of Psychiatry and Neurology, Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA

*Correspondence: javier.maeso@mssm.edu

INTRODUCTION

G protein-coupled receptors (GPCRs) were assumed to exist and function in the plasma membrane as monomeric proteins that became activated by binding of one agonist ligand to one receptor molecule (Bourne et al., 1990). However, although previous findings based on rather indirect measures such as radioligand binding had suggested a direct interaction of two receptors with each other (Limbird et al., 1975; Ferre et al., 1991), it was the application of a protein-protein interaction assay by bioluminescence resonance energy transfer (BRET) that revealed the phenomenon of molecular proximity between beta2-adrenergic receptors in living cells (Angers et al., 2000). Since then, this topic has been a major subject of research and numerous in silico and in vitro studies have suggested expression of family A GPCRs as homodimers and higher-order homomers in heterologous expression systems. However, the demonstration that reconstitution of a single beta2-adrenergic receptor molecule into lipoprotein particles leads to efficient activation of G proteins raised concerns about the functional significance of family A GPCR homomers (Whorton et al., 2007), and this is currently a controversial topic (for an extensive review on GPCR homodimers/homomers, see Milligan, 2013; see also Bouvier and Hebert, 2014; Lambert and Javitch, 2014).

Another fundamental yet relatively independent question is that related to expression of different GPCR subtypes as heteromers. It is well accepted that the family C GABAB receptor needs two protomers (GABAB1-R1 and GABAB2-R2) to reach the plasma membrane as a functional dimeric receptor (Jones et al., 1998; Kaufmann et al., 1998; White et al., 1998). On the other hand, although multiple lines of evidence indicate that family A GPCR heteromers may exist, particularly in tissue cultures (González-Maeso, 2011; Ferre et al., 2014), only relatively recent studies started to test this formulation in whole animal models.

FAMILY A GPCR HETEROMERS IN WHOLE ANIMAL MODELS

One of the main limitations of the classical techniques used to define GPCR heteromeric formation is the translation of findings obtained in cellulo into physiological or behavioral assays in whole animal models. In this context, co-immunoprecipitation is an approach commonly used to examine protein-protein interaction in native tissue (Milligan and Bouvier, 2005). GPCR antibodies are usually neither specific nor sensitive and therefore validation assays in knockout mice are often required (Fribourg et al., 2011; Moreno et al., 2012). Considering this, it is also clear that demonstration of co-immunoprecipitation in native tissues does not imply the existence of a heteromeric assembly, as they may form part of same protein complex through for example PDZ domain-binding motifs at the end of the C-terminal tails of both receptor types together with adaptor proteins (Magalhaes et al., 2010). Remarkably, there are only a few studies that have investigated GPCR heteromeric formation in living animals, and due to the lack of biophysical methods applicable to study protein-protein interactions in preclinical models, their experimental approaches were mostly focused on signaling and behavioral outcomes rather than on the existence of molecular proximity between different GPCR subtypes.

Although it does not measure molecular proximity, an attractive approach to define whether heteromeric formation is involved in behavioral phenotypes is the use of peptides that disrupt receptor complex formation. These peptides tested in vivo are usually selected according to findings previously obtained in heterologous expression systems. As an example, it was demonstrated that the Gq-coupled dopamine D1 receptor and the Gz/o-coupled dopamine D2 form a receptor complex that induces Ca2+ release via a Gq11-dependent pathway (Lee et al., 2004), and that the region of Met-257—Glu-271 (intracellular loop 3; D2IL3-29-2) but not Asn-243—Ile-256 (intracellular loop 3; D2IL3-29-1) of the dopamine D2 receptor can pull-down the dopamine D1 receptor. Based on a Tat-tagged peptide approach, it was shown that intracerebroventricular administration of the peptide D2IL3-29-2, which disrupts heteromeric formation between dopamine D1 and D2 receptors in vitro, induces antidepressant-like effects in rats (Pei et al., 2010). More recent findings using serial deletions and point mutations further demonstrate that dopamine D1 receptor carboxyl tail residues Glu-404 and Glu-405 are critical in mediating the interaction with the D2 receptor, and that administration of a disrupting peptide Tat-D1 modulates depression-like behavior in rats such as forced swim test (Hashi et al., 2014). A similar approach was used to block the association as a GPCR heteromer between the mu-opioid receptor isoform MOR1D and the gastrin-releasing peptide receptor (GRPR) in the spinal cord (Liu et al., 2011). The authors demonstrated...
that the C-terminus of MOR1D is critical for MOR1D-GPRP heteromeric formation. Using a Tat-fusion peptide, they also found that a motif consisting of seven amino acids of the MOR1D C-terminus (RNEEPSS) attenuates morphine-induced scratching, but not morphine-induced analgesia.

