3rd G Protein-Coupled Receptors

An ASPET Colloquium

Sponsored by:
The Division for Molecular Pharmacology

April 27-28, 2007
Washington, D.C.

Organizers:
Kim A. Neve, Ph.D. & Olivier Civelli, Ph.D.

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3rd G Protein Coupled Receptors Colloquium

Friday-Saturday, April 27-28, 2007
Washington DC Convention Center, Room 204 A-C

Organized by: Kim A. Neve, Ph.D. and Olivier Civelli, Ph.D.
Sponsored by the ASPET Division for Molecular Pharmacology

Friday, April 27

7:30 am	Continental breakfast
8:10 am	The structural basis for GPCR oligomerization: Implications for activation **Jonathan A. Javitch,** *Columbia University College of Physicians and Surgeons*
8:50 am	Heterooligomerization of Class A GPCRs creates novel signaling units distinct from their constituent GPCR homooligomers **Susan R. George**, *University of Toronto*
9:30 am	GPCR ligand binding and release: Insights and mysteries **David L. Farrens**, *Oregon Health and Science University*
10:10 am	Coffee break and posters in room 206
10:40 am	G proteins and their accessory proteins **Stephen M. Lanier**, *Medical University of South Carolina*
11:20 am	Is Helix VIII of G Protein-Coupled Receptors (GPCRs) a Lipid-Activated Signalling Sensor? **John Huynh**, *Baker Heart Research Institute, Australia*
11:50 am	Lunch
1:20 pm	Interactions between GPCRs and receptor tyrosine kinases **Kevin J. Catt**, *NICHD, NIH*
2:00 pm	GPCRs, arrestins, and ubiquitination **Sudha K. Shenoy**, *Duke University Medical Center*
2:40 pm	Allosteric agonists and allosteric modulators are differentially affected by mutations within the allosteric binding pocket of M2 muscarinic acetylcholine receptors. **Karen Gregory**, *Monash University, Australia*
3:10 pm	Coffee break and posters in room 206
3:40 pm	Multiplexing resonance energy transfer approaches to study GPCR signaling complexes in living cells **Michel Bouvier,** *University of Montreal*
4:20 pm	Use of genetically engineered mice to unravel the functions of dopamine receptors **Emiliana Borrelli**, *University of California, Irvine*
5:00-7:00 pm	Wine & cheese reception/poster presentations in room 206

Saturday, April 28

7:30 am	Continental breakfast
8:20 am	Kisspeptin and GPR54 in the regulation of puberty and reproduction **Ursula Kaiser,** *Brigham & Womens Hospital, Harvard Medical School*
9:00 am	GPCRs in arousal and anxiety **Rainer K. Reinscheid**, *University of California, Irvine*
9:40 am	Vasoactive intestinal polypeptide receptor 2 female knockout mice are resistant to diet-induced obesity **Mari Candelore,** *Merck & Company*
9:55 am	Dynorphin A activates bradykinin receptors to maintain neuropathic pain **Josephine Lai,** *University of Arizona Health Sciences Center*
10:10 am	Break
10:40 am	The role of GPR30 in estrogen signaling **Eric R. Prossnitz**, *University of New Mexico Health Sciences Center*
11:20 am	Therapeutic benefits of inverse agonism at cannabinoid receptors **Douglas Greene**, *Sanofi-aventis*
12:00 noon	Lunch
1:30 pm	Novel aspects of the melanocortin receptors **Roger D. Cone**, *Oregon Health and Science University*
2:10 pm	Leucocyte chemoattractant receptors: New molecules and new concepts **Marc Parmentier,** *Free University of Brussels*
2:50 pm	Break
3:20 pm	**Special Lecture:** The function and regulation of G protein coupled glutamate receptors in the neural network **Shigetada Nakanishi**, *Osaka Bios*cience Institute
4:30 pm	End of Meeting

ASPET gratefully acknowledges the educational grants from AstraZeneca, Johnson & Johnson, Merck Research, Wyeth Research, and the ASPET Division for Neuropharmacology, with support for the Keynote Lecture on day 2 by the ASPET Division for Molecular Pharmacology.

3rd G Protein Coupled Receptors Colloquium
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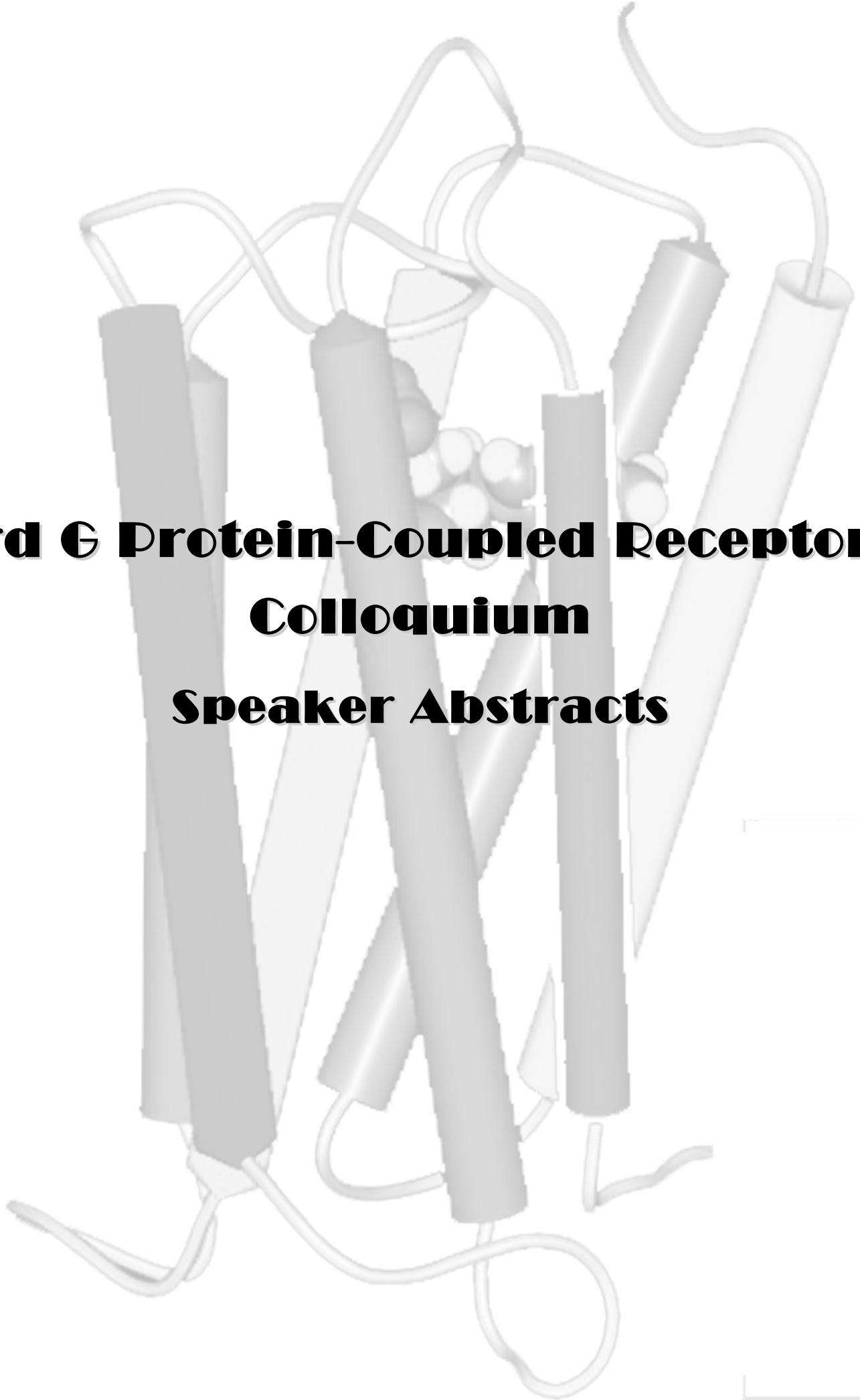

3rd G Protein-Coupled Receptors Colloquium

Speaker Abstracts

The structural basis for GPCR oligomerization: Implications for activation

Jonathan A. Javitch
Columbia University

G protein-coupled receptors are widely thought to exist as dimers on the cell surface, and limited experimental evidence suggests that they are organized into higher-order oligomeric forms. In the dopamine D2 receptor we previously demonstrated by crosslinking of substituted cysteines that the fourth transmembrane segment (TM4) contributes to a symmetrical dimer interface. The susceptibility to crosslinking of these cysteines was differentially altered by the presence of agonists and inverse agonists. The TM4 dimer interface in the inverse agonist-bound conformation is consistent with the dimer of the inactive form of murine rhodopsin modeled by Liang et al. with constraints from atomic force microscopy (AFM). Crosslinking of a different set of cysteines in TM4 was slowed by inverse agonists and accelerated in the presence of agonists; crosslinking of the latter set locked the receptor in an active state. Thus, a conformational change at the TM4 dimer interface is part of the receptor activation mechanism. The AFM model suggested a TM1-TM1 interface between rows of dimers. We have now demonstrated such a second symmetrical interface at the extracellular end of TM1. In contrast to the TM4 interface, we observed no significant effect of agonists or antagonists on crosslinking of the TM1 interface. When we combined a TM1 and a TM4 cysteine into the same receptor, we observed crosslinking to dimer, trimer, tetramer, and higher-order forms, consistent with the organization of the receptor into a tetramer or higher-order array. Using an inducible expression system to vary the range of receptor expression more than 100-fold, we found that crosslinking is independent of expression level, which argues strongly against collisional crosslinking and for a higher-order organization involving symmetrical contacts in TM4/5 and TM1. We will discuss various models of GPCR organization in the light of our data.

Heterooligomerization of Class A GPCRs creates novel signaling units distinct from their constituent GPCR homooligomers

Susan R. George, Christopher So, Asim Rashid, Ahmed Hasbi and Brian F. O'Dowd
Departments of Pharmacology and Medicine
University of Toronto and the Centre for Addiction and Mental Health
Toronto, ON Canada.

Heterooligomerization has novel implications for GPCR signal transduction, chief of them being that of increasing the repertoire of signaling pathways and effector mechanisms available for endogenous ligands. In examples such as the opioid or dopamine receptor heterooligomers. where a novel signaling system is accessed through differential coupling to G proteins, there is a diversity of signaling mechanisms generated that is very distinct from that of the constituent homooligomers. For dopamine D1 and D2 receptor heterooligomers we have shown co-localization of the receptors in neurons, co-immunoprecipitation from brain, formation of heterooligomers in the endoplasmic reticulum trafficked to the plasma membrane, with linkage to Gq-linked intracellular calcium release that is readily evident in brain as well. The D1 and D2 receptor homooligomers largely function through activation of Gs/olf and Go/i respectively. The opioid mu and delta receptor heterooligomer we have found selectively activates Gz, whereas the homooligomers activate Gi proteins. The affinity of the mu-delta heterooligomer for Gz was found to be greater than for Gi; the converse was true for the homooligomers. In each case, there are unique features of the binding pocket that results in novel pharmacology being generated. These GPCR heterooligomers have considerable significance for understanding the physiology of dopamine and opioid receptor systems in brain. They will also be potential novel targets for GPCR drug discovery once their physiological functions have been fully elucidated.

Funded by the National Institute on Drug Abuse, the Canadian Institutes of Health Research and the Canada Research Chairs Program.

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3. Rashid A.J., So C.H., Kong M.M.C., Furtak T., Cheng R., O'Dowd B.F. and **George S.R.** The D1-D2 dopamine receptor heterooligomer is coupled to rapid activation of Gq/11 in the striatum: Discovery of a novel agonist selectively targeting the complex. *Proceedings of the National Academy of Science* 104: 654-659, 2007

GPCR ligand binding and release: Insights and mysteries

David L. Farrens
Oregon Health and Science University

The rhodopsin crystal structures provide a wealth of insights, yet mysteries remain. For example, in all of the structures, the ligand (retinal) is covered by a tight "lid" formed by the N-terminus and interhelical loops. How does the retinal get past all of this and get into and out of the binding pocket? Futhermore, what structural changes are involved in forming an active, signaling receptor? How is G-protein and arrestin binding coupled to ligand binding and release? And finally, how do different oligomeric states of rhodopsin (and other GPCRs) affect all these processes? We are trying to address these questions using some relatively novel biochemical and spectroscopic methods. I will discuss some of our recent methods and results (see references below), and entertain some thoughts and speculations concerning some of these mysteries.

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G Proteins And Their Accessory Proteins

Stephen M. Lanier, Ph.D.
Medical University of South Carolina

In addition to GPCR-mediated activation of G-protein signaling, nature has evolved creative ways to utilize the Gαβγ heterotrimer or Gα and Gβγ subunits for processing signals within the cell. In such situations, the G-protein subunits (Gα and Gβγ) may actually be complexed with alternative binding partners independent of the typical heterotrimeric Gαβγ. Such regulatory accessory proteins include receptor independent activators of G-protein signaling or AGS proteins that may influence the nucleotide binding properties of Gα and/or the interaction of G-protein subunits. Such entities may also regulate the transfer of signal from receptor to G-protein. The regulatory mechanisms operational with AGS proteins reveal unexpected diversity in the "G-switch" signaling mechanism and cellular functions regulated by G-proteins. AGS1-10 were identified in a yeast-based functional screen for receptor-independent activators of the pheromone response pathway. Three groups of AGS proteins can be defined based on their functional and biochemical properties. One group appears to influence nucleotide exchange whereas Group II influences subunit interactions independent of nucleotide exchange. Both Groups I and II interact with Gα subunits; whereas, Group III AGS proteins appear to interact with Gβγ subunits. Group II AGS proteins possess one or more signature G-protein regulatory or GPR motifs that stabilize the GDP bound conformation of Gα within the Gi/Go family. Areas of focus in the field include biochemical studies to define the mechanism and structural basis of the G-protein regulation, functional studies in various model systems and broad efforts to define modes of signal input to such entities as well as the downstream effectors involved in the signaling cascade. The concepts advanced with the studies of AGS and other accessory proteins reveal unexpected avenues for therapeutic manipulation of G-protein signaling as well as an expanded role for G-proteins in disease and physiological adaptation.

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Speaker selected from submitted abstracts

Is Helix VIII of G Protein-Coupled Receptors (GPCRs) a Lipid-Activated Signalling Sensor?

John Huynh, Len. K. Pattenden , Marie-Isabel Aguilar & Walter G. Thomas
Baker Heart Research Institute, Australia

The inactive GPCR structure of the bovine rhodopsin receptor [1] confirmed an additional helix VIII (H8) within the proximal carboxyl-terminus, positioned parallel to the plasma membrane. We identified H8 (Y302 to P322) of the angiotensin II type 1 receptor (AT1AR) interacts with high affinity to membranes [2,3]. This, with emerging evidence that the plasma membrane participates with GPCR regulation [4,5], poses the question: does membrane-binding of H8 constitute as a conserved conformational switch to control GPCR signalling and function?

We predict that a possible detethering of H8 away from the membrane may occur during the activation of the AT1AR. Rhodopsin structures reveal two residues within TM7 and H8 that may assist positioning H8 towards the membrane in the inactive state - sustaining this TM7-H8 interaction may affect receptor activation. To investigate this interplay, we introduced:

(1) Cysteine residues to form a disulfide bond between TM7 and H8.
(2) An unnatural palmitoylation site to anchor H8 to the membrane.

Results show H8 may not detether from the membrane, but undergo a subtle conformational change. An alanine scan conducted along H8 identified residues important for membrane tethering and receptor function. Some mutations displayed a reduced binding capacity (F304 to K311, P321A and P322A) and residue K308A resulted in no ligand binding despite the formation of mature glycosylated receptors. To sequentially identify the influence of each residue on folding, receptor maturation, cell-surface trafficking, receptor ligand affinity and specific membrane-lipid targeting, mutant receptors are subjected to ligand binding, internalisation and signalling assays, in conjunction with surface plasmon resonance assays of H8 peptides using liposomal surfaces with diverse lipid compositions.

Preliminary results show that H8 and its positioning with respect to membranes are crucial for GPCR signalling and function. Subtle conformational changes may allow certain residues within H8 to form specific and structured associations with components of the membrane.

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Interactions between GPCRs and Receptor Tyrosine Kinases

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The seminal observation that transactivation of the epidermal growth factor receptor (EGFR) can occur during GPCR-induced signaling opened a novel and still expanding field of cell regulation. The EGFR and its related erbB/HER receptor tyrosine kinases (RTKs) are directly activated by EGF, TGFα and other ligands, and are transactivated by a variety of GPCRs. Ullrich et al. found that GPCRs for ET-1, LPA, thrombin (1996) as well as angiotensin II, bombesin, bradykinin, and carbachol (2004), activate specific metalloproteinases (MMPs) that cause transactivation of the EGFR in human kidney and bladder cancer cells. Consequently, cellular responses to GPCRs via EGFR signaling can include survival, differentiation, proliferation and migration. Also, ligand-independent EGFR activation occurs in certain tumors that express mutant forms of the receptor. Many of the 40 known MMPs/ADAMs have been implicated in the transactivation of RTKs by GPCRs. Also, at least 20 other GPCRs, and certain RTKs, have been found to transactivate the EGFR. In addition to the EGFR, several other RTKs including the PDGFR, IGF-1R, FGFR-1, Trk_A and c-ME, are also activated by GPCRs. Recognition of the frequent role of EGFR overexpression and/or mutations in carcinogenesis has prompted the development of monoclonal antibodies and low-MW RTK inhibitors for use in the treatment of HER2-positive breast cancers and non-small cell lung cancers.

Reciprocal actions of RTKs on GPCRs also occur, but are much less common than the GPCR-induced activation of RTKs. These include actions of insulin on α_I and β_2 adrenergic receptors, with phosphorylation of specific tyrosine residues, and regulation of α_{IB} adrenergic receptors by TGF-β via an R(S/T)kinase. Also, in addition to angiotensin II-induced activation of the EGF receptor, the agonist-activated EGFR has been found to form a caveolin-dependent signaling complex with the angiotensin AT_1 receptor in cells that endogenously express the two receptors.

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GPCRs, arrestins, and ubiquitination

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Ubiquitination, a post-translational modification of proteins has been implicated in the endocytosis of several cell surface receptors. For several GPCRs, namely, the β2-adrenergic receptor (β2AR), the Vasopressin V2 receptor (V2R), the Chemokine receptor CXCR4, the Protease activated receptor 2 and the Neurokinin1 receptor, agonist-stimulated receptor ubiquitination is necessary for lysosomal targeting and receptor degradation, but not for receptor internalization [1].

β-arrestins, the multifunctional adaptor proteins originally discovered as desensitizers of G protein signaling, are now appreciated for their novel roles in trafficking and signaling of GPCRs [2]. β-arrestins not only recruit ubiquitinating enzymes to cell surface receptors, but are also themselves ubiquitinated upon receptor activation. For the β2AR, receptor internalization is facilitated by β-arrestin ubiquitination as carried out by the E3 ubiquitin ligase, Mdm2. Interestingly, receptors such as the β2AR, trigger transient β-arrestin ubiquitination, dissociate from β-arrestin during endocytosis and transiently and diffusely activate extra cellular-signal regulated kinases (ERK 1 and 2) in the cytosol and nucleus. In contrast, receptors such as the V2R trigger persistent β-arrestin ubiquitination, co-internalize with β-arrestin, and strongly and persistently activate a pool of endosomal ERK. β-arrestin ubiquitination appears to regulate its ability to assemble, endocytose and stabilize signalosomes containing the receptors and activated pERK. Receptor and β-arrestin ubiquitination thus serve as distinct and crucial regulatory steps that modulate GPCR life cycle and signal transduction.

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Speker selected from submitted abstracts

Allosteric agonists and allosteric modulators are differentially affected by mutations within the allosteric binding pocket of M2 muscarinic acetylcholine receptors

Karen J. Gregory, Lauren T. May, Vimesh A. Avlani, Patrick M. Sexton and Arthur Christopoulos

Muscarinic acetylcholine receptors (mAChRs) contain at least one allosteric site that is topographically distinct from the acetylcholine (ACh)-binding orthosteric site. Although numerous studies have investigated the structural basis of allosteric modulation at these receptors, far less is known about allosteric ligands that activate the receptor in their own right. We generated a number of M2 mAChRs harbouring mutations within the second extracellular loop and transmembrane domains 2 and 7 (Y80A, 172EDGE175->QNGQ, Y177A, T423A) and investigated their impact on the function of putative allosteric agonists (McN-A-343 and N-desmethylclozapine (NDMC)) and the interaction between ACh and prototypical allosteric modulators (gallamine and C7/3-phth) as assessed by receptor mediated phosphorylation of extracellular-signal regulated kinases 1 and 2 (ERK1/2). Very high negative co-operativity was observed between ACh and the allosteric modulators, gallamine and C7/3-phth, across all mutant M2 receptors. In agreement with previous studies, allosteric site mutations (Y177A, 172EDGE175->QNGQ, 172EDGE175->QNGQ +Y177A+T423A) significantly reduced the affinity of gallamine and C7/3-phth. In contrast, Y80A significantly increased the affinity of C7/3-phth, but didn't change the affinity of gallamine. Significant enhancements of efficacy and potency of McN-A-343 and NDMC were observed at Y177A. These findings suggest that allosteric agonists utilize a different binding mode to prototypical (non-agonistic) modulators at M2 mAChRs.

Multiplexing resonance energy transfer approaches to study GPCR signalling complexes in living cells

Michel Bouvier
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A growing body of evidence suggests that G protein-coupled receptors (GPCR) signalling is controlled by the formation of dynamically regulated multimeric complexes. In addition to their cognate G proteins, various scaffolding and signalling partners can be recruited to the receptors thus determining the selectivity and efficacy of the signal transduction. Although the occurrence of these complexes has been well investigated in vitro, their ontogeny and their dynamic regulation in space and time in living cells are still poorly understood. Such understanding is crucial to apprehend the consequences that these newly appreciated complexes may have on drug discovery. To directly assess the real-time assembly of GPCR signalling complexes in living cells, we used a combination of biochemical and biophysical approaches. In particular, multiplexing Bioluminescence and Fluorescence Resonance Energy Transfer (BRET and FRET) techniques with bimolecular fluorescence complementation (BiFC) allowed to monitoring the assembly of multiple partners simultaneously. In addition to reveal the existence of unanticipated complexes, these studies indicate that different ligands may favour the formation of distinct signalling complexes thus providing a molecular explanation for the phenomenon of ligand biased efficacy. These results will be discussed in the context of their potential impact on drug discovery.

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Use of genetically engineered mice to unravel the functions of dopamine receptors

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Dopamine (DA) is a major neuromodulator of the central nervous system, where it regulates diverse physiological functions ranging from locomotion to hormone synthesis and release. DA signalling is also stimulated in the response to drugs of abuse, underlying its participation in the establishment of addiction. DA elicits its control through the binding to membrane receptors which transduce the signal inside the neurons. Five different DA receptors (D1 to D5) have been isolated, they all belong to the family of seven transmembrane domain G-protein coupled receptors. Dysfunctions of the DA signalling has been evoked in major neurological and psychiatric human diseases further stimulating the research on how these key receptor work in health and disease. Our research focuses on dopamine D2 receptors, one of the lading actors of the dopaminergic system. These proteins control not only post-synaptic dopamine-mediated responses, but also the presynaptic control of DA synthesis and release. Interestingly, two isoforms of dopamine D2 receptors are present in the brain, both isoforms are generated from the same gene by a mechanism of alternative splicing. We have addressed the study of the function of these isoforms by generating genetically engineered mice in which the expression of the dopamine D2 receptors is either abolished or modified. The biochemical, molecular and behavioral analyses of these mice are clarifying the physiological role of these proteins in specific D2-mediated functions. In particular, we have been able to demonstrate that the two isoforms, D2L and D2S, have different functions in vivo. D2L appears to have mainly post-synaptic activities while D2S has preponderant presynaptic release-modulating functions. Using these mice we have recently assessed the role of D2S and D2L in the stimulation of intracellular signaling pathways to psychostimulants. Our results show different involvment of each dopamine D2 isoform in D2-dependent physiological effects.

Kisspeptin and GPR54 in the regulation of puberty and reproduction

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The G protein-coupled receptor, GPR54, and its endogenous ligands, kisspeptins (derived from the *Kiss1* gene), were first identified on the basis of their ability to inhibit tumor invasion and metastasis by regulating cell proliferation and motility. More recently, the identification of loss-of-function mutations in the gene encoding GPR54 has highlighted a previously unrecognized pathway in the physiologic regulation of puberty and reproduction. Patients with loss-of-function mutations in GPR54 have hypogonadotropic hypogonadism, and mice lacking GPR54 similarly fail to undergo puberty and have low levels of sex steroids and gonadotropic hormones. These observations have led to the discovery of an important role for the kisspeptin-GPR54 system in activation of hypothalamic GnRH release, thereby serving as a pivotal factor in the pubertal activation of the reproductive cascade. Studies in animal models have demonstrated localization of KiSS-1 mRNA and protein in the hypothalamus, colocalization of GPR54 in GnRH neurons, GnRH-dependent activation of LH and FSH release by intracerebroventricular or peripheral administration of kisspeptin, and increased hypothalamic KiSS-1 expression at the time of puberty. In addition, kisspeptin has been implicated in the induction of the preovulatory GnRH/LH surge. Stimulation of GPR54 leads to activation of phospholipase C with Ca^{2+} mobilization, phosphatidylinositol turnover, and stimulation of ERK phosphorylation, suggestive of coupling with proteins of the G_q class. We have shown that loss-of-function mutations interfere with activation of these signaling pathways by kisspeptins. Conversely, we hypothesized that gain-of-function mutations in GPR54 may be a cause of gonadotropin-dependent precocious puberty (GDPP). We have identified a missense mutation in the carboxyterminal tail of the receptor, R386P, in a girl with idiopathic GDPP. In a heterologous cell system transiently transfected with either wild type (WT) or R386P GPR54, no differences in inositol phosphate (IP) accumulation were detected for the R386P mutant compared to WT GPR54 following stimulation with increasing doses of kisspeptin, indicating no effect of the mutation on ligand binding affinity or maximal kisspeptin responsiveness. Furthermore, no constitutive activity was detected. However, a time-course study demonstrated a slower rate of decline in IP levels in cells transfected with the R386P mutant, resulting in significantly higher intracellular IP levels following 12 hours or longer of kisspeptin stimulation. This prolonged stimulatory effect was confirmed by measurement of another downstream effector of GPR54 signaling, ERK phosphorylation. These differences suggest a decreased rate of receptor desensitization following ligand stimulation, thereby resulting in a greater, more prolonged cellular response to a given exposure to kisspeptin, to contribute to the phenotype of precocious puberty. Modulation of the activity of GPR54 is a potential target for the treatment of pubertal and other reproductive disorders. To this end, we are currently performing a high throughput screen of a small molecule compound library to identify potential GPR54 agonists and antagonists.

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GPCRs in arousal and anxiety

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For many decades, research on arousal/sleep or anxiety disorders has focused on classical neurotransmitter systems, such as GABA or the noradrenergic system, and most therapeutically used drugs modulate either one of these. Only recently it became apparent, that neuropeptides play critical roles at the neurochemical basis of these behaviors. At the center of attention are currently the orexin/hypocretin peptides as modulators of arousal (1) and corticotropin-releasing factor (CRF) as an integrator of emotional responses and stress (2), but there are numerous reports of additional and overlapping functions for both of these neuropeptide systems. In addition, a growing number of other peptide transmitters have recently been implicated in arousal or its behavioral counterpart sleep (e.g. thyrotropin-releasing hormone, ghrelin, neuropeptide Y [NPY]) and even more examples of anxiolytic or anxiogenic activities have been discovered for other neuropeptides, such as NPY, vasopressin, cholecystokinin, nociceptin/orphanin FQ, galanin or melanin-concentrating hormone, to name a few. Whether any of these peptides is functionally involved in the pathogenesis of sleep or anxiety disorders, or whether they might yield valuable therapeutic targets for novel types of drugs to treat these conditions remains to be seen.

We have recently discovered that a newly found neuropeptide, termed neuropeptide S (NPS), promotes profound arousal while suppressing all stages of sleep (3). In addition, NPS also possesses anxiolytic-like properties by reducing defensive behaviors of rodents to potentially fearful or unfamiliar environments. This pharmacological spectrum is fairly unique as arousal-promoting compounds such as classical stimulants are usually anxiogenic in the same tests while anxiolytics of the benzodiazepine class are well known for their sedative effects. We have begun to study the pharmacology, neuroanatomy and functional interaction of the NPS system with other transmitters in the brain in order to understand its physiological functions and validate its potential as a drug target. We found that NPS is synthesized in only a few brainstem nuclei, most prominently in a previously uncharacterized nucleus found in between the noradrenergic locus coeruleus and Barrington's nucleus. NPS is co-localized with other excitatory transmitters such as glutamate, CRF and acetylcholine (4), and activation of NPS receptors causes increases in intracellular Ca^{2+} and cAMP, underlining its excitatory role. Recent studies from other groups have demonstrated anorectic effects of NPS and implicate interactions between the NPS system, orexin/hypocretin and/or CRF. Obviously, our knowledge about the physiological functions of NPS is at a very early stage but the promising results obtained thus far warrant further attention and research into this exciting new transmitter molecule.

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Speaker selected from submitted abstracts

Vasoactive intestinal polypeptide receptor 2 female knockout mice are resistant to diet-induced obesity

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Vasoactive Intestinal Polypeptide Receptor 2 (VPAC2) is G protein-coupled receptor (GPCR) present at high levels in white adipose tissue (WAT) and pancreatic islets in humans. A recent publication has shown that a VPAC2-selective peptide enhances glucose-stimulated insulin secretion in both rat and human islets. VPAC2 knockout (KO) mice present anomalies in circadian rhythm and immunomodulation. Another study showed that VPAC2 KO mice on chow diet had increased metabolic rate. In the study presented here, KO mice were characterized at 20 weeks of age following eleven weeks on a high fat diet to ascertain the role of this receptor in insulin sensitivity and glucose homeostasis. Surprisingly, VPAC2 KO female mice weighed less than their wild type (WT) counterparts (31.8 ± 0.8 *vs*. 50.4 ± 1.6 g) while male KO mice were not different from controls (43.6 ± 2 vs. 47.6 ± 1.3 g). Associated with the reduced body weight, female but not male KO mice demonstrated enhanced glucose disposal following an OGTT and marked insulin sensitivity during an ITT compared to wild type controls. In addition, female VPAC2KO mice showed enhanced ambulatory behavior during the early dark phase when compared to female WT and to male (KO and WT) mice. These data show that VPAC2 KO female mice are resistant to dietary induced obesity with improved glucose tolerance and increased insulin sensitivity.

Speaker selected from submitted abstracts

Dynorphin A activates bradykinin receptors to maintain neuropathic pain

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Endogenous opioid neuropeptides include the enkephalins, the endorphins and the dynorphins. While the physiological actions of enkephalins and endorphin peptides are the result of inhibitory effects, predominantly on neuronal cells, to elicit analgesia, the physiological role of the dynorphins remains more obscure. Dynorphin A(1-17) elicits excitatory actions that are not mediated by opioid receptors. Intrathecal dynorphin A(1-17) produces little antinociceptive actions; higher doses induce excitatory effects including hyperalgesia, loss of muscle tone, paralysis, and severe motor dysfunction. Dynorphin A stimulates the release of excitatory amino acids from cultured neurons and induces an increase in intracellular calcium. In spinal cord slices, dynorphin A enhances the evoked release of pronociceptive transmitters such as calcitonin gene related peptide (CGRP) and intrathecal infusion of dynorphin A stimulates the release of excitatory amino acids and prostaglandin E2 in the spinal cord, demonstrating pronociceptive actions of this peptide. Our data demonstrate that dynorphin A directly binds to bradykinin receptors and stimulates calcium influx via the L-type and P/Q type voltage sensitive calcium channels. This agonist action of dynorphin is not predicted by structural similarity between dynorphin and bradykinin, or between opioid receptors and the bradykinin receptors. The coupling mechanisms of these two classes of receptors are also different. This novel action of dynorphin at the bradykinin receptors underlies the hyperalgesia produced by pharmacological administration of dynorphin by the spinal route in rats and mice. Blockade of spinal B1 or B2 receptor also reverses persistent neuropathic pain but only when there is sustained elevation of endogenous spinal dynorphin, which is required for maintenance of neuropathic pain. These data reveal a mechanism for endogenous dynorphin to promote pain through its agonist action at bradykinin receptors and suggest new avenues for therapeutic intervention.

The role of GPR30 in estrogen signaling

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Estrogen is a critical hormone in the development, normal physiology and pathophysiology of numerous human tissues. Although the functions of estrogen have traditionally been ascribed to the nuclear receptor family of soluble transcription factors, recent studies demonstrate that the G protein-coupled receptor, GPR30, binds estrogen and mediates rapid cellular responses to this steroid [1]. Unlike conventional GPCRs, GPR30 is localized predominantly in the endoplasmic reticulum, where signaling is initiated upon ligand binding. Signaling initiated by GPR30 is mediated by transactivation of epidermal growth factor receptors at the cell surface. Effector functions appear to be regulated by a combination of this transactivation and direct signaling events triggered by GPR30. With the recent description of a GPR30-specific agonist [2], studies are now able to begin to dissect the functions of GPR30 vs. classical estrogen receptors. Recent work shows that GPR30 is capable of mediating gene expression and cellular proliferation [3]. In addition, GPR30 is overexpressed in both breast [4] and endometrial [5] cancers. In the latter, GPR30 overexpression is inversely correlated with survival, suggesting GPR30 to be an important prognostic indicator.

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Inverse Agonist vs. Full Antagonist Properties of Rimonabant in Systems with Overexpressed vs. Native CB1 Receptors

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In CHO cells overexpressing human CB1 receptors, rimonabant antagonizes MAP kinase activation induced by a CB1 agonist but also decreases per se MAP kinase activity (Bouaboula et al. J Biol Chemistry 1997, 272:22330-22339). These observations led to the hypothesis that under certain conditions in heterologous systems, rimonabant may behave as an inverse agonist. Whether or not such preparations are endocannabinoid-free is still a matter of debate. Functional studies using in vitro systems expressing native CB1 receptors demonstrated that rimonabant reverses the inhibitory effect exerted by known cannabinoid agonists on adenylyl cyclase activity and on electrically evoked contractions of the mouse vas deferens. In vivo, rimonabant was able to cross the blood-brain barrier after peripheral administration and produced a long lasting occupancy of CB1 receptors. In rodents, rimonabant antagonized the classical responses induced by cannabinoid receptor agonists (hypothermia, ring-immobility, antinociception, and cannabinoid cue). Thus, rimonabant behaves in vitro and in vivo as a potent and selective antagonist of the cannabinoid CB1 receptor. Several preclinical studies have shown that the impact of rimonabant is minimal or nonexistent in the absence of pretreatment with or concomitant administration of a CB1 agonist, suggesting that it does not behave as an inverse agonist. In aggregate these findings provide little evidence that rimonabant has inverse agonist properties in vivo. However, in the literature rimonabant has been referred to as an antagonist with inverse agonist properties.

Novel aspects of the melanocortin receptors

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The MC3-R remains the most enigmatic of the melanocortin receptors with regard to its physiological functions. The receptor is expressed both in the CNS and in multiple tissues in the periphery, and is an inhibitory autoreceptor on POMC neurons. Evidence for a role for the MC3-R as an inhibitory autoreceptor on arcuate POMC neurons derives from four findings **1)** coexpression of MC3-R mRNA with POMC and NPY in the arcuate, **2)** inhibition of the spontaneous firing of POMC neurons after application of a MC3-R specific agonist[1], **3)** stimulation of food intake by peripheral administration of a MC3-R–specific agonist[2], and **4)** demonstration of an enhanced inhibition of food intake and weight loss after IL-1 administration in the MC3-R null mouse[3]. While the MC3-R may act to suppress the anorexic POMC neurons, paradoxically, an obesity syndrome results when the receptor is deleted from the mouse genome[4]. Of course, the MC3-R obesity syndrome is quite unusual, particularly when compared with diet-induced obese (DIO) and MC4-R null obesity models. In these latter models, animals demonstrate hyperphagia, increase in lean mass, increase in adipose mass across all depots, hyperlipidemia, hyperinsulinemia, hepatic steatosis, and pro-inflammatory changes including increased expression of chemokine (C-C) ligand 2 (CCL2)/monocyte chemoattractant protein-1 (MCP-1) in adipose tissue. Thus, the MC4-R null and DIO animals serve as models for human metabolic syndrome described above. In contrast, deletion of the MC3-R produces an obesity syndrome with a loss in lean mass and increased adipose mass. Compared with the MC4-R null, hyperphagia is not observed in the MC3-R null, even on high fat chow. Body temperature and resting energy expenditure appear normal in the MC3-R null, as does diet-induced thermogenesis, arguing against a defect in energy expenditure accounting for the obesity in this model. Remarkably, the MC3-R null and MC4-R null mice both develop considerable obesity, yet the MC3-R null mice appear resistant to the pro-inflammatory changes and symptoms of metabolic syndrome seen in other models of obesity in the mouse.