The question of whether GPCR heteromers exist ex vivo has been addressed using time-resolved Förster resonance energy transfer FRET (TR-FRET) in plasma membrane preparations of mouse brain. It was found that the dopamine D$_2$ receptor and the ghrelin receptor (GHSR1a) co-localize in mouse striatum, hippocampus and hypothalamus (Kern et al., 2012). When membrane preparations from hypothalamus were incubated with red-ghrelin (acceptor fluorophore) and an anti-D$_2$ receptor antibody together with a europium cryptate-labeled secondary antibody (donor fluorophore), a significantly TR-FRET signal was observed. Although TR-FRET signal is eliminated in hypothalamic membrane preparations of GHSR1a knockout mice, which supports specificity, these findings were observed ex vivo in plasma membrane preparations and further investigation will be necessary to confirm the existence of GHSR1a-D$_2$ heteromeric formation in hypothalamus in vivo.

Another indirect approach to test whether GPCR heteromeric formation affects behavioral phenotypes is the use of chimeric constructs that according to previous findings with receptor mutants that do not form heterocomplexes in vitro or in cellulo assays disrupt GPCR heteromeric assembly. Similarly, the use of viral-mediated over-expression or transgenic animals could translate into animal models previous findings with receptor mutants that do not form heterocomplexes in vivo or in cellulo. However, a more precise understanding of such structural assembly obtained in rodent models will be necessary to fully define whether GPCR heteromers exist and function in vivo. Some of these strategies include the use of FRET (McGinty et al., 2011) of BRET (Dragulescu-Andrasi et al., 2011) imaging of protein-protein interactions in living mice.

An important challenge in the fields of GPCR research and molecular pharmacology is to develop an integrated understanding of how various mechanisms communicate with each other to ultimately orchestrate the formation of heteromeric complexes between some but not all GPCR subtypes. Potential mechanisms that are critical for this interaction specificity include specific pairs of residues that are critical for this interaction specificity include specific pairs of residues that are independent of GPCR heteromeric formation. Detailed measurement of such molecular proximity in vivo, as well as the processes that control GPCR heteromerization in whole animal models, will require further study.

**LIMITATIONS, FUTURE DIRECTIONS, AND CONCLUDING REMARKS**

Although a wealth of data from in vitro and in cellulo models have established the important role of GPCR heteromers in mediating precise and distinct roles in signaling cascades, their influence in the establishment of complex behavioral phenotypes remains to be fully elucidated. For instance, certain physiological and behavioral outcomes could conceivably be altered in the presence of peptides that according to in vitro or in cellulo assays disrupt GPCR heteromeric assembly. Similarly, the use of viral-mediated over-expression or transgenic animals could translate into animal models previous findings with receptor mutants that do not form heterocomplexes in vivo or in cellulo.

Although these events have been proposed to represent a demonstration of GPCR heteromeric expression, thereby suggesting a new target for drug design, their conclusions in animal models were based largely on indirect approaches that measured phenotypes affected by manipulations such as chimeric constructs or Tat-tagged peptides that impact heteromeric organization in vitro. Consequently, it remains unclear as to whether different GPCR subtypes exist in close molecular proximity in vivo and in whole animal models, or alternatively if these phenotypes result from signaling mechanisms that are independent of GPCR heteromeric formation. Detailed measurement of such molecular proximity in vivo, as well as the processes that control GPCR heteromerization in whole animal models, will require further study.