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Leucocyte chemoattractant receptors: New molecules and new concepts

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Over the recent years, we have characterized the natural ligands of previously orphan G protein-coupled receptors. ChemR23 is related to chemokine receptors and is expressed in dendritic cells and macrophages. Its natural ligand, chemerin, was purified and characterized from a human inflammatory fluid. Chemerin is secreted as a precursor of low biological activity, which, upon proteolytic cleavage of its C-terminal domain, is converted into a potent and highly specific agonist of ChemR23 (Wittamer et al. 2003, 2005). Chemerin is abundant in a diverse set of human inflammatory fluids. Chemerin is structurally and evolutionary related to the cathelicidin precursors (anti-bacterial peptides), cystatins (cysteine protease inhibitors) and kininogens. Chemerin promotes calcium mobilization and chemotaxis of immature dendritic cells and macrophages in a ChemR23-dependent manner. Chemerin appears therefore as a potent chemoattractant protein of a novel class, which requires proteolytic activation, and is specific for antigen-presenting cells. The mouse chemerin/ChemR23 system was also studied. The binding and functional parameters were very similar to those observed in human, with full cross-reactivity between the human and mouse components. As for human chemerin, it was found that the carboxy-terminal nonapeptide of mature mouse chemerin keeps high potency on ChemR23, and structure-function analysis demonstrated that the four hydrophobic residues and the glycine are essential for this activity. A knock out model is being used in order to determine the role of ChemR23 in disease models.

We also identified recently a natural ligand for FPRL2, a human receptor of the formyl peptide receptor (FPR) family. F2L, a 21 amino acid peptide derived from heme-binding protein (HBP), was isolated from a spleen extract and activates FPRL2 in the low nanomolar range. It promotes calcium mobilization and chemotaxis of monocytes and monocyte-derived DCs (Migeotte et al. 2005). We have recently determined, among the seven mouse FPR family members, the mouse counterpart of FPRL2. By testing the whole set of mouse receptors, we identified Fpr2 as the only mouse receptor responding to F2L. Mouse neutrophils, which express Fpr2, in addition to macrophages and dendritic cells, respond to human and mouse F2L in both calcium flux and chemotaxis assays. Neutrophils from mice genetically deficient in Fpr2 failed to respond to F2L (Gao et al. 2007).

Besides, we investigate how chemoattractant receptors function in native cells as a result of their oligomerization properties. Indeed, most G protein-coupled receptors are able to form homo- and heterodimers, but the functional consequences of this process remain often unclear. Using BRET assays, we demonstrated that CCR5 and CCR2 heterodimerize with the same efficiency as they homodimerize. Stimulation by their respective ligands does not influence receptor homo- or heterodimerization. No cooperative signaling was observed following co-stimulation of the two receptors. However, we showed that CCR5-specific ligands efficiently prevented MCP-1 binding when CCR5 and CCR2b were co-expressed. Similar observations were made for the CCR2-selective ligand MCP-1 (El Asmar et al. 2005). We later showed that the rate of radioligand dissociation from one unit of the heterodimer in “infinite” tracer dilution conditions is strongly increased in the presence of an unlabelled chemokine ligand of the other unit (Springael et al. 2006). Taken together, our observations suggest that a receptor dimer can only bind a single chemokine, and that the interaction between chemokine receptor heterodimer is of allosteric nature. This concept has more recently been extended to other chemokine receptor couples, and the mechanisms behind the observations are being studied.

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The function and regulation of G protein-coupled glutamate receptors in the neural network

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Neurotransmitter glutamate is essential for a variety of neuronal functions. Glutamatergic neurotransmission is mediated by ionotropic receptors (iGluRs) and G protein-coupled metabotropic receptors (mGluRs). iGluRs are glutamate-gated cation channels and are subdivided into AMPA/kainate and NMDA receptors. We elucidated the molecular entities of the family of mGluRs and NMDA receptors by a functional cloning that combined the Xenopus oocyte expression system and electrophysiology. mGluRs consist of 8 different subtypes and are classified into three groups: group 1 (mGluR1 and 5), group 2 (mGluR2 and 3), and group 3 (mGluR4, 6, 7, and 8)(1). mGluRs posses an unusually large extracellular glutamate-binding domain and the three-dimentional structures of an active and an inactive form of mGluR1 are disclosed by X-ray crystallography. Individual mGluRs are distinctly expressed at both subcellular levels and neuronal cell types and bear specialized functions in various brain regions. It remains, however, poorly understood how mGluRs and iGluRs act distinctly or cooperatively in glutamatergic neurotransmission. We first discuss synaptic mechanisms underlying light and dark segregation at photoreceptors-bipolar cells in the retina, indicating that mGluR6 and AMPA receptors mediate light-induced ON and dark-induced OFF responses, respectively, thus contributing to discrimination of light and dark signals(1). This segregative role of mGluR and iGluR in synaptic transmission seems to be rather unusual. We show that inhibitory group 2 mGluR, most likely mGluR2, acts cooperatively with excitatory AMPA receptors at mitral cell-granule cell synapses in the accessory olfactory bulb and is crucial for odorant discrimination and olfactory memory formation. The dual function of mGluR and iGluR in glutamatergic transmission is also seen at cerebellar granule cell-Golgi cell synapses and is important for spatiotemporal regulation of granule cell excitability(2,3). The roles and integrative mechanisms of mGluR and iGluR in synaptic transmission will be discussed in my presentation.

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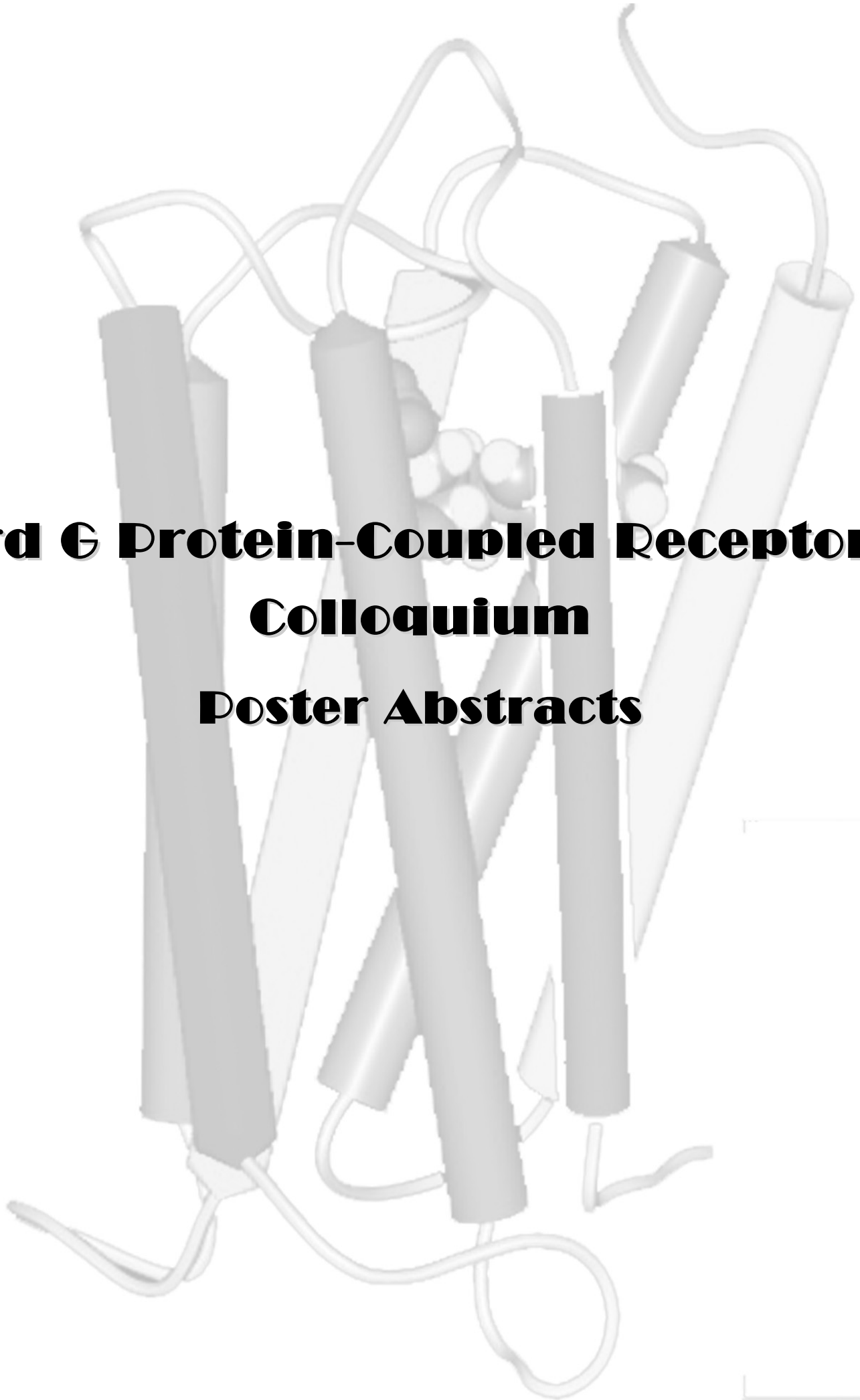

3rd G Protein-Coupled Receptors Colloquium

Poster Abstracts

Obestatin is not the cognate ligand for GPR39

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Using a bioinformatic approach, Zhang *et al*. (Science 310, 996, 2005) reported the identification of a novel neuropeptide designated obestatin, which is derived from the ghrelin precursor. They subsequently purified obestatin from a rat stomach extract and showed that the synthetic replicate of the peptide suppresses food intake and decreases body-weight gain in mice. Finally, the authors reported that obestatin is a cognate ligand for the orphan G protein–coupled receptor GPR39.

In order to further characterize whether obestatin is the natural ligand for GPR39, we transfected CHO cells with human GPR39 cDNA corresponding to the GPR39 sequence. Two distinct stably-transfected CHO cell lines expressing either the Flag- or the EGFP-tagged human GPR39 receptor were established. We then synthesized human obestatin and thoroughly characterized the synthetic peptide by mass spectrometry and microsequencing. The effects of this peptide on various cellular functional assays were then evaluated in parrallel with those of a commercial source of synthetic human obestatin.

In presence of the commercially available [^{125}I]-obestatin at a concentration of 5×10^{-10} M, no specific binding was observed in GPR39-transfected CHO cell membranes and in crude membranes from pituitary, a tissue known to express GPR39. In addition, incubation of CHO cells stably-expressing GPR39 receptor with human obestatin, at concentrations which range from 10^{-9} to 10^{-6} M for 20 minutes or 16 hours did not induce any significant increase in cAMP formation whereas treatment of the cells with 10 µM forskolin provoked a robust stimulation of cAMP production. The effect of obestatin on intracellular calcium mobilization was then studied and compared with that of ATP used as an internal control. Incubation of CHO cells stably expressing either the Flag- or the EGFP-tagged human GPR39 receptor with human obestatin, at doses ranging from 10^{-9} to 10^{-6} M did not modify intracellular calcium concentration in either one of the two cell lines. The agonistic activity of obestatin on GPR39 was further evaluated using an internalization assay that has successfully been used for other GPCRs notably for the ghrelin 1a receptor that belongs to the same receptor subfamily as GPR39. Incubation of CHO cells stably expressing the fluorescent GPR39 receptor with obestatin, at concentrations ranging from 10^{-9} to 10^{-6} M for 20 minutes did not promote internalization of GPR39. Finally, intracerebroventricular injection of obestatin (8 nmol per kg body weight) in fasting mice for 16 hours had no significant effect on food consumption during the first hour following administration of the peptide. During the two subsequent hours, food intake was slightly reduced compared to control animals. However, measurement of cumulative food intake during the 3 hours following the injection of obestatin did not reveal any significant anorexigenic effect of the peptide.

Taking together, these results provide evidence that obestatin is not the endogenous ligand of the orphan receptor GPR39 although we found that the peptide given centrally provokes a modest inhibitory effect on food intake.

Dopamine D2 receptor regulates reactive oxygen species production in the kidney

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Alterations in D2-like dopamine receptor function have been reported in essential hypertension. A polymorphism in exon 6 of the D2 receptor (D2R) gene is associated with elevated blood pressure and a Taq1 polymorphism is associated with human essential hypertension. We have previously shown that disruption of D2R in mice (D2-/-) results in high blood pressure. We hypothesized that the increased blood pressure in D2-/- is related to increased NADPH oxidase activity and reactive oxygen species (ROS). We determined the mRNA expression of the NADPH oxidase subunits p22phox, p40phox, p47phox, p67phox, Rac1, Rac2, Nox1, Nox2, and Nox4 by RT-PCR in kidneys of D2-/- and their wild type littermates, D2+/+. The mRNA expression of Nox1, Nox2 and Nox4 was increased 30±3, 14±1 and 29±2%, respectively in D2-/- ($p<0.05$ vs D2+/+, n=5 per group).The mRNA expression of the other subunits tested was similar in both groups. The protein expression of Nox1, Nox2 and Nox4, determined by western blot, was increased in kidneys of D2-/- mice by 45±8, 89±10 and 55±5%, respectively ($p<0.05$ vs D2+/+). We also found that the expressions of both mRNA and protein of the constitutive antioxidant enzyme heme-oxigenase-2 were decreased in kidneys of D2-/- mice by 28±3 and 64±7% respectively ($p<0.05$ vs D2+/+). However, the expression of the inducible heme-oxigenase-1 was unchanged. The urinary excretion of 8-isoprostane (77±15 vs 35±8 ng/mg creatinine [D2+/+]; $p<0.05$, n=9), a marker of oxidative stress, and activity of renal NADPH oxidase (121,300±15,310 vs 81,200±9,870 [D2+/+] LU/mg protein; $p<0.05$; lucigenin assay) were also increased in D2-/- mice. Treatment with hemin, an inducer of heme oxygenase (50 □mol, IP, for 24 h), normalized blood pressure in anesthetized D2-/- mice (systolic: vehicle: 123±5; hemin: 89±6 mmHg; $p<0.05$; n=5) but had no effect in D2+/+ mice (systolic: vehicle: 103±2; hemin: 100±1 mmHg; n=5). Treatment with apocynin, a NADPH oxidase inhibitor (3 mg/k/day, via osmotic mini-pump, 10 days), also normalized blood pressure in D2-/- mice (systolic: D2-/-, 96±2; D2+/+, 96±1 mmHg; n=5 per group). Treatment of mouse renal proximal tubule cells in culture for 24 h with quinpirole (1□M), a mixed D2/D3 agonist, decreased the protein expression of Nox1, Nox2 and Nox 4 by 15±2, 16±3 and 12±1% respectively (n=6 per group). The effect of quinpirole was completely abolished by the selective D2 receptor antagonist L-741,262 (1□M) but not by GR103,691 (1□M), a selective D3 receptor antagonist. Treatment of mouse renal proximal tubule cells in culture for 24 h with quinpirole also decreased the activity of NADPH oxidase by 15±1% ($p<0.05$; n=6 per group), an effect that was completely blocked by the D2 receptor antagonist but not by the D3 receptor antagonist. Our results show that the D2R is involved in the regulation of ROS production and NADPH oxidase activity and expression suggest that altered D2R function, by direct and indirect mechanisms, may result in increased oxidative stress and hypertension.

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Real-time analysis of agonist-induced activation of PAR1 / Gαi1 protein complex measured by BRET in living cells

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Presenter: **Mohammed Akli Ayoub**

G protein-coupled receptors (GPCRs) transmit extracellular signals into the cells by activating heterotrimeric G proteins, a process that is often followed by receptor desensitization. Monitoring such a process in real time and in living cells will help better understand how G protein activation occurs. Energy transfer-based approaches (FRET and BRET) were recently shown to be powerful methods to monitor GPCRs-G proteins association in living cells. Here, we used a BRET technique to monitor the coupling of the protease-activated receptor 1 (PAR1) to Gαi1 protein. A specific constitutive BRET signal can be measured between non-activated PAR1 and the Gαi1 protein expressed at physiological level. This signal is insensitive to Pertussis toxin (PTX) and PAR1 antagonist and likely reflects the pre-assembly of these two proteins. The BRET signal rapidly increases upon receptor activation in a PTX-sensitive manner and then the signal returns to the basal level after few minutes. The desensitization of the BRET signal is concomitant with ß-arrestin-1 recruitment to the receptor, consistent with the known rapid desensitization of PARs. The agonist-induced BRET increase was dependent on the insertion site of fluorophores in proteins. Taken together, our results show that BRET between GPCRs and Gα proteins can be used to monitor the receptor activation in real time and in living cells. Our data also revealed that PAR1 can be part of a pre-assembled complex with Gαi1 protein, resulting either from a direct interaction between these partners or from their co-localization in specific microdomains, and that receptor activation likely results in rearrangements within such complexes.

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Development of a Time-resolved Fret High-throughput Assay to Identify Inhibitors of the RGS/GalphaO Interaction

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Regulator of G-protein Signaling (RGS) proteins are important regulatory molecules in the transduction of G-Protein Coupled Receptor (GPCR) signaling. By increasing the rate of intrinsic GTPase activity of the G alpha subunit, RGS proteins effectively shorten the duration and decrease the magnitude of signals from heterotrimeric G protein subunits. It has been proposed (Nat. Rev. Drug Disc. 1:187, 2002) that small molecule inhibitors of RGS proteins may provide a novel mechanism for therapeutic intervention in diseases that stem from deficiencies in GPCR signaling. To this end, we developed a high throughput time-resolved fluorescence resonance energy transfer (TR-FRET) assay to quantify the interaction between RGS4 and Galpha O. The assay utilizes a lanthanide-chelate donor covalently attached to Galpha O and an AlexaFluor-488 acceptor attached to RGS4. The Kd for binding in the presence of GDP:AlF4- is 35+/-4 nM with much lower affinity with GDP alone. Z' values of 0.50-0.88 were obtained in 384-well plate measurements. High throughput chemical screens using this approach are underway. Supported by NIH R01-GM39561 & T32-GM008597 (Chemical Biology Interface Training Program).

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Mu Opioid receptor Activation without arrestin recruitment

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Opioid receptor agonists such as morphine, methadone and fentanyl activate mu opioid receptors to mediate analgesia in vivo. Each agonist stimulates G protein coupling to Gi alpha proteins and in turn inhibits adenylyl cyclase. Opioid agonists also stimulate ERK activation via actions at the mu opioid receptors. Morphine differs from methadone and fentanyl in that it promotes very little phosphorylation of the receptor and leads to weak beta-arrestin recruitment and little receptor internalization. These limitations of morphine binding can be overcome, however, by the overexpression of GPCR kinases (GRKs). Studies in mice lacking beta-arrestin2 indicate that the interaction between the mu opioid receptor and beta-arrestin2 impact on the physiological responsiveness to the drug as morphine-induced behaviors are dramatically altered in these animals (enhanced analgesia, reduced tolerance, reduced side effects). However, analgesic responses to drugs such as fentanyl and methadone, which robustly phosphorylate receptors, recruit beta-arrestins and internalize receptors, are not altered in these mice. These findings suggest that eliminating the mu opioid receptor- arrestin interactions may be therapeutically beneficial. Here we discuss novel mu opioid receptor ligands, derived from the active hallucinogen from Salvia divinorum, salvinorin A, that do not lead to the recruitment of beta-arrestins, even with GRK overexpression. We anticipate that such drug development will be a useful tool for determining the role of beta-arrestins in mu opioid receptor-mediated signaling and may hopefully lead to the development of improved opioid analgesics.

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Importance of Carboxil Terminus in the Regulation of Alpha1A and Alpha1B-Adrenergic Receptors

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Adrenoreceptors (ARs) are a heterogeneous group of hormone/neurotransmitter receptors that mediate the central peripheral actions of the natural adrenergic amines, adrenaline and noradrenaline, comprising three major types: alpha1-, alpha2- and beta-ARs. It is well known that alpha1-adrenoceptors are mainly coupled to Gq/11 to stimulate phospholipase C activity. Three alpha1-ARs have been cloned the alpha1A-, alpha1B-, and alpha1D-ARs. These receptors are differentially regulated by activation of protein kinase C (PKC), in a process called heterologous modulation. alpha1A-ARs are less sensitive to heterologous modulation than alpha1B-ARs. The primary goal of this work was study the roles(s) of the carboxy termini of both ARs through construction of chimeric receptors, in which the carboxyl terminus domains were interchanged, and truncated mutant of alpha1A-AR lacking the last 118 amino acids. These ARs were transfected in HEK293 cells. Both chimeric receptor and truncated mutant showed partial desensitization in response to TPA, as evidence by noradrenalne-stimulated increase in intracelular calcium. Nervertheless, the alpha1AB chimeric receptors increase their phosphorylation state in reponse to TPA, much more than the alpha1BA chimeric receptor. Interestingly, the truncated alpha1A ARs increase their phosphorylation more than alpha1A ARs. These data further emphasize the importance of the carboxyl terminus of alpha1-ARs in receptor phosphorylation.

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Vasoactive Intestinal Polypeptide Receptor 2 Female Knockout Mice are Resistant to Diet-Induced Obesity

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Vasoactive Intestinal Polypeptide Receptor 2 (VPAC2) is G protein-coupled receptor (GPCR) present at high levels in white adipose tissue (WAT) and pancreatic islets in humans. A recent publication has shown that a VPAC2-selective peptide enhances glucose-stimulated insulin secretion in both rat and human islets. VPAC2 knockout (KO) mice present anomalies in circadian rhythm and immunomodulation. Another study showed that VPAC2 KO mice on chow diet had increased metabolic rate. In the study presented here, KO mice were characterized at 20 weeks of age following eleven weeks on a high fat diet to ascertain the role of this receptor in insulin sensitivity and glucose homeostasis. Surprisingly, VPAC2 KO female mice weighed less than their wild type (WT) counterparts (31.8 ± 0.8 *vs*. 50.4 ± 1.6 g) while male KO mice were not different from controls (43.6 ± 2 vs. 47.6 ± 1.3 g). Associated with the reduced body weight, female but not male KO mice demonstrated enhanced glucose disposal following an OGTT and marked insulin sensitivity during an ITT compared to wild type controls. In addition, female VPAC2KO mice showed enhanced ambulatory behavior during the early dark phase when compared to female WT and to male (KO and WT) mice. These data show that VPAC2 KO female mice are resistant to dietary induced obesity with improved glucose tolerance and increased insulin sensitivity.

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Role of Group-Conserved Residues in the Helical Core of beta2-adrenergic receptor

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G protein coupled receptors (GPCRs) belonging to class A contain several highly conserved (>90%) amino acids in their transmembrane helices. Results of mutational studies of these highly conserved residues suggest a common mechanism for locking GPCRs in an inactive conformation and for their subsequent activation upon ligand binding. Recently, a second set of sites in the transmembrane helices has been identified where amino acids with small side chains, such as Gly, Ala, Ser, Thr, and Cys, are highly conserved (> 90%) when considered as a group. These group-conserved residues have not been recognized as having essential structural or functional roles. To determine the role of group-conserved residues in the Beta2-adrenergic receptor (b2-AR), amino acid replacements guided by molecular modeling were carried out at key positions in transmembrane helices H2 to H4. The most significant changes in receptor expression and activity were observed upon replacement of the amino acids, Ser161 and Ser165, in H4. Substitution at these sites by larger residues lowered the expression and activity of the receptor, but did not affect specific binding to the antagonist ligand dihydroalprenolol. A second site mutation, V114A, rescued the low expression of the S165V mutant. Substitution of other group-conserved residues in H2 – H4 by larger amino acids lowered receptor activity in the order, Ala128, Ala76, Ser120 and Ala78. Together these data provide the first comprehensive analysis of group-conserved residues in a class A GPCR, and allow insights into the roles of these residues in GPCR structure and function.

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Interaction of ETA and ETB endothelin receptors expressed in HEK-293 cells

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Endothelin-1 (ET-1) constricts blood vessels by acting at ETA (ETAR) and ETB (ETBR) receptors on vascular smooth muscle cells (VSMCs). VSMCs express both receptors complicating studies of the localization and function of each receptor. We investigated the localization and function of wild type or epitope-tagged ETAR and ETBR expressed in HEK-293 cells. Receptor localization was studied using an ELISA method and confocal microscopy. 125ET-1 displacement assays were used to study ligand binding. An ETAR antagonist (ABT-627) was inactive in ETBR cells and an ETBR agonist (S6c) was inactive in ETAR cells. ETAR cells had lower affinity for ET-3 than ETB cells while ET-3 produced a biphasic displacement curve in ETA/BR cells. ETA/B cells exhibited 300 fold lower affinity than ETA cells for ABT-627 but only 1 fold lower affinity than ETB cells for S6c. ET-1 treatment (100 nM, 2 h) reduced membrane receptor levels by 21% in ETA cells and 70% in ETB cells. In ETA/B cells, ET-1 treatment reduced membrane ETAR and ETBR by 21% and 47% ($P < 0.05$ vs. ETB cells). ETAR and ETBR co-localized with the membrane marker pan-cadherin in ETA/B cells. ETAR co-immunoprecipitated with ETBR in protein extracts from ETA/B cells. We conclude that ETAR and ETBR interact to increase membrane expression and to decrease agonist-induced internalization of ETBR. This interaction modifies ETAR and ETBR function in VSMCs that co-express the receptors.

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Identification of histamine H3 receptor antagonists

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Histamine H3 receptors (H3R) regulate the release of histamine and other neurotransmitters in the brain. H3R have been shown to modulate many functions, including feeding, sleep, and cognition, making them attractive drug targets. We have developed a functional cell-based assay, R-SAT® (Receptor Selection and Amplification Technology) which is predictive of agonist and antagonist activity at H3R. Using R-SAT, we have screened over 250,000 small molecule compounds for antagonist activity at H3R. Over 10 non-imidazole chemotypes were identified with potent human H3 activity. Most of these chemistries also displayed similar potencies at the rat H3 receptor with weak or no detectable activity at human H1, H2, or H4 receptors. These compounds can also antagonize histamine-induced intracellular release of calcium at H3R, and some compounds can inhibit basal cAMP activity at H3R. Differences in the affinities of these compounds for various H3R splice variants were also observed. Moreover, one of these compounds, H3-381, significantly reduced food intake in fasted rats. These novel and selective compounds may be useful leads for the development of H3R antagonist therapeutics.

Heterodimerization of AT1 and DOR1 confers differential transactivation

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Presenting Author: **Ying-Hong Feng**

Activation of type-1 angiotensin (Ang) II receptor (AT1) induces IP1 production via Gαq pathway and Jak2/Stat phosphorylation via Y319IPP motif of the receptor. Stimulation of delta1 opioid receptor (DOR1) with agonist DPDPE inhibits cAMP production via Gαi pathway. Both AT1 and DOR1 are G protein-coupled receptors (GPCRs) that often form receptor dimers for distinct signaling and actions for which the mechanisms are poorly understood. The objective of this study was to determine whether AT1 and DOR1 dimerize and understand how dimerization modulates their signaling properties. Methods: bimolecular fluorescence complementation (BiFC), Western blot, and functional assays of IP1 and cAMP were applied to detect the heterodimerization and signaling of AT1 and DOR1 in COS-1, CHO and H9c2 cells. Results: heterodimerization of AT1 and DOR1 was detected in COS-1 cells using BiFC technology. ER-trapping BiFC assay detected that the spontaneous heterodimerization took place in the ER and pre-treatment of the cells with agonist or antagonist of the receptors altered little magnitude of the heterodimerization. Co-expression of DOR1 inhibited up to 50% of basal and Ang II-stimulated IP1 production with little effect on AT1-mediated activation of Jak2 and Stat3. Reciprocally, co-expression of AT1 attenuated the DOR1-mediated inhibition of cAMP production in the presence of DOR1 agonist DPDPE. Stimulation of DOR1 with DPDPE in CHO cells induced little phosphorylation of Jak2, Stat3, and GSK3β. Surprisingly, co-expression of AT1 in CHO cells empowered DOR1 to trigger phosphorylation of Jak2, Stat3, and GSK3β in the presence of DPDPE. Consistently, AT1 specific antagonist candesartan inhibited DOR1-induced phosphorylation of Jak2, Stat3, and GSK3β in both CHO cells expressing exogenous AT1 and DOR1 receptors, and in H9c2 cells expressing endogenous AT1 and DOR1 receptors, suggesting that AT1 was differentially transactivated by DOR1 through heterodimerization. Conclusion: These results suggest that AT1 and DOR1 can undergo spontaneous heterodimerization in the ER and the heterodimerization can reciprocally modulates the signaling of the dimerized receptors. Heterodimerization may serve as a mechanism to arm a receptor deficient in activating a signaling pathway with the signaling structural machinery from another receptor through differential transactivation of the partner receptor. This may represent a general paradigm for GPCRs and even other proteins.

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Fibronectin activates ADAM17/TACE by competing with alpha5beta1 integrin binding

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We have shown previously that pro-fibrotic 5-HT induces epidermal growth factor receptor (EGFR) transactivation and renal mesangial cells proliferation through TNFalpha-converting enzyme (TACE) activation and heparin-binding epidermal growth factor (EGF) shedding. As cellular functions are also orchestrated by interactive signals of the extracellular matrix (ECM), and the matrix receptor integrins, in this study we aimed to explore how they modulate TACE activity. Our hypothesis was that TACE activity is modulated by integrin/ECM binding, because TACE possesses a disintegrin domain. We first employed a cyclic RGD peptide, which completely attenuated 5-HT-induced phosphorylation of EGFR and ERK (pERK) showing that integrins and RGD-containing matrix molecules can be involved in the cross-talk. Next, we analyzed the effect of integrin antibodies, and found that anti-beta1, -alphaV, -beta3 and -beta5 modulated 5-HT-induced pERK. However, integrins can be essential for the activation of the EGFR itself. Thus, we performed co-immunoprecipitation studies and confirmed increased assembly of EGFR with beta3 integrin upon 5-HT stimulation. We also found that TACE associates with beta1 integrin in resting mesangial cells and that this complex significantly decreases upon 5-HT-stimulation. The interaction between TACE and beta1 integrin was confirmed by surface plasmon resonance spectroscopy, which showed that purified alpha5beta1 integrin strongly binds to surface-bound recombinant TACE. As several glomerular diseases are characterized by increased expression of alpha5beta1 integrin and its matrix ligand fibronectin (FN), we investigated how this RGD-containing ECM molecule interacts with TACE. We employed an enzyme activity assay using quenched fluorogenic TACE substrate and recombinant TACE, together with FN and/or purified alpha5beta1 integrin. We found that TACE activity is dose dependently increased by alpha5beta1 integrin or FN alone. However, the molecules showed competitive interaction when used in combination. Further studies are needed to analyze the functions of these molecules in living cells.
In conclusion: our data support the role of integrin ligation in 5-HT2A-EGFR crosstalk, and strongly suggest that beta1 integrin and the matrix molecule fibronectin regulate the activity of TACE.

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A Homogenous, High Through-Put Luminescent cAMP Assay to Monitor Modulation of Gs and Gi Protein Coupled Receptors

Said A. Goueli, Jolanta Vidugiriene, and Kevin Hsiao

Presenting Author: **Said A. Goueli**

G Protein Coupled Receptors represent a large validated target for Drug discovery research. They are classified into three, main groups based on the G protein associated with the receptor. The Gs is coupled to activation of adenylate cyclase and Gi is coupled to inhibition of adenylate cyclase while Gq is coupled to activation of phospholipase . There are two main strategies to monitor the activation of GPCR, a reporter based assays and cAMP accumulation based assays. We report here on a new assay for monitoring modulation of GPCR that are linked to activation or inhibition of adenylate cyclase (Gs or Gi). The assay can be used to monitor cAMP accumulation or depletion in the cell upon treatment with agonists or antagonists of Gs or Gi coupled receptors. The assay is homogenous, and amenable to high through screening of modulators of GPCR. A value of Z' higher than 0.7 attests to the robustness of the assay and is carried out in 96-, and 384-, and 1536-well plates format. The assay is antibody-free and thus it is not affected by the quality or integrity of antibodies. It is based on luminescence and thus it does not suffer from fluorescence interference of library compounds. We have successfully generated EC50 values for agonists and IC50 values for antagonists of Gs and Gi-coupled receptors that are similar to those reported in the literature. The assay is easy to use, and can be carried out in less than 60 minutes and does not require expensive instrumentation for signal detection. The signal output is relatively stable for few hours and thus can be used for screening large number of plates. This luminescent assay is fast, homogenous, and reliable as indicated by high Z' values, making it an attractive screening tool for identifying agonist or antagonists that modulate Gs and Gi coupled receptors.

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Allosteric agonists and allosteric modulators are differentially affected by mutations within the allosteric binding pocket of M2 muscarinic acetylcholine receptors

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Muscarinic acetylcholine receptors (mAChRs) contain at least one allosteric site that is topographically distinct from the acetylcholine (ACh)-binding orthosteric site. Although numerous studies have investigated the structural basis of allosteric modulation at these receptors, far less is known about allosteric ligands that activate the receptor in their own right. We generated a number of M2 mAChRs harbouring mutations within the second extracellular loop and transmembrane domains 2 and 7 (Y80A, 172EDGE175->QNGQ, Y177A, T423A) and investigated their impact on the function of putative allosteric agonists (McN-A-343 and N-desmethylclozapine (NDMC)) and the interaction between ACh and prototypical allosteric modulators (gallamine and C7/3-phth) as assessed by receptor mediated phosphorylation of extracellular-signal regulated kinases 1 and 2 (ERK1/2). Very high negative co-operativity was observed between ACh and the allosteric modulators, gallamine and C7/3-phth, across all mutant M2 receptors. In agreement with previous studies, allosteric site mutations (Y177A, 172EDGE175->QNGQ, 172EDGE175->QNGQ +Y177A+T423A) significantly reduced the affinity of gallamine and C7/3-phth. In contrast, Y80A significantly increased the affinity of C7/3-phth, but didn't change the affinity of gallamine. Significant enhancements of efficacy and potency of McN-A-343 and NDMC were observed at Y177A. These findings suggest that allosteric agonists utilize a different binding mode to prototypical (non-agonistic) modulators at M2 mAChRs.

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Identification and characterization of dopamine receptor interacting proteins

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It is evident that dopamine receptors (DARs) do not exist as singular independent units within the synaptic membrane, but rather are part of a large macromolecular complex of interacting proteins. These interacting proteins may be transmembrane or cytosolic, and can play a variety of roles in receptor function, including trafficking, targeting and localization, ligand selectivity, association with downstream signaling machinery, specificity of cellular response, and desensitization of receptor response/internalization. We have employed a co-immunoprecipitation assay for D1 and D2 DARs (from mouse brain and transfected cell lines), coupled with mass spectrometry (MS) sequencing to identify interacting partners. Negative controls were used to limit false positive identification of interacting proteins, including non-immunized antibodies, mock transfected cells, and knockout animals. Following immunoprecipitation of the DARs from cells or brain tissue, the protein complex was separated using 1D gel electrophoresis and stained with colloidal Coomassie dye to detect proteins. Independent bands were excised, de-stained, trypsinized, and subjected to MS-based peptide sequencing, which to date has yielded detection and subsequent peptide-matching to more than 50 proteins by searching against a non-redundant protein database. In addition to the discovery of novel protein interactors, MS analysis also positively identified both the D1 and D2 DARs. Novel interacting proteins identified through these experiments include the chaperone protein calnexin, various subunits of the Na+,K+-ATPase (NKA), and the regulatory protein sorting nexin 25 (SNX25). Studies on these proteins have revealed that calnexin acts as both an ER retention protein and surface trafficking chaperone protein for DARs, critically regulating receptor trafficking and expression at the cell surface. Conversely, SNX25 appears to act on a different stage of the DAR cycle, causing enhanced DAR expression and function, possibly through differential sorting or internalization of the receptor. Experiments with the NKA have revealed that the DARs and NKA can reciprocally regulate one another, providing a control mechanism for both receptor signaling and cellular ion balance. These data offer insight into the cellular components of the DAR signalplex, and indicate that interacting proteins can influence the DARs at all stages of receptor processing and function.