Although a wealth of data from in vitro and in cellulo models have established the important role of GPCR heteromers in mediating precise and distinct roles in signaling cascades, their influence in the establishment of complex behavioral phenotypes remains to be fully elucidated. For instance, certain physiological and behavioral outcomes could conceivably be altered in the presence of peptides that according to in vitro or in cellulo assays disrupt GPCR heteromeric assembly. Similarly, the use of viral-mediated over-expression or transgenic animals could translate into animal models previous findings with receptor mutants that do not form heterocomplexes in vivo or in cellulo. However, a more precise understanding of such structural assembly obtained in rodent models will be necessary to fully define whether GPCR heteromers exist and function in vivo.

Some of these strategies include the use of FRET (McGinty et al., 2011) of BRET (Dragulescu-Andrasi et al., 2011) imaging of protein-protein interactions in living mice.

An important challenge in the fields of GPCR research and molecular pharmacology is to develop an integrated understanding of how various mechanisms communicate with each other to ultimately orchestrate the formation of heteromeric complexes between some but not all GPCR subtypes. Potential mechanisms that are critical for this interaction specificity include specific pairs of residues that govern heteromeric formation, clustering of GPCRs in membrane microdomains, and crosstalk between receptors and a plethora of multidomain scaffolding proteins. Another important question to be addressed by future research is the molecular basis through which GPCR heteromers affect G protein function. For example, it has been shown that drugs that activate the G$_{q/11}$-coupled 5-HT$_2$A receptor induce both G$_{q/11}$- and G$_{i/o}$-dependent signaling in HEK293 cells co-expressing 5-HT$_2$A and the G$_{i/o}$-coupled mGlu2 receptor as a GPCR heteromer (Gonzalez-Maeso et al., 2008). Although findings in knockout mice suggest that co-expression of 5-HT$_2$A and mGlu2 receptors is necessary to activate G$_{q/11}$ and G$_{i/o}$ by 5-HT$_2$A...
agonists in mouse frontal cortex membrane preparations (Fribourg et al., 2011), whether heteromeric formation is needed in living mice for this signaling crosstalk remains unknown. Similarly, more work is required both in cellulo and in animal models to solve whether $G_{q/11}$ and $G_{i/o}$ simultaneously or sequentially couple to the 5-HT$_{2A}$-mGlu2 heteromeric receptor complex upon agonist binding to one of the two promoters.

Another significant limitation to our current understanding of GPCR heteromeric function is the lack of knowledge about physical stability of family A GPCR heteromers in animal models. Previous findings in HEK293 cells convincingly demonstrate that the alpha1B-adrenergic receptor forms higher-order oligomers, and that receptor oligomerization is required for receptor maturation and plasma membrane delivery (Lopez-Gimenez et al., 2007). On the other hand, results based on an experimental approach that recruits beta2-adrenergic receptors into artificial domains on the surface of living HEK293 cells suggest that the components of family A GPCR homomers interact transiently (Fonseca and Lambert, 2009; Gavalas et al., 2013). A similar conclusion has been reached using total internal reflection fluorescence microscopy (TIRFM) to visualize individual molecules in isolated CHO cells—the authors observed a transient association and dissociation of muscarinic M$_1$ receptor dimers in real time (Hern et al., 2010). Much further work is needed to characterize where along the pathway from synthesis to maturation and degradation do GPCR heteromers form. It also remains uncertain the stability of family A GPCR heteromers both in vitro and in whole animal models. Many studies examining homomeric GPCR interfaces report that residues of both TM1 and TM4 form symmetrical interfaces that lead to higher order species in heterologous expression systems (Guo et al., 2005, 2008), and this has been supported further by a number of recent crystal structures (Wu et al., 2012; Huang et al., 2013). However, it remains to be fully elucidated whether different homomeric and heteromeric organizations (e.g., squares and/or parallelograms) might exist in native tissue. These are all key questions that require further technical advances.