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Different Signal Amplification in Gs- and Gi-Coupled Pathways

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Presenting Author: **Peter Hein**

Signal transduction on G protein coupled receptors allows for amplification of the signal. Specifically, few activated receptors can lead to a full effector response, a phenomenon which is termed spare receptors or receptor reserve. We used fluorescence resonance energy transfer (FRET)-based assays for receptor / G protein interaction and G protein activation together with assays for effector activation to directly compare amplification processes in Gs and Gi coupled systems in single cells. When stimulating HEK293 cells transfected with appropriately tagged A2A adenosine receptors, Gs protein subunits or an EPAC-based cAMP sensor, we measured receptor / Gs interaction, Gs activation and wholecell cAMP accumulation. For a Gi coupled system cells were transfected with alpha2A adrenergic receptors, Gi proteins or GIRK channels, and GIRK channel activation was used to assess effector activation. Again, receptor / Gi interaction, Gi activation and GIRK channel activation were measured. Comparing these results, we observed a substantial higher shift in EC50 curves for the Gi compared to the Gs coupled system. We conclude that signal transduction processes involving different G protein systems may be differently organized with regard to their ability to amplify the signal.

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The effect of coupling states on CCR5 antagonist pharmacology: compound-specific stabilization of an uncoupled receptor conformation which binds CCL5 with high affinity

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Several small molecule CCR5 antagonists are in clinical trials as antiviral therapies for HIV-1 infection. In this report, we investigate the pharmacology of vicriviroc, SCH-C, aplaviroc and maraviroc and how they interact with different CCR5 conformations. All the compounds displaced 125I-CCL3 and 125I-CCL4 from hCCR5. 125I-CCL5 binding was blocked by vicriviroc, SCH-C and maraviroc but only partially inhibited by aplaviroc. We hypothesized that aplaviroc may have different intrinsic efficacy at coupled (R*) and uncoupled CCR5 (R). We uncoupled CCR5 from its G protein transducers with GTP-gamma-S which suppressed CCL3, CCL4 and CCL5 binding although CCL5 binding was least affected. SCH-C, maraviroc and vicriviroc effectively inhibited 125I-CCL5 binding from uncoupled receptor while aplaviroc demonstrably enhanced the binding of the agonist to a previously uncharacterized population of uncoupled CCR5 (R'). In control incubations, 125I-CCL5 bound to the receptor with two distinct affinities (Kd ~ 25 pM; BMAX ~ 140 fmol/mg & Kd ~ 0.25 nM; BMAX ~ 110 fmol/mg). In co-incubations with GTP-gamma-S/aplaviroc, 125I-CCL5 bound at a single high affinity site (Kd = ~ 20 pM, BMAX ~ 230 fmol/mg). Maraviroc and vicriviroc did not stabilize this receptor conformation although the compounds displaced 125I-CCL5 from CCR5 R', presumably by competing with aplaviroc. The compounds were docked to a bovine rhodopsin-based homology model of CCR5 using a flexible docking algorithm guided by mutagenesis data. The modeled compound-receptor interactions indicate that the agents bind in a pocket formed by helices 2, 3, 6 and 7 and overlap in binding mode but aplaviroc is unique in accessing a pocket containing L285, G286, and H289. Taken together, these data show that aplaviroc stabilizes the previously unrecognized CCR5 conformation which is not G protein-coupled but binds CCL5 with high affinity.

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Interaction between α_1 and α_2 adrenergic receptors in mesenteric veins and HEK293 cells

Alexandra Hlavacova and James J. Galligan

Presenting Author: **Alexandra Hlavacova**

Norpinephrine (NE) released by sympathetic nerves constricts arteries and veins. Veins are more sensitive to NE than arteries and veins, but not arteries, are resistant to desensitization caused by NE. This might occur because α_2 adrenergic receptors (AR) indirectly contribute to NE constrictions of veins, but not arteries. α_{1D}AR and α_{2C}AR mediate NE-induced venous contraction. Therefore, we studied receptor interactions in HEK-293 cells expressing α_{1D}AR and α_{2C}AR. Immuocytochemistry revealed that α_{1D}AR were localized intracellularly but co-expression with α_{2C}AR did not alter this cellular localization. Similar results were obtained when α_{1D}AR was co-expressed with α_{2A}AR and α_{2B}AR. Co-expression with β_2AR enhanced membrane expression of α_{1D}AR. Functional interactions were investigated using calcium imaging techniques. NE caused an increase in calcium in α_{1D}AR expressing cells, these responses were not altered by co-expression with any α_2-AR. Co-expression of β_2AR with α_{1D}AR caused an increase in NE-induced calcium signals. α_{1D}AR desensitization caused by NE was not altered by co-expression with α_2ARs. These findings suggest, that α_{1D} and α_2AR co-expression alone does not account for enhanced NE sensitivity and resistance to desensitization in veins compared to arteries. Intracellular signaling mechanisms might contribute to artery-vein differences in adrenergic reactivity.

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PI-3K differentially regulates protease activated receptor-mediated platelet activation in humans through Rap1

Michael Holinstat, W. James Hudson and Heidi E. Hamm

Presenting Author: **Michael Holinstat**

Thrombin induces platelet activation in humans through two G protein coupled receptors (GPCRs), PAR1 and PAR4. A better understanding of how thrombin regulates platelet function following receptor activation will aid in the development of new anti-platelet drugs. To address how thrombin regulates platelet activation through its two GPCRs, we investigated the differential signaling pathways activated by the PARs in human platelets. Our findings establish a PAR1 specific signaling pathway that requires PI-3K for normal platelet activation which is not required for PAR4-mediated platelet function.

To determine how PI-3K signals downstream of PAR1, we looked at the small G protein Rap1 (which is known to play a role in platelet activation) as well as platelet aggregation, secretion and formation of phosphatidyinositol 3 phosphate. Inhibiting PI-3K with LY294002 resulted in an attenuation and subsequent reversal of PAR1-mediated platelet aggregation and Rap1 activation while having no observable affect on PAR4. Further, PAR1-induced aggregation and Rap1 activation was fully inhibited of AKT, while no inhibition was observed following stimulation of PAR4. Finally, inhibition of PAR1-mediated platelet aggregation in the presence of LY294002 was rescued by addition of a PAR4 specific activating peptide. A testable model of PAR1 versus PAR4 mediated platelet activation will allow for the identification of new targets for anti-platelet drug intervention without the classical side effects of excessive bleeding.

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The mu opioid receptors in rat caudate putamen and thalamus are different in N-glycosylation and lipid rafts association

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We characterized the mu opioid receptor (MOR) in brain by western blot with antibodies against a C-terminal peptide of the MOR using brain tissues from MOR-knockout (K/O) mice as controls. The MOR in the rat and mouse caudate putamen (CPu) and thalamus migrated as diffuse and broad bands, which were absent in the cerebellum and brains of MOR-K/O mice. The electrophoretic mobility of MOR differed in the two brain regions with median relative molecular masses (Mr's) of 75 kDa (CPu) vs. 66 kDa (thalamus) for the rat, and 74 kDa (CPu) vs. 63 kDa (thalamus) for the mouse, which was due to its differential N-glycosylation. Rat CPu or thalamus membranes were homogenized, sonicated in a detergent-free 0.5 M Na_2CO_3 buffer and fractionated through continuous sucrose-density gradients. MOR in CPu was found mainly associated with low-density cholesterol- and ganglioside M1 (GM1)-enriched fractions (lipid rafts), while the MOR in the thalamus was present in rafts and non-rafts without preference. Cholesterol reduction by methyl-□-cyclodextrin decreased DAGMO-induced [^{35}S]GTP□S binding in rat CPu membranes to a much greater extent than in thalamus membranes. The possibility that the heterogeneity in N-glycosylation and rafts-distribution of MOR in the two brain regions may be related is discussed.

RGS2, RGS6, and GAIP differentially regulate Lysophosphatidic Acid Signaling in Ovarian Cancer Cells

Jillian H. Hurst, April L. Brown, Paul A. Henkel, and Shelley B. Hooks

Presenting Author: **Jillian H. Hurst**

Lysophosphatidic acid (LPA) is a bioactive phospholipid that is produced by and stimulates ovarian cancer cells, promoting adhesion, migration, invasion, and survival. LPA signaling pathways are therefore attractive targets for new drug therapies for ovarian cancer. The effects of LPA are mediated by cell surface G-protein coupled receptors (GPCRs) that activate different heterotrimeric G-proteins, which in turn trigger cell signaling pathways that result in the cancer-promoting processes described above. G-proteins are regulated downstream of the receptor by Regulators of G-protein Signaling (RGS) proteins, which function in cells to turn G-proteins off by accelerating their endogenous GTPase activity. To characterize the role of RGS proteins in the regulation of LPA signaling we first determined the effects of various RGS proteins on exogenously expressed members of the classic LPA receptor family, LPA1, LPA2, and LPA3 in an assay measuring the production of the second messenger inositol phosphate (IP assay). We found that LPA1 signaling was inhibited by RGS2; LPA2 by RGS2, RGS6, and GAIP; and LPA3 by RGS2 and GAIP. Signaling from the three receptors was differentially sensitive to pertussis toxin, indicating different G-protein coupling of receptors. In order to determine the significance of this activity in ovarian cancer cells, we probed the ovarian cancer cell lines OVCAR-3, SKOV-3, and CAOV-3 and immortalized ovarian surface epithelial (IOSE) cells for the expression of RGS proteins. We found that transcripts encoding RGS2 and RGS6 were expressed in each of the ovarian cell lines, while GAIP was not expressed. Finally, expression of RGS proteins had distinct effects on LPA signaling in SKOV-3 and CAOV-3 cells. Signaling in SKOV-3 cells was attenuated by overexpression of RGS2, while RGS2, RGS6, and GAIP reduced signaling in CAOV3 cells.

Is Helix VIII of G Protein-Coupled Receptors (GPCRs) a Lipid-Activated Signalling Sensor?

John Huynh, Len. K. Pattenden , Marie-Isabel Aguilar & Walter G. Thomas

Presenting Author: **John Huynh**

The inactive GPCR structure of the bovine rhodopsin receptor [1] confirmed an additional helix VIII (H8) within the proximal carboxyl-terminus, positioned parallel to the plasma membrane. We identified H8 (Y302 to P322) of the angiotensin II type 1 receptor (AT1AR) interacts with high affinity to membranes [2,3]. This, with emerging evidence that the plasma membrane participates with GPCR regulation [4,5], poses the question: does membrane-binding of H8 constitute as a conserved conformational switch to control GPCR signalling and function?

We predict that a possible detethering of H8 away from the membrane may occur during the activation of the AT1AR. Rhodopsin structures reveal two residues within TM7 and H8 that may assist positioning H8 towards the membrane in the inactive state - sustaining this TM7-H8 interaction may affect receptor activation. To investigate this interplay, we introduced:

(1) Cysteine residues to form a disulfide bond between TM7 and H8.
(2) An unnatural palmitoylation site to anchor H8 to the membrane.

Results show H8 may not detether from the membrane, but undergo a subtle conformational change. An alanine scan conducted along H8 identified residues important for membrane tethering and receptor function. Some mutations displayed a reduced binding capacity (F304 to K311, P321A and P322A) and residue K308A resulted in no ligand binding despite the formation of mature glycosylated receptors. To sequentially identify the influence of each residue on folding, receptor maturation, cell-surface trafficking, receptor ligand affinity and specific membrane-lipid targeting, mutant receptors are subjected to ligand binding, internalisation and signalling assays, in conjunction with surface plasmon resonance assays of H8 peptides using liposomal surfaces with diverse lipid compositions.

Preliminary results show that H8 and its positioning with respect to membranes are crucial for GPCR signalling and function. Subtle conformational changes may allow certain residues within H8 to form specific and structured associations with components of the membrane.

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Functional dissociation between apelin receptor signaling and endocytosis : implications for the effects of apelin on arterial blood pressure.

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Apelin was recently isolated as the endogenous ligand of the human orphan APJ receptor, a G protein-coupled receptor which shares 31 % amino-acid sequence identity with the angiotensin II type 1 receptor. The predominant molecular forms of apelin naturally occuring *in vivo,* in brain and plasma, are apelin 17 (K17F) and the pyroglutamyl form of apelin 13 (pE13F). Apelin has been shown to be involved in the regulation of body fluid homeostasis and cardiovascular functions. We investigated the structure-activity relationships of apelin at the rat apelin receptor, tagged at its C-terminal end with enhanced green fluorescent protein and stably expressed in CHO cells. We compared the abilities of N- and C-terminal deleted fragments of K17F (KFRRQRPRLSHKGPMPF) to bind with high affinity to the apelin receptor, to inhibit cAMP production and to induce apelin receptor internalization. The first five N-terminal and the last two C-terminal amino acids of K17F were not essential for apelin binding or cAMP response. In contrast, deletion of the arginine in position 6 drastically decreased binding and cAMP response. The full-length sequence of K17F was the most potent inducer of apelin receptor internalization because successive N-terminal amino-acid deletions progressively reduced this process. Moreover, the removal of the phenylalanine in position 17 at the C-terminus of K17F abolished internalization, since, K16P binds with high affinity to the apelin receptor and strongly inhibits cAMP production evoked by forskolin, but does not induce apelin receptor endocytosis. These data indicate that apelin receptor signaling (coupling to Gi) and endocytosis are functionally dissociated, suggesting that the binding of different apelin fragments might stabilize the apelin receptor in different conformational states. In order to specify the residues in the apelin receptor which interact with the C-terminal Phe of K17F we built a three-dimensional (3D) model of the apelin receptor by homology, using as a template the experimentally validated 3D model of the cholecystokinin receptor type 1 and by docking pE13F into this receptor model. This 3D model has revealed that the C-terminal Phe in apelin is embeded in a receptor cavity constituted by aromatic side-chain residues (F255 and W259). Preliminary experiments on site-directed mutagenesis of these residues (F255/A and W259/A) showed that K17F binds with the same affinity to these mutated receptors as compared to the wild type apelin receptor whereas K17F does not induce internalization of these mutated proteins, confirming the critical role of the C-terminal Phe of K17F in receptor internalization process. Finally, we investigated *in vivo* the role of the C-terminal Phe in K17F on the hypotensive effect of apelin. For this purpose we evaluated the effects of different K17F-deleted fragments injected by intravenous route on arterial blood pressure in normotensive Wistar Kyoto rats. We showed that apelin fragments, that did not induce receptor internalization but kept their ability to activate receptor coupling to Gi, did not decrease arterial blood pressure. Our data showed that the hypotensive effect of apelin peptides correlates best with the ability of these ligands to trigger apelin receptor internalization. Thus, the hypotensive effect of apelin seems to be controlled by apelin receptor endocytosis, which is probably required for initiation of a second wave of signal transduction G protein-indenpendent. Our results suggest the possibility of the development of biaised agonists of the apelin receptor capable of activating only one signal transduction pathway and therefore to produce a specific biological action. The design and the development of such compounds may offer new potential therapeutic avenues for the treatment of water retention and cardiovascular disorders.

IMPORTANCE OF THE HUMAN CB1 CANNABINOID RECEPTOR RESIDUE D2.63 FOR MODULATING THE SIGNAL TRANSDUCTION PROCESS

Ankur Kapur, Daniel Fleischer, and Mary E. Abood

Presenting Author: **Ankur Kapur**

To date, two cannabinoid receptors, CB1 and CB2, have been conclusively identified by molecular cloning and pharmacological characterization. The cannabinoid receptors belong to the Class A rhodopsin-like superfamily of G-protein coupled receptors (GPCRs). Structurally diverse cannabinoid ligands bind at the CB1 receptor suggesting the existence of multiple ligand recognition sites. These binding sites are contributed predominantly by distinct noncontiguous regions of the hydrophobic transmembrane helixes (TMHs).

Previous studies with GPCRs have identified a highly conserved, negatively charged aspartate at position 2.50 (from TMH 2) to be crucial for ligand binding and/or receptor function. D2.50 (D163 in CB1 and D80 in CB2) were demonstrated to be important for G-protein coupling and signal transduction and not ligand binding.

In this study, we investigated the role of the negatively charged aspartate residue (from TMH 2) at position 2.63 (D176) in CB1 receptor function by replacing it with glutamate (D2.63E) or asparagine (D2.63N). D2.63 is unique; whereas it is highly conserved in all species of the CB1 receptor, an asparagine residue is present at the equivalent position in the CB2 receptor. This conserved residue in the CB1 receptor is located closer to the top of TMH 2 towards the extracellular region making it accessible to ligands.

HEK293 cells stably expressing recombinant hCB1 wild-type or mutant receptors were used to investigate the consequences of mutating D2.63 (to D2.63N and D2.63E) in radioligand binding studies and GTPgammaS functional assays. Our results suggest this aspartate residue at position 2.63 is not obligatory for ligand recognition in CB1 receptor; however, it plays an important role in modulating agonist-stimulated receptor activation. Not surprisingly, the charge conserved substitution, D2.63E, resembled the wild-type CB1 receptors. Furthermore, this divergent residue, D2.63 (N2.63 in CB2) is not responsible for the differential interaction of ligands in CB1 and CB2 receptors. In conclusion, the presence of a negatively charged residue at the location 2.63, rather than the residue aspartate per se plays a role in modulating the CB1 receptor signal transduction process.

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In vivo analysis of brain AT1 angiotensin receptor signaling pathways

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AT1 receptors mediate pressor and dipsogenic actions of angiotensin II (Ang II) in the brain. This study examined intracellular signaling pathways of brain AT1 receptors in vivo, using selective inhibitors and Ang II, administered intracerebroventricularly. Vehicle or inhibitor (2 µl) was given 15 min prior to Ang II (100 pmol/2 µl artificial CSF) to adult, male Sprague-Dawley rats implanted with radiotelemetric blood pressure transducers. Increases in mean arterial blood pressure (BP) and volume of water drunk (WV) in response to Ang II were monitored. U-73122 (2 nmoles) a phospholipase C inhibitor, but not the inactive analog U-73433 (n=4), attenuated the effects of Ang II (BP: 41.7 %; WV: 56.5 %; $p<0.05$ n = 6). Chelerythrine (100 pmol) a protein kinase C (PKC) inhibitor also inhibited the effects of Ang II (BP: 20.1% $p<0.01$; WV: 37.5% $p<0.05$ n=8). PD-98059 (1 nmol) a specific MAPKK inhibitor attenuated only the pressor effect of Ang II (28.2% $p<0.05$ n=6). Neither KN-93 (40 pmol, n=6) a Ca2+/Calmodulin-kinase II (CaCMK II) inhibitor nor wortmannin (40 pmol, n=5) a PI3-kinase inhibitor affected the responses to Ang II. The results confirm that the PLC/PKC pathway is the dominant mediator of brain AT1 receptor responses, as in the periphery. The differential effects of the MAPKK inhibitor on pressor and dipsogenic responses suggests that that MAPK cascade may mediate pressor, but not dipsogenic effects of Ang II in the brain.

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LYSOPHOSPHATIDIC ACID REGULATES EPIDERMAL GROWTH FACTOR RECEPTOR BINDING IN AIRWAY EPITHELIAL CELLS VIA ERK1/2 AND PROTEIN KINASE C

Karen M. Kassel, Anna C. Jacobs, and Myron L. Toews

Presenting Author: Karen M. Kassel

We showed that lysophosphatidic acid (LPA) induces a rapid and sustained decrease in EGF receptor (EGFR) binding in BEAS-2B airway epithelial cells (FASEB J 19:A527). We hypothesized that the rapid and sustained decreases would be mediated by different signaling pathways. BEAS-2B cells were treated for 15 min or 18 hr and then incubated with 125I-EGF on ice to measure cell surface EGFR binding. Cells were also treated for 5 min and assessed for ERK phosphorylation. LPA, the direct PKC activator PMA, and the Ca2+ ionophore ionomycin caused rapid and sustained decreases in EGFR binding and stimulated ERK phosphorylation. The MEK inhibitor U0126 blocked ERK phosphorylation and the 15-min decrease in binding for LPA, PMA, and ionomycin, but not the 18-hr decrease in binding. PKC inhibition blocked the 15-min decrease in binding and ERK phosphorylation by PMA and ionomycin, but only slightly inhibited LPA. In contrast, PKC inhibition completely blocked the 18-hr decrease in binding by LPA, PMA, and ionomycin. The Ca2+ chelator BAPTA-AM blocked the ionomycin-induced rapid decrease in EGFR binding and ERK phosphorylation, but not that induced by LPA or PMA. These data confirm that LPA uses distinct mechanisms to mediate the rapid vs sustained decreases in EGFR binding in BEAS-2B cells. The rapid decrease is primarily mediated via MEK/ERK whereas the sustained decrease involves PKC. Supported by an AHA fellowship to KMK and by NE DHHS.

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Biochemical analysis of Gaq-PLCb interaction using recombinant Gaq proteins

Takeharu Kawano and Tohru Kozasa

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The activation of PLCb isozymes by Gaq plays critical functions in a variety of physiological processes. Both DAG and IP3 that are produced from PIP2 by PLCb serve as crucial second messengers in intracellular signal transduction. However the biochemical analysis of PLCb activation by Gaq has been severely limited by the lack of method to generate recombinant Gaq in large scale. Recently, we were successful to solve this problem by constructing a chimera of Gai1 and Gaq (Gai/q), in which the N-terminal 36 residues of Gaq were replaced with the corresponding region of Gai1. When His6-Gai/q chimera was expressed in Sf9 cells, the protein was recovered from a soluble fraction and the yield was improved up to about 10~40-fold of that of wild type Gaq. Using the chimera, we have determined the atomic structure of Gai/q-GRK2-Gbg complex (Tesmer et al., Science, 2005). However, this chimera could not activate PLCb possibly because N-terminus of Gaq is missing in the construct. To investigate the mechanism of Gaq -PLCb interaction, we created a new Gai/q chimera, which retains 9th and 10th cysteine residues of Gaq that have been shown to be critical for PLCb activation. We also introduced GRK2-insensitive mutations (T257E and Y261L) into the new chimera in order to characterize the significance of such residues on functional association with PLCb.

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Inhibitors of phosphoinositide 3-kinase cause defects in the postendocytic sorting of β2-adrenergic receptors

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Phosphatidylinositol 3-kinase inhibitors have been shown affect endocytosis or subsequent intracellular sorting in various receptor systems. Agonist-activated β2-adrenergic receptors undergo desensitization by mechanisms that include the phosphorylation, endocytosis and degradation of receptors. Following endocytosis, most internalized receptors are sorted to the cell surface, but some proportion is sorted to lysosomes for degradation. It is not known what governs the ratio of receptors that recycle versus receptors that undergo degradation. To determine if phosphatidylinositol 3-kinases regulate β2-adrenergic receptor trafficking, HEK293 cells stably expressing these receptors were treated with the phosphatidylinositol 3-kinase inhibitors LY294002 or wortmannin. We then studied agonist-induced receptor endocytosis and postendocytic sorting, including recycling and degradation of the internalized receptors. Both inhibitors amplified the internalization of receptors after exposure to the β-agonist isoproterenol, which was attributable to the sorting of a significant fraction of receptors to an intracellular compartment from which receptor recycling did not occur. The initial rate of β2-adrenergic receptor endocytosis and the default rate of receptor recycling were not significantly altered. During prolonged exposure to agonist, LY294002 slowed the degradation rate of β2-adrenergic receptors and caused the accumulation of receptors within rab7-positive vesicles. These results suggest that phosphatidylinositol 3-kinase inhibitors (1) cause a misrouting of β2-adrenergic receptors into vesicles that are neither able to efficiently recycle to the surface nor sort to lysosomes, and (2) delays the movement of receptors from late endosomes to lysosomes.

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Opsin is dimeric in COS1 cells: amino acids at the dimeric interface

Parvathi Kota, Philip J Reeves, Uttam L RajBhandary and H Gobind Khorana

Presenting Author: **Parvathi Kota**

Rhodopsin in the disc membranes of rod outer segments serves as the dim light photoreceptor and is a prototypic member of a G-protein-coupled receptor family. Several members of this family appear to form and function as oligomers. Atomic force microscopy indicates that rhodopsin is present as dimers in the native membranes. We have expressed the protein, opsin, in COS1 cells and have studied its molecular state by using fluorescence resonance energy transfer and by intermolecular cross-linking following site-directed cysteine mutagenesis. To observe fluorescence resonance energy transfer, N-terminus of the genes for the cyan or yellow fluorescent proteins was fused to the C-terminus of the opsin gene and the resulting fused genes were expressed in COS1 cells. The emission spectra in situ of the expressed proteins were recorded and fluorescence resonance energy transfer was then calculated. This indicated intermolecular interaction between opsin molecules in COS1 cells. To identify the amino acids involved in the interaction, those predicted by molecular modeling to be at the dimer interface were mutated one at a time to cysteine and dimer formation was measured by the rate of disulfide bond formation in the presence of cupric orthophenanthroline. The mutants W175C and Y206C formed the dimers most rapidly showing that the two amino acids were at the interface in the dimer.

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Dynorphin A activates bradykinin receptors to maintain neuropathic pain

Josephine Lai, Miaw-chyi Luo, Qingmin Chen, Shouwu Ma, Luis Gardell, Michael Ossipov and Frank Porreca

Presenting Author: **Josephine Lai**

Endogenous opioid neuropeptides include the enkephalins, the endorphins and the dynorphins. While the physiological actions of enkephalins and endorphin peptides are the result of inhibitory effects, predominantly on neuronal cells, to elicit analgesia, the physiological role of the dynorphins remains more obscure. Dynorphin A(1-17) elicits excitatory actions that are not mediated by opioid receptors. Intrathecal dynorphin A(1-17) produces little antinociceptive actions; higher doses induce excitatory effects including hyperalgesia, loss of muscle tone, paralysis, and severe motor dysfunction. Dynorphin A stimulates the release of excitatory amino acids from cultured neurons and induces an increase in intracellular calcium. In spinal cord slices, dynorphin A enhances the evoked release of pronociceptive transmitters such as calcitonin gene related peptide (CGRP) and intrathecal infusion of dynorphin A stimulates the release of excitatory amino acids and prostaglandin E2 in the spinal cord, demonstrating pronociceptive actions of this peptide. Our data demonstrate that dynorphin A directly binds to bradykinin receptors and stimulates calcium influx via the L-type and P/Q type voltage sensitive calcium channels. This agonist action of dynorphin is not predicted by structural similarity between dynorphin and bradykinin, or between opioid receptors and the bradykinin receptors. The coupling mechanisms of these two classes of receptors are also different. This novel action of dynorphin at the bradykinin receptors underlies the hyperalgesia produced by pharmacological administration of dynorphin by the spinal route in rats and mice. Blockade of spinal B1 or B2 receptor also reverses persistent neuropathic pain but only when there is sustained elevation of endogenous spinal dynorphin, which is required for maintenance of neuropathic pain. These data reveal a mechanism for endogenous dynorphin to promote pain through its agonist action at bradykinin receptors and suggest new avenues for therapeutic intervention.

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Identification of distinct, ligand-specific structural changes in a G protein-coupled receptor

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Presenting Author: **Jian Hua Li**

G protein-coupled receptors (GPCRs) represent by far the largest class of cell surface receptors. The conformational changes in GPCR structure induced by GPCR ligands (agonists, neutral antagonists, or inverse agonists) are not well understood at present. In this study, we employed an in situ disulfide cross-linking strategy to monitor ligand-induced conformational changes in a series of cysteine (Cys)-substituted mutant M3 muscarinic acetylcholine receptors. We demonstrated that muscarinic agonists inhibited disulfide cross-linking in the A91C/T549C and F92C/F550C double Cys mutant M3 receptors. In striking contrast, inverse muscarinic agonists enhanced disulfide bond formation in the same receptor constructs. Given the predicted localization of the Cys residues present in these mutant receptors, our data strongly suggest that muscarinic agonists trigger a separation of the N-terminal segment of the cytoplasmic tail from the cytoplasmic end of transmembrane domain I (TM I), whereas inverse muscarinic agonists increase the proximity between these two receptor regions. These findings provide a structural basis for the opposing biological effects of muscarinic agonists and inverse agonists. This study also provides the first piece of direct structural information as to how the receptor conformations induced by these two functionally different classes of ligands differ at the molecular level.

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Effect of alpha-2C adrenergic receptor Del322-325 polymorphism on cyclic AMP production in HEK293 cells

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The human alpha-2C adrenergic receptor is involved in multiple signaling pathways including inhibition of adenylyl cyclase. A polymorphism of this receptor results in the deletion of four amino acids within the third intracellular loop. Previous studies in CHO cells have shown reduced downstream signaling by the alpha-2C-Del322-325 receptor compared to that of the wild type receptor. The focus of our studies is to compare differences in G protein-coupling of these two receptors in HEK293 cells. Saturation studies with [^{3}H]RX821002 showed the affinities of the Del322-325 and wild type receptors to be similar (1.0 vs. 0.9 nM). Functional assays in the form of inhibition of cyclic AMP production revealed that the receptor density of the cells plays a critical role in determining the efficacy and potency of various agonists, including UK-14-304, clonidine, NU-117117, NU-118415, and NU-173892. The possibility of functional selectivity of a wide variety of agonists is being investigated.

The Effects of beta-Amyloid Plaque Accumulation on G-Protein Coupled Receptor Kinase 5 in Alzheimer's Disease

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Alzheimer's disease (AD) is a neurodegenerative disease characterized by loss of cholinergic neurons as well as muscarinic receptor-G-protein uncoupling and dysfunction (1,2). Muscarinic G-protein coupled receptors (mGPCRs) are regulated by the enzyme G-protein coupled receptor kinase (GRK). It has been speculated that lack of GRK5 activity may produce desensitization of muscarinic receptors that could eventually lead to cholinergic hypofunction (3). Suo et al reported that GRK5 was altered in brains from transgenic mice that overproduced beta-amyloid (4). In their study, GRK5 was translocated from the membrane to the cytosolic fraction, making it non-functional. The objective of this study was to determine whether GRK5 was also altered in the brains of humans with AD. We have observed uncoupling of muscarinic receptors from G-proteins even in brain tissues from non-demented control subjects who have varying degrees of Alzheimer-like pathology, in that they display varying levels of neuritic plaques which stain for beta-amyloid. Therefore, a second objective was to determine whether changes in GRK5 occurred even before the development of dementia, in age-matched controls with varying amounts of neuritic plaques. Brain samples from patients with documented AD were compared with those of non-demented controls. The controls were divided into three groups: those with no neuritic plaques, those with few plaques (plaque count less than 7.25), and those with many plaques (plaque count greater than 7.25). Loss of cholinergic input was assessed by measuring choline acetyltransferase (ChAT) activity. Western blot analysis was employed to determine the level of GRK5 in samples from temporal lobe, in the same manner used by Suo et al (3). Levels of GRK5 in the cytosolic and the membrane fraction were also assessed. There was no significant change in the amount of GRK5 in the total tissue sample in the four groups: control, control with some plaques, control with many plaques, and AD. Membrane GRK5 was also not significantly changed. There was a trend towards decreased cytosolic levels of GRK5 as the number of plaques increased in the non-demented controls and in AD, but this again was not significant. There was a statistically significant decrease in ChAT activity with increasing plaque level ($p<0.001$), in both non-demented controls and AD, as well as a significant correlation between ChAT activity and the level of membrane and cytosolic GRK5 ($p<0.05$). It is possible that the small decrease seen cytosolic GRK5 resulted from loss of cholinergic neurons. However, in contrast to the results of Suo et al (3), translocation of GRK5 from the membrane to the cytosol did not occur in brain samples from patients with AD. This suggests that uncoupling of muscarinic receptors, which occurred in AD as well as in non-demented controls with plaques, did not result from alterations in the level of GRK5.

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The role of Angiotensin II AT1AR internalization in the Angiotensin II-induced expression of cyclooxygenase 2 (COX-2) in rat aortic vascular smooth muscle cells

Thomas A. Morinelli, Aleksander Baldys, John R. Raymond and Michael E. Ullian

Presenting Author: **Thomas A. Morinelli**

The presence of G protein-coupled receptors (GPCRs) within the nuclear area has been a subject of much debate over the past 20 years. Several recent investigations demonstrate not only the trafficking of receptors to the nucleus subsequent to GPCR stimulation but also nuclear localization of unstimulated GPCRs. Questions still exist on the mechanism and the function of the nuclear localization of ligand-activated GPCRs. Previously, we used a Green Fluorescent Protein (GFP) construct of both a wild-type and site-directed mutant of the angiotensin II (AII) AT1aR to demonstrate 1) the active nuclear localization of this receptor, 2) a putative nuclear localization motif in the carboxy-terminal tail of the receptor and 3) association between nuclear localization of the receptor and activation of transcription for the cyclooxygenase 2 (COX-2) gene, PTGS-2 (Morinelli et al, AJP:Cell, in press 2007). The hypothesis of the present study is that AT1aR internalization is a first step in the nuclear localization of the endogenous AT1aR of rat aortic vascular smooth muscle cells (RASMC) and the resultant activation of COX-2 expression. Monolayers of cultured RASMC exposed to AII demonstrated both time- and concentration-dependent increases in COX-2 protein expression as determined by immunoblotting, reaching a maximum 1.5 fold increase over unstimulated cells at 3 hours with 100 nM AII.

The initiation of GPCR internalization occurs as the ligand-bound receptor localizes to clathrin-coated pits at the plasma membrane. Subsequent dynamin-mediated endocytosis produces vesicles containing ligand/receptor complexes that traffic through the cytoplasm. To determine the influence of receptor internalization on the activation of COX-2 expression by AII, we examined the ability of sucrose, an inhibitor of clathrin assembly, one of the earlier steps of receptor internalization, and cytochalasin B, an inhibitor of actin polymerization that is involved in subsequent stages of receptor trafficking, to alter the AII-induced COX-2 expression in RASMC. To demonstrate the effectiveness of these agents on inhibiting receptor internalization, we used our previously characterized HEK-293 cells stably expressing the wild-type AT1aR/GFP construct and laser scanning confocal microscopy. Sucrose pretreatment (0.4M, 15 minutes) of these cells prevented AT1aR/GFP internalization after exposure to AII. Cytochalasin B pretreatment (5 μg/ml, 15 minutes) also prevented the AT1aR/GFP internalization of the receptor but did not prevent the apparent clustering of the AT1aR/GFP construct at the plasma membrane. The effect of these agents on AT1aR internalization after exposure to AII was confirmed through radioligand binding assays employing [125I]-AII. Sucrose significantly reduced AT1aR internalization as measured by the amount of acid-resistant [125I]-AII associated with the cell monolayer after 5 minutes exposure to agonist (44.3% ± 2.7 % internalized receptor for control vs. 30 % ± 2.2 %* for sucrose pretreatment; *$p<0.05$ vs. control N=3). Interestingly, cytochalasin B pretreatment significantly increased the amount of acid-resistant ligand associated with the cell monolayer (73 % ± 1.9 %, N=3), confirming laser scanning confocal imaging showing clustering of the receptor at the plasma membrane. Both sucrose and cytochalasin B significantly reduced the AII-induced expression of COX-2 (6.2 ± 1.9 fold increase over basal for AII vs. 0.85* ± 0.3 for sucrose/AII and 1.8*± 0.6 for cytochalasin B, respectively; *$p<0.05$ vs. AII alone, N=5). In summary, inhibition of AII-activated AT1aR internalization by either sucrose, an inhibitor of clathrin assembly, or by cytochalasin B, an inhibitor of actin polymerization, blocked the ability of AII to stimulate the expression of COX-2. Thus, AT1aR internalization from the cell surface is the initial step in the pathway for nuclear localization of the receptor and resultant activation of the transcription for COX-2. In addition, these data suggest that the source of the nuclear-localized receptor is the plasma membrane. (Supported by Dialysis Clinic Incorporated and the Department of Veterans Affairs)

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A Dopamine D_2 Receptor Mutant Capable of G Protein-mediated Signaling but Deficiemt in Arrestin Binding

Hongxiang Lan, Yong Liu, Vsevolod V. Gurevich, and Kim A. Neve

Presenting Author: **Kim A. Neve**

We explored non-phosphorylated arrestin-binding sites of D_2 and D_3 dopamine receptors by assessing the ability of purified arrestins to bind to glutathione S-transferase (GST) fusion proteins containing the second and third intracellular loops (IC2 and IC3) of the receptors. Arrestin-3 bound to IC3 of both D_2 and D_3 receptors, and to D_2-IC2 with lower affinity. Mutagenesis of GST-IC3 identified an important determinant of the binding of arrestin in the N-terminal region of IC3. Alanine mutations of this determinant (IYIV212-215) in the full-length D_2 receptor generated a signaling-biased receptor with intact ligand binding and G-protein coupling and activation, but deficient in receptor-mediated arrestin-3 translocation to the membrane and agonist-induced receptor internalization in 293 cells. This mutation also decreased arrestin-dependent activation of extracellular signal-regulated kinases (ERKs) by the D_2 receptor without altering pertussis toxin-sensitive, G protein-mediated activation of ERKs, and decreased agonist-induced association of arrestin with the D_2 receptor. The finding that non-phosphorylated D_2-IC3 and D_3-IC3 have similar affinity for arrestin is consistent with previous suggestions that the differential effects of D_2 and D_3 receptor activation on membrane translocation of arrestin and receptor internalization are at least in part due to differential phosphorylation of the receptors. In addition, these results indicate that the sequence IYIV212-215 in IC3 of the D_2 receptor is required to form a binding site for arrestin. (MH045372 and VA Merit Review)

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CHO cell Y receptor oligomers, but not monomers or agonist internalization, depend on functional Gi alpha subunits

Steven L. Parker, Michael S. Parker, Ambikaipakan Balasubramaniam and Floyd R. Sallee

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A role for fast Gi alpha subunits in organization of GPCRs was indicated by a number of studies, and our recent work with a human Y2 receptor (hY2R) expression demonstrated a tight co-regulation of activity, and even of levels, of the receptor and of Gi alpha subunits in CHO cells (Parker et al., Regul Pept 139:128). We extended the examination of this relationship to human Y1 receptor (hY1R) and mouse Y4 receptor (mY4R) especially in terms of receptor oligomerization and cycling. Characterization of receptor dimers by gradient centrifugation was possible due to the tight attachment of primary agonists to the receptors. Pertussis toxin at $\geq$ 4 ng/ml over 24 h (with hY1R and hY2R) or 36 h of culture (with mY4R) caused $\geq$ 90% loss of function of Gi alpha subunits, and $\geq$ 85% loss of Y agonist binding. There also was a large loss of immunoreactive hY2R, and a decrease of immunoreactive Gi3 alpha. The loss of Y receptor and Gi functionality (as assessed by Y agonist binding, inhibition of adenylate cyclase, and stimulation of [35S]GTP-gamma-S binding) was essentially stable over at least two cell passages after removal of external toxin. After solubilization, the Y receptor dimers labeled by [125I] agonists sedimented at ~180 kDa and were associated with G-proteins containing Gi alpha subunits, but not binding [35S]GTP-gamma-S. The solubilized control monomers were associated largely with Gi3 alpha subunit labeled by GTP-gamma-S. The dimers represented at least 60% of the total for all three Y receptors, and were rapidly lost to PTX treatment (half-period ~ 4 h), in parallel to loss of Gi function. However, in the virtual absence of dimers, the rate of internalization of Y agonists was less than 40% reduced, and there was an increased interaction with Gq alpha subunits. The Gi-associating oligomers thus constitute the bulk of Y receptors expressed in CHO cells, and at least with the hY2R also include the receptor reserve (activated by agonists, shearing or alkylators). The upkeep of this population appears to depend on Gi chaperoning. In the presence of PTX, monomers that significantly associate with Gq and / or follow Gi -independent routes of intracellular processing are the main cycling form of Y receptors in CHO cells. Further work should clarify to what extent this applies in the presence of functional Gi subunits.

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Postnatal maturation modulates GPCR mediated IP3 release in ovine cerebral arteries

William J. Pearce and James M. Williams

Presenting Author: **William J. Pearce**

A key feature of late fetal and early postnatal development is a dramatic change in the efficiency of pharmacomechanical coupling for many G-protein receptor agonists. This shift in reactivity can be explained only in part by changes in receptor density and agonist affinity, suggesting that the efficiency of coupling between ligand binding and second messenger production is altered during postnatal maturation. To explore the specific hypothesis that the size of the IP3 signal resulting from activation of a single receptor is physiologically regulated during postnatal maturation, we examined 5HT-induced IP3 production in middle cerebral arteries from fetal and adult sheep. IP3 mass was measured after exposure to 5HT at varied concentrations and durations. Due to the small size and multiple 5HT receptor types of sheep middle cerebral arteries, 5HT2a affinity and density were determined by quantitative receptor autoradiography using 3H-Ketanserin and 5HT in saturation and competition binding assays. Using the affinity values measured, we calculated fractional receptor occupancy, which we converted to pmol receptor bound per gram wet wt using measured density values. The areas beneath the IP3 time course cuvres were plotted against their corresponding values of pmol receptor bound per gram wet weight to obtain a relation between the size of the IP3 signal and the number of receptors activated. The slope of this relation was termed receptor gain with units of fmol IP3-min per pmol receptor bound. In fetal cerebrals, pKa, Bmax, and gain averaged 6.3, 3.14 pmol/g w.w., and 587 fmol-IP3-min per pmol receptor bound. Adult values were 5.9, 5.84, and 93. Thus, maturation down-regulated 5HT-induced IP3 production in ovine cerebral arteries. These data demonstrate that postnatal changes in pharmacomechanical coupling involve multiple aspects of GPCR signaling, including receptor density, agonist affinity, and the efficiency of coupling between GPCR activation and second messenger activation. These data also suggest that postnatal maturation may involve changes in PLC-beta, IP3 turnover, and/or the GTPase activity of the alpha subunit. In turn, these data indicate that the effects of postnatal maturation on the abundance and activity of RGS proteins may be well warranted.

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Site-directed mutagenesis in tandem with selective ligands to better understand the tertiary structure of GPCRs

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The human cannabinoid CB2 receptor is an increasingly important therapeutic target for neuropathic pain, inflammation, cancer treatment, as well a variety of other physiological disorders. In lieu of the significance of this G-protein coupled receptor, it is essential for researchers to investigate the manor in which cannabinergic ligands interact with the receptor's binding domain(s). A number of high affinity covalent ligands (pharmacophores conjugated to isothiocyanate (NCS)) have been designed and synthesized in order to biochemically map the receptor's binding site(s) including: AM-841, AM-1336, AM-4772 as well as other novel high affinity covalent probes. These probes bind irreversibly to specific amino residues which incorporate sulfhydryl or unprotonated amino side chains by nucleophilic addition. Cysteine is the most preferred, however at pH above 10, well above the physiological pH, unprotonated lysine may couple with NCS. Using a global set of CB2 cysteine substitution (C to S) mutant transgenic cell lines, I am attempting to identify key cysteine residue in CB2 which covalently attach to specific ligands. These data will provide detail as to how the compound docks into its specific binding domain.

Revisiting the classification of beta-arrestin usage: Insights from bioluminescence resonance energy transfer (BRET)

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G protein-coupled receptors (GPCRs) that interact with beta-arrestins have been classified into two distinct classes (Oakley *et al*., 2000). According to this scheme, GPCRs of Class A are believed to associate with beta-arrestin 2 with higher affinity than beta-arrestin 1. These interactions appear to be transient in nature, with the receptor dissociating from arrestin at or near the plasma membrane without the arrestin entering receptor-containing vesicles. Receptor dephosphorylation and recycling then occurs rapidly. In contrast, Class B GPCRs are believed to interact with beta-arrestins 1 and 2 with similar high affinity. These interactions seem to result in the formation of a stable receptor-arrestin complex that internalises into endocytic vesicles. Receptor recycling is then proposed to occur slowly, if at all. However, the somatostatin receptor sst2A is an example of a putative Class B receptor that recycles rapidly (Tulipano *et al.*, 2004), implying that certain receptors may exhibit characteristics of both receptor classes.

Bioluminescence resonance energy transfer (BRET) technology allows receptor-arrestin interactions to be measured in live cells, in real-time, at 37 C, extending and complementing the spatial information obtained from confocal microscopy (Pfleger *et al.* 2006a,b). Furthermore, the recently validated extended BRET (eBRET) now enables these interactions to be monitored for prolonged time periods (Pfleger *et al.* 2006c), with signals resulting either from stable receptor-arrestin complexes or a steady state of multiple transient interactions over time. Our studies using eBRET and BRET dose-response assays, supported by internalisation and recycling ELISAs and confocal microscopy, provide novel insights into the current classification scheme. GPCRs used to illustrate our observations include the thyrotropin-releasing hormone receptors, the orexin receptors and the angiotensin II receptor type 1a.

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The role of the tumor supressor- p53 in alpha1D- adrenergic receptor (AR) induced apoptosis in vascular smooth muscle cells

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Presenting Author: **M. T. Piascik**

Activation of the alpha1-AR with phenylephrine (PE) promoted a rapid increase in reactive oxygen species (ROS) in fibroblasts stably transfected with each of the alpha1-ARs as well as in human aortic smooth muscle cells (HASMC). RT –PCR, showed that each of the alpha1-ARs was expressed in the HASMC. The alpha1A-AR selective antagonist WB 4104 blocked the ROS increase only in fibroblasts expressing the alpha1A-AR. BMY 7378, a selective alpha1D-AR antagonist, blocked ROS generation only in fibroblasts expressing the alpha1D-AR. In HASMC, BMY 7378, but not WB 4104, blocked generation of ROS. This indicates that in HASMC it is the alpha1D-AR that is coupled to the generation of ROS. In HASMC, PE stimulated the translocation of the tumor suppressor protein p53 to the mitochondria. The translocation of p53 was blocked by the alpha1D-AR antagonist BMY 7378. As p53 is involved in activating programmed cell death, we examined apoptosis in HASMC. A 24hr treatment with PE induced apoptosis in HASMC. PE stimulated apoptosis was blocked by BMY 7378 but not WB 4101. PE activated apoptosis was also blocked by the p53 antagonist pifithrin alpha. These data show that 1) While each of the alpha1-ARs can couple to ROS generation in stably transfected fibroblasts, there is a specificity of coupling in mammalian cells that express all three receptors and the alpha1D-AR is linked to ROS generation in HASMC, 2) The alpha1D-AR can promote the mitochondrial translocation of p53, 3) The alpha1D-AR can produce apoptosis in HASMC, 4) the Alpha1D-AR appears to activate an apoptotic pathway that involves p53. These are the first data linking a G-protein coupled receptor to the multifunctional tumor suppressor p53.

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Cytoprotective characterization of a â-arrestin/small heat shock protein signaling complex

Erin B. Harmon, Lalida Rojanathammanee, Bryon D. Grove, Masaru Miyagi, and James E. Porter

Presenting Author: **James E. Porter**

Heat shock proteins (HSP) represent an emerging model for the coordinated, multistep regulation of apoptotic signaling events. Although certain aspects of the biochemistry associated with HSP cytoprotective effects are known, little information is found describing the regulation of HSP in response to harmful stimuli. While screening human urothelial (UROtsa) cells for non-canonical â adrenergic receptor (AR) signaling pathways using mass spectroscopy techniques, an agonist-dependent interaction with â-arrestin and the small HSP 27 was observed in vitro. Formation of this â-arrestin/HSP27 complex in response to the selective âAR agonist isoproterenol, was subsequently confirmed in situ by immunofluorescent co-localization studies. Using TUNEL analysis to detect apoptosis, pre-incubation of UROtsa cells with isoproterenol was found to be sufficient for protection against programmed cell death initiated by staurosporine. RNA interference strategies confirmed the necessity for both HSP27 and â-arrestin to confer this cytoprotective consequence of âAR activation using this cell model. As a result, these studies represent the first description of an agonist-dependent relationship between a small HSP and â-arrestin to form a previously unknown anti-apoptotic fisignalosomefl.

The evolutionary modification of the npxxy(x)5,6f motif to npxxy(x)5,6l in the cannabinoid cb1 receptor leads to structural changes in the helix 8 region that may contribute to maximal activity and to differential coupling

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The NPXXY(X)5,6F motif in rhodopsin (Rho), which links by aromatic interaction Y7.53(306) to F7.60(313), has been proposed to provide structural constraints for the Rho inactive state. The cannabinoid CB1 receptor, a Class A GPCR, lacks the NPXXY(X)5,6F motif, having a Leu instead of Phe at 7.60. The goal of the studies described here was to investigate the structural consequences of the introduction of a Phe at position 7.60 in CB1. We contrasted these studies with results for the substitution of Ile at 7.60, the corresponding residue in the CB2 receptor. In order to explore the structural, as well as dynamical characteristics of this region, we carried out a series of NAMD2 multi-nanosecond molecular dynamics simulations on WT CB1, and the L7.60F and L7.60I mutants, in a fully hydrated POPC phospholipid Bilayer environment. These studies suggest that the packing of Hx8 with TMH1 is different in each case. The hydrogen bonding patterns along the helix backbones of each Hx8 also are different, as are the geometries of the elbow region of Hx8 (R7.56(400)-K7.58(402)). R7.56 (a residue of primary importance in determining apparent WT CB1 affinity for G protein) was found to exist in a helical region in each of the mutants, but to exist in a non-helical region in WT CB1. Taken together, these results suggest that G protein coupling may be altered for the L7.60F and L7.60I mutants relative to WT CB1.

While agonist and antagonist binding were found to be unaffected by the L7.60F and L7.60I mutations, the two mutant receptors differed from wild-type in their ability to regulate G-proteins in the [35S]GTP-gamma-S binding assay. The L7.60F receptor exhibited attenuated stimulation by agonists WIN-55,212-2 and CP-55,940 but not HU-210, whereas the L7.60I receptor exhibited impaired stimulation by all agonists tested. Reconstitution experiments with pertussis toxin-insensitive G proteins revealed loss of coupling to N-type calcium channels for G-alpha-i3 but not G-alpha-0A in the L7.60I mutant. Furthermore, reconstitution of the WT and mutant receptors with G-alpha-i3 but not G-alpha-0A enhanced the basal facilitation ratio at N-type calcium channels, suggesting that G-alpha-i3 may be responsible for CB1 tonic activity. This study suggests that the evolutionary modification to NPXXY(X)5,6L contributes to maximal activity of CB1 receptor, and provides a molecular basis for the differential coupling observed with chemically different agonists. [Support: Grants DA09978, DA05274 (MEA) DA00489, DA03934 (PHR)]

Functional rescue of intracellularly retained MC4-R mutants responsible for early onset morbid obesity using pharmacological chaperone strategy

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The melanocortin-4 receptor (MC4-R) plays a pivotal role in the control of energy homeostasis. Blockade of MC4-R signaling leads to hyperphagia, reduced energy expenditure and ultimately, obesity in humans and rodents. Mutations in the MC4-R may be responsible for 4 to 6% of cases of severe obesity in children and are the most common monogenic cause of obesity to date. Intracellular retention of the receptor has been proposed to be the most frequent consequence of the mutations, with 80% of childhood obesity-related MC4-R mutations leading to partial or complete retention in the biosynthetic pathway. Intracellular retention of misfolded mutant receptors is a common cause of loss-of-function phenotypes for G-protein-coupled receptors (GPCRs). This retention is the result of the recognition of incompletely or misfolded protein by the cell's quality control system within the endoplasmic reticulum (ER) and Golgi. The fact that heterozygous null mutations result in an obese phenotype in both humans and mice indicates that the regulatory system is sensitive to quantitative variation in MC4R function and that this receptor might represent a tightly regulated point in the homeostatic control of body weight. Thus, increasing cell surface expression of intracellularly-retained mutant receptor could have an effect on the phenotype. The present study therefore was aimed at characterizing the subcellular distribution of obesity-related mutant forms of hMC4-R and testing a pharmacological approach to increase their cell surface expression.

Ten different point mutations described in hMC4R that cause early onset obesity in humans were selected [S58C, E61K, N62S, I69K, I125K, T162I, R165W, R165Q, C271Y, P299H] based on prevalence in the human population and broad coverage of different receptor domains. Among the MC4R agonists and antagonists developed recently, four compounds were chosen (2 antagonists and 2 agonists) to test for their ability to act as pharmacological chaperones, facilitating the folding and cell-surface expression of MC4R-mutants.Ten mutant constructs and WT hMC4R, each carrying a 3xHA tag at the N-terminus and the yellow fluorescent protein (EYFP) at their C-terminus, were transiently transfected into HEK293T cells. The relative cell surface expression of the receptors was assessed by flow cytometry through the differential monitoring of cell surface HA immuno-reactivity and total cellular EYFP fluorescence. Signaling activity was determined by measuring cAMP accumulation in MC4-R expressing cells following stimulation with the agonist NDP-α MSH. Under basal conditions, nine mutant receptors [S58C, E61K, N62S, I69K, T162I, R165W, R165Q, C271Y, P299H] were largely retained intracellularly, leading to marginal or undetectable signaling activity. Surprisingly, we found that the relative cell surface expression of the mutant hMC4R (I125K) was similar to the WT hMC4R ; however, I125K MC4R is non-functional, possibly due to deficient coupling efficacy. Treatment with the four compounds tested led to total or partial rescue in cell surface expression and signaling activity of the mutant receptors. Interestingly, the ability of the different compounds to promote cell surface expression and functional rescue varied; some compounds worked better for specific mutations but at least one of the antagonist compounds showed a broader activity profile.

These results demonstrate the potential application of pharmacological chaperones for the treatment of genetic obesity in humans, via restoration of mutant melanocortin 4 receptor function.

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Characterization and mechanistic investigation of CCG-4986, a small molecule RGS4 Inhibitor

DL Roman, JN Talbot, RA Roof, RK Sunahara, JR Traynor, RR Neubig

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Regulators of G protein signaling (RGS) Proteins accelerate the intrinsic GTPase activity of GTP-bound G-alpha subunits, promoting the reformation of the inactive g protein heterotrimer. Our aim is to demonstrate the feasibility of RGS proteins as novel pharmacological targets for modulating G protein coupled receptor signaling. We recently reported (Mol Pharm, Jan 2007) the discovery of CCG-4986 (methyl N-[(4-chlorophenyl) sulfonyl]-4-nitrobenzenesulfinimidoate), a small molecule inhibitor of RGS4 identified in a high throughput screen or Galpha/RGS4 binding. CCG-4986 binds directly to RGS4 with a Kd of ~3uM as demonstrated by quenching of RGS4 intrinsic fluorescence. It inhibits the RGS4/G alpha interaction, functionally inhibits RGS4 GTPase accelerating activity, and inhibits RGS4 action on mu-opioid receptor mediated adenylate cyclase regulation on permeablized C6 cells. CCG-4986 exhibits specificty, as it does not inhibit G alpha binding or GTPase acceleration by RGS7 or RGS8, the most similar RGS4 family member. Surprisingly, the actions of CCG-4986 were found to be irreversible, as washing steps do not reduce the
inhibition of RGS4 by CCG-4986. Furthermore, high concentrations of sulfhydryl reagents can reverse the inhibition, suggesting covalent modification of cysteines on RGS4. This should facilitate studies to determine the site of action of CCG-4986 on RGS4 and the identification of additional inhibitor compounds by computer-based docking studies.

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Peptide Ligands of Regulators of G-Protein Signaling 4 (RGS4) Identified by Screening of a Focused One-Bead, One-Compound

Rebecca A. Roof, David L. Roman, Katarzyna Sobczyk-Kojiro, Anjanette Turbiak, Henry I. Mosberg, and Richard R. Neubig

Presenting Author: **Rebecca A. Roof**

The objective of this study is to identify peptide inhibitors of Regulator of G-Protein Signaling 4 (RGS4) that are more potent than our lead compound, YJ34 (Ac-Val-Lys-[Cys-Thr-Gly-Ile-Cys]-Glu-NH2, S-S). YJ34 was rationally designed to inhibit RGS activity by mimicking the switch 1 region of Gαi, based on the RGS4-Gαi crystal structure. We synthesized a focused One-Bead, One-Compound peptide library which retains structural features known to be necessary for YJ34 activity. The library of 2.5 million peptides was screened and peptide beads with increased binding to a fluorescently labelled RGS4 were isolated. The hit sequences identified were resynthesized and verified for binding fluorescent RGS4 in a flow cytometry assay. One of the verified hits decreases the intrinsic fluorescence of RGS4 with Kd = 50 nM, and another blocks the interaction of RGS4 with Gαo with IC50 = 40 μM. In conclusion, even though the library was constrained to have structural similarities to our lead compound, we have identified peptides that have different functions and possibly different modes of binding RGS4. Future directions include more complete functional evaluation and determining if peptides of similar structures can compete for binding.

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A novel strategy to identify proteins that interact with the M3 muscarinic receptor in vivo using the split-ubiquitin system in yeast

Erica Rosemond, Bo Li, Sally McMillin, Jurgen Wess.

Presenting Author: **Erica Rosemond**

The five muscarinic acetylcholine receptors (M1-M5) are widely expressed throughout the central and peripheral nervous systems. These receptors are prototypic members of the superfamily of G protein-coupled receptors (GPCRs). Recent evidence suggests that the function of many GPCRs can be modulated by different classes of receptor-interacting proteins. The objective of the present study was to identify proteins that can interact with the M3 muscarinic receptor, a subtype that is found in most regions of the CNS. Recently, the split-ubiquitin yeast two-hybrid system has emerged as a novel approach to study membrane protein interactions. Using this system we screened a human brain cDNA library, which led to the identification of 69 proteins predicted to interact with the M3 receptor in yeast. Most of these proteins have not been previously identified to interact with the M3 receptor. Of considerable interest the candidates include other GPCRs and membrane proteins, several members of the tetraspanin family, various signaling molecules, proteolipid protein-domain containing proteins and recently cloned proteins of unknown function. It is expected that the results from this screen will provide more insight into the activation, regulation and/or the physiological function of the M3 muscarinic receptor.

The interaction between M2 and M3 muscarinic receptor subtypes in mediating stomach contraction is sub-additive

Alan S. Braverman, Ronald J. Tallarida and Michael R. Ruggieri

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Receptor subtypes exist for most G-protein coupled receptors and multiple subtypes may coexist in the same tissue. For muscarinic receptors, most smooth muscles contain a heterogenous population of M2 and M3 subtypes. Generally M2 receptors outnumber M3 receptors by about 3 to 1. As determined by classical Schild analysis, subtype selective antagonist affinity is most consistent with contraction being mediated by the M3 subtype. However, in esophageal smooth muscle and in some human bladders, antagonist affinities suggests that M2 receptor also contribute to the contractile response. In addition a contractile function for the M2 receptor subtype has been demonstrated following induced pathologies including the hypertrophied rat bladder, inflamed rat colon, and in the lower esophageal sphincter from a model of esophagitis. Findings in knockout mice indicate that the M2 receptor is capable of mediating a contractile response approximately 20-40 % of the contractile response mediated by the M3 receptor subtype. The present study was performed to determine whether the M2 and M3 receptor subtypes interact to mediate contraction in wild type stomachs.

Cumulative carbachol concentration effect responses were determined for wild type, M2 and M3 KO stomach body smooth muscle. Using M2 and M3 dissociation constants (KD) for carbachol (obtained from the psychoactive drug screening program data base) the concentration values were converted to fractional receptor occupation.

The resulting occupation-effect relations showed maximum effects of 21.6 % and 88.6% of the KCl maximum for the M2 and M3 subtypes, respectively. These occupation-effect relations allow the determination of the expected additive isobole (occupation pairs for constant effect) for any effect (E) level between zero and the 100%. This analysis used E = 40% of the KCl maximum and yielded an additive isobole for this effect, thereby providing a curve against which the experimental value (occupation pair) in the wild type can be compared. Fractional receptor occupancies for the expected additive effect were calculated from this isobole and yielded values 0.148 for M2 and 0.0076 for M3. The actual values determined experimentally in the wild type were found to be significantly greater, namely, 0.337 for M2 and 0.0221 for M3. Therefore, these occupation values indicate that the required carbachol concentration to attain this effect was 1.40 μM, whereas the calculated additive concentration from the isobole = 0.476 μM, a value approximately one third of the experimental. This greater than expected receptor occupancy indicates that the interaction between these mutually occupied receptors is sub-additive.

GPCR oligomerization involves the participation of all transmembrane domains

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Presenting Author: **Saurabh Sen**

Membrane bound cell surface receptors such as GPCRs are involved in the communication between the external environment and biological factory housed within the cell. Oligomerization of these GPCRs is now widely accepted but the mechanism of how GPCRs form larger oligomeric structures is elusive. Examples from literature suggest that the lipid-exposed portions of the transmembrane helices (TMs) constitute a major portion of the oligomerization interface. To comprehensively study the contributions of the lipid-facing residues of a GPCR to receptor function and oligomerization, we systemically generated chimeric C5a receptors in which five to six lipid-exposed residues in TM1, TM2, TM4, TM5, TM6 or TM7 were exchanged with the cognate residues from the angiotensin AT1 receptor, another member of the rhodopsin family of GPCRs which, as shown here, does not form oligomers with the C5a receptor. Selection of the lipid-facing residues was based on the X-ray structure of bovine rhodopsin and supported with our molecular model of the TM bundle of the C5a receptor. This novel approach preserved the intrahelical interactions known to stabilize the TM bundle while only altering those residues expected to mediate protein-protein interactions, such as in receptor oligomerization. Using fluorescence micrographs, we demonstrated that all of the lipid-facing chimeras were retained in the endoplasmic reticulum; but they are structurally competent enough to bind ligand and activate G proteins. Furthermore, the oligomerization studies revealed a complex set of interactions involving all of the TM helices. We hypothesize that GPCRs constitutively associate into larger oligomers in the ER, a process that involves multiple receptor interactions, and that this oligomerization phenomenon is necessary for transport to the plasma membrane. These studies highlight the importance of the lipid-facing residues in determining the transport competence of GPCRs, outlining a broad molecular signature profile for the oligomerization domain, and suggest that the ability to form larger-oligomeric structures using multiple transmembrane domains may be necessary for cell surface expression.

Monomeric and Dimeric Rhodopsins in Nanoscale Lipid Bilayers and Their Interaction with Transducin

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The trans-membrane signaling activities of G-Protein Coupled Receptors (GPCRs) can be modulated by formation of specific oligomeric complexes of the receptor. Indeed, the inherent activities and physiological relevance of dimeric GPCRs has been intensely debated in recent years. Resolving the specific activities of monomeric and dimeric GPCRs is important not only because of the underlying mechanistic details of trans-membrane signaling and coupling with the relevant G-proteins but also due to the potential of receptor cross-talk giving rise to a complexity and potential richness in the physiologically relevant cellular processes. In order to provide a definitive answer to the relative activities of monomeric and dimeric rhodopsin (Rho), we took advantage of the Nanodisc system of discoidal phospholipid bilayers (BioTechniques 40, 601-610, 2006). The size of these self-assembled membrane mimetics can be controlled through choice of the encircling amphipathic membrane scaffold protein (MSP) "belt". Using a 12 nm diameter Nanodisc, we generated a monodisperse sample of monomeric Rho together with 200 POPC phospholipids. By increasing the ratio of receptor to lipid/MSP during self-assembly we could favor generation of a dimeric Rho species. Both monomer and dimer Rho-Nanodiscs were separated in sucrose density gradients to afford pure receptor populations with a defined association state. We measured the activity of Rho by transducin dependent conversion to the MetaII form as well as the kinetics of transducin activation via tryptophan fluorescence. Interestingly, monomeric Rho displayed twice the active MetaII form as the dimeric sample, based on the number of receptors present. Additionally, transducin activation by the dimeric sample was ~ 1/2 that of the monomeric preparation. These results unambiguously demonstrate that: (1) monomeric Rho is active in photoinduced coupling to its G-protein and (2) the dimeric sample can interact with only a single transducin. Such results have important implications to the questions of signaling cross-talk. Supported by NIH grant GM33775 (SGS).

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The importance of preserving 125I-angiotensin II in brain AT1 receptor binding assays

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Angiotensin II (Ang II) receptors are G protein-coupled receptors (GPCRs) that have a variety of functions and transduction mechanisms. They have been the subject of numerous studies that define mechanisms of GPCRs. The Ang II receptor subtype that mediates most of the actions of Ang II is the AT1 subtype. However, a controversy has arisen regarding the endogenous agonist of brain AT1 receptors. Studies involving inhibition of brain aminopeptidases have sparked a debate as to whether Ang II or Ang III is the endogenous agonist of brain AT1 receptors (Reaux-Le Goazigo et al., Curr Hypertens. Rep. 2005, 7:1522). This begs the question: Does Ang II bind to the brain AT1 receptor? And, if so, but with no efficacy, is it an AT1 receptor antagonist? In brain angiotensin receptor binding studies radioligand degradation continues to be a major problem. Our objective was to design a procedure whereby binding of 125I-Ang II to rat brain hypothalamic AT1 receptors could be determined with minimal metabolic degradation. After 1 hr incubation at 24oC in 5 mM EDTA, 150 mM NaCl, 0.1 mM bacitracin and 50 mM NaPO4 (pH 7.2) buffer (with 10 μM PD123319 added to inhibit AT2 receptor binding) HPLC analysis indicated that only 3.1±0.8% of the bound radioligand was 125I-Ang II, 2.9±1.1% was 125I-Ang III and 82±3.2% was 125I-tyrosine. Specific (3 μM SI Ang II displaceable) binding was not significantly different from zero. With the addition of o-phenanthroline (1 mM), puromycin (3 mM), phenylmethylsulfonyl fluoride (PMSF, 1 mM) and glutamate phosphonate (3 mM) to the incubation buffer bound radioligand was 70.2±0.8% 125I-Ang II, 15.8±2.5% 125I-Ang III and only 6.5±1.2% other fragments. Specific binding was 630±140 pmol/mg wet weight with KD = 3.3±1.6 nM. These results indicate that under conditions which protect 125I-Ang II from degradation, it binds to brain AT1 receptors. This suggests that Ang II is capable of eliciting pressor and dipsogenic effects in the brain via the AT1 receptor without the necessity of its conversion to Ang III.

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Prenatal exposure to cocaine produces unique developmental and long-term adaptive changes in dopamine D1 receptor activity and subcellular distribution

Gregg Stanwood

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Low-dose intravenous cocaine administration to pregnant rabbits causes permanent structural alterations in DA-rich cerebral cortical areas, substantially reduced dopamine D1 receptor coupling to Gs protein, and cognitive function. The developmental influences of reduced D1-Gs coupling, and the underlying cellular basis, are unknown. Using primary neuronal cultures derived from the medial frontal cortex and striatum of in utero saline- and cocaine-exposed embryos, spontaneous neurite outgrowth of in utero-exposed cortical neurons was greater than in control neurons. In contrast, striatal neurons exposed to cocaine in utero exhibited an entirely opposite adaptive response, with diminished spontaneous neurite outgrowth compared to saline-exposed controls. Control neurons isolated from the two structures also exhibited opposite regulatory responses to the D1 receptor agonist SKF38393, inhibiting outgrowth in cortical cultures, and stimulating outgrowth in striatal cultures. The agonist was ineffective in modulating neurite outgrowth of neurons from either structure isolated from cocaine-exposed fetuses, reflecting the reduced D1-Gs coupling. Total D1 receptor number was indistinguishable in neurons from the cocaine- and saline-exposed animals, but cell imaging and receptor binding of differentially isolated membranes showed that the lack of responsiveness was due to greatly reduced cell surface localization of D1 receptors. These data suggest that prenatal exposure to cocaine causes a novel, long-lasting adaptive response in the subcellular distribution of D1 receptors, resulting in alterations in signaling capacity that have developmental and behavioral consequences.

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Identification of residues in the binding pocket of GPR40 that contribute to potent agonist interaction

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GPR40 belongs to a new sub-family of G protein-coupled receptors (GPCRs), the free fatty acid (FFA) receptors. GPR40 (aka FFAR1) has been implicated in the pathophysiology of type 2 diabetes and is a drug target because of its role in FFA-mediated enhancement of glucose-stimulated insulin release. The receptor is activated by medium to long chain FFAs, but the potencies are low. Small molecule agonists have been developed that have shown improved potency in the receptor. In order to delineate the molecular determinants contributing to binding, we developed a homology model of GPR40 liganded by GW9508, a small molecule agonist, or linoleic acid, a C18 polyunsaturated FFA, and used it to identify 12 residues within the putative binding pocket. The 12 residues were positively charged, aromatic or hydrophobic residues, and we subjected them to site-directed mutagenesis. Our results suggest that R183, N244 and R258 act as anchor residues for the carboxylate group on linoleic acid and GW9508. Furthermore, H86, Y91, H137, L186 and Y240 may contribute to aromatic and/or hydrophobic interactions with GW9508 that are not present, or only relatively weak with linoleic acid. The greater number of putative interactions between GW9508 and GPR40 compared to linoleic acid and GPR40 may explain the higher potency of GW9508. These data may prove useful in understanding the mechanism of activation of this recently discovered receptor and for the development of pharmacological agents, such as that using a receptor based-pharmacophore approach.

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Differential involvement of EPAC and PKA in cAMP-mediated inhibition of human lung fibroblast proliferation and contraction

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Inappropriate proliferation and contraction of airway smooth muscle cells and lung fibroblasts contribute to the airway wall remodeling that occurs in asthma and COPD. Identifying mechanisms that inhibit these responses could point to new therapeutic strategies. We previously presented evidence for involvement of the exchange protein directly activated by cAMP (EPAC) rather than the cAMP-dependent protein kinase (PKA) in the inhibition of proliferation of human airway smooth muscle (HASM) cells by beta-2 adrenergic (â2AR) agonists and other cAMP-elevating agents [FASEB J. 20:A171.9, 2006]. We hypothesized that EPAC would similarly be involved in cAMP inhibition of fibroblast proliferation but that PKA might mediate the cAMP inhibition of fibroblast contraction. For proliferation assays, HFL1 human fetal lung fibroblasts were stimulated with epidermal growth factor (EGF, 60 ng/ml) in the absence or presence of various cAMP-related agents and [3H]thymidine incorporation was measured. For contraction assays, the ability of fibroblasts plated in three-dimensional collagen gels to contract the gels was measured with similar agents. Effects of these agents on PKA was assessed by in vitro activity assays. The â2AR agonist albuterol and the adenylyl cyclase (AC) activator forskolin (Fsk) both inhibited EGF-stimulated mitogenesis but the AC-inactive analog dideoxy-Fsk did not, implicating cAMP involvement. The EPAC-selective analog 2-methyl-8-chlorophenylthio-cAMP (2Me8CPT) inhibited mitogenesis at concentrations that had little or no effect on PKA, whereas the PKA-selective analog N6-benzoyl-cAMP (6Bz) did not inhibit mitogenesis at concentrations that markedly activated PKA. In contrast, fibroblast-mediated collagen gel contraction was inhibited by 6Bz but not by 2Me8CPT. These data implicate EPAC as the likely mediator for cAMP inhibition of HFL1 mitogenesis, similar to results with HASM cells, but they implicate PKA in the cAMP inhibition of contraction of these cells. Agents that selectively modulate EPAC vs. PKA could thus provide approaches to selectively control proliferation vs. contraction for therapeutic benefit in diseases involving airway remodeling. Funded By Nebraska Department of Health and Human Services and American Heart Association.

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ERK1/2 is activated by Gi2, GoB and GoA in relaxin family peptide receptor 3 (RXFP3) expressing cell lines

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Relaxin-3 is the most recently identified member of the relaxin/insulin peptide family. It is a neuropeptide that activates cell signalling via its cognate G protein-coupled receptor, the relaxin family peptide receptor 3 (RXFP3) (Bathgate et. al., 2006). In CHO-K1 and HEK293T cells recombinantly expressing human RXFP3 receptors (CHO-RXFP3 and HEK-RXFP3), the time course of ERK1/2 activation was rapid and transient (peak activation 2-5 min) with maximum response 8.91+/-0.98 fold/basal (HEK-RXFP3) and 7.31+/-0.53 fold/basal (CHO-RXFP3), following stimulation with H3 relaxin