In conclusion, although a range of approaches has been applied and this has led to a general appreciation that GPCR heteromers affect receptor trafficking, pharmacology and function in cellulo, much more work is needed to probe the role of GPCR heteromerization in vivo. These advances in GPCR heteromeric research are now occurring at a rapid pace and promise to greatly contribute to the future of molecular medicine.

ACKNOWLEDGMENT
Supported by R01MH084894 to Javier González-Maeso.

REFERENCES
Aars, S., Salhabour, A., Joly, E., Hilairet, S., Chelsky, D., Dennis, M., et al. (2000). Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc. Natl. Acad. Sci. U.S.A. 97, 3684–3689. doi: 10.1073/pnas.97.7.3684
Baba, K., Benleulmi-Chaouchoua, A., Journe, A. S., Kamal, M., Guillaume, J. L., Dussaud, S., et al. (2013). Heteromeric MT1/MT2 melanin receptors modulate photoreceptor function. Sci. Signal 6, ra89. doi: 10.1126/scisignal.2004302
Bourne, H. R., Sanders, D. A., and McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348, 125–132. doi: 10.1038/348125a0
Bouvier, M., and Hebert, T. E. (2014). CrossTalk proposal: weighing the evidence for Class A GPCR dimers, the evidence favours dimers. J. Physiol. 592, 2439–2441. doi: 10.1113/jphysiol.2014.272252
Dragulescu-Andrasi, A., Chan, C. T., De, A., Massoud, T. F., and Gambhir, S. S. (2011). Bioluminescence resonance energy transfer (BRET) imaging of protein-protein interactions within deep tissues of living subjects. Proc. Natl. Acad. Sci. U.S.A. 108, 12060–12065. doi: 10.1073/pnas.110923108
Ferre, S., Casado, V., Devi, L. A., Filizola, M., Jockers, R., Lohse, M. J., et al. (2014). G protein-coupled receptor oligomerization revisited: functional and pharmacological perspectives. Pharmacol. Rev. 66, 413–434. doi: 10.1124/pr.113.008052
Ferre, S., von Euler, G., Johansson, B., Fredholm, B. B., and Fuxe, K. (1991). Stimulation of high-affinity adenosine A2 receptors decreases the affinity of dopamine D2 receptors in rat striatal membranes. Proc. Natl. Acad. Sci. U.S.A. 88, 7238–7241. doi: 10.1073/pnas.88.16.7238
Fonseca, J. M., and Lambert, N. A. (2009). Instability of a class a G protein-coupled receptor oligomer interface. Mol. Pharmacol. 75, 1296–1299. doi: 10.1124/mol.108.053876
Fribourg, M., Moreno, J. L., Holloway, T., Provasi, D., Baki, L., Mahajan, R., et al. (2011). Decoding the Signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs. Cell 147, 1011–1023. doi: 10.1016/j.cell.2011.09.055
Gavalas, A., Lan, T. H., Liu, Q., Correa, I. R. Jr., JVitch, J. A., and Lambert, N. A. (2013). Segregation of family A G protein-coupled receptor protomers in the plasma membrane. Mol. Pharmacol. 84, 346–352. doi: 10.1124/mol.113.086868
González-Maeso, I. (2011). GPCR oligomers in pharmacology and signaling. Mol. Brain 4:20. doi: 10.1186/1756-6606-4-20
González-Maeso, I., Ang, R. L., Yuen, T., Chan, P., Weisstaub, N. V., Lopez-Gimenez, J. F., et al. (2008). Identification of a serotonin/glutamate receptor complex implicated in psychosis. Nature 452, 93–97. doi: 10.1038/nature06612
González-Maeso, I., Weisstaub, N. V., Zhou, M., Chan, P., Ivic, L., Ang, R., et al. (2007). Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling pathways to affect behavior. Neuron 53, 439–452. doi: 10.1016/j.neuron.2007.01.008
Guo, W., Shi, L., Filizola, M., Weinstein, H., and Javitch, J. A. (2005). Crossstalk in G protein-coupled receptors: changes at the transmembrane homodimer interface determine activation. Proc. Natl. Acad. Sci. U.S.A. 102, 17495–17500. doi: 10.1073/pnas.0508955102
Guo, W., Urizá, E., Králiková, M., Mobarcé, J. C., Shi, L., Filizola, M., et al. (2008). Dopamine D2 receptors form higher order oligomers at physiological expression levels. EMBO J. 27, 2293–2304. doi: 10.1038/embj.2008.153
Hanks, J. B., and González-Maeso, J. (2013). Animal models of serotonergic psychedelics. ACS Chem. Neurosci. 4, 33–42. doi: 10.1021/cn300138m
Hasibi, A., Perreault, M. L., Shen, M. Y., Zhang, L., To, R., Fan, T., et al. (2014). A peptide targeting an interaction interface disrupts the dopamine D1-D2 receptor heteromer to block signaling and function in vitro and in vivo: effective selective antagonism. FASEB J. doi: 10.1096/fj.14-254037. [Epub ahead of print].
Hern, J. A., Baig, A. H., Mashanov, G. I., Birdsal, B., Corrie, J. E., Lazareno, S., et al. (2010). Formation and dissociation of M1 muscarinic receptor dimers seen by total internal reflection fluorescence imaging of single molecules. Proc. Natl. Acad. Sci. U.S.A. 107, 2693–2698. doi: 10.1073/pnas.0907915107
Huang, J., Chen, S., Zhang, J. I., and Huang, Y. C. (2013). Crystal structure of oligomeric beta1-adrenergic G protein-coupled receptors in ligand-free basal state. Nat. Struct. Mol. Biol. 20, 419–425. doi: 10.1038/nsmb.2504
Jones, K. A., Borowsky, B., Tam, J. A., Craig, D. A., Durkin, M. M., Dai, M., et al. (1998). GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. Nature 396, 674–679. doi: 10.1038/335438a
Kaufmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., Beck, P., et al. (1998). GABA(B)-receptor subtypes assemble into functional heteromeric complexes. Nature 396, 683–687. doi: 10.1038/25360
Kern, A., Albarran-Zeckler, R., Walsh, H. E., and Smith, R. G. (2012). Apo-ghrelin receptor forms heteromers with DRD2 in hypothalamic neurons and is essential for anorexigenic effects of DRD2 agonism. Neuron 73, 317–332. doi: 10.1016/j.neuron.2011.10.038
Lambert, N. A., and Javitch, J. A. (2014). CrossTalk opposing view: weighing the evidence for class A GPCR dimers, the jury is still out. J. Physiol. 592, 2443–2445. doi: 10.1113/jphysiol.2014.272997

www.frontiersin.org
October 2014 | Volume 5 | Article 226 | 3
González-Maeso (2013). The prevalence, maintenance and relevance of GPCR oligomerization. Mol. Pharmacol. 84, 158–169. doi: 10.1124/mol.113.084780

Milligan, G., and Bouvier, M. (2005). Methods to monitor the quaternary structure of G protein-coupled receptors. FEBS J. 272, 2914–2925. doi: 10.1111/j.1742-4658.2005.04731.x

Moreno, J. L., Holloway, T., Albizu, L., Sealfon, S. C., and Gonzalez-Maeso, J. (2011). Metabotropic glutamate mGlu2 receptor is necessary for the pharmacological and behavioral effects induced by hallucinogenic 5-HT2A receptor agonists. Neuron. Lett. 493, 76–79. doi: 10.1016/j.neulet.2011.01.046

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 August 2014; paper pending published: 17 September 2014; accepted: 21 September 2014; published online: 09 October 2014.

Citation: González-Maeso J (2014) Family a GPCR heteromers in animal models. Front. Pharmacol. 5:226. doi: 10.3389/fphar.2014.00226

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology. Copyright © 2014 González-Maeso. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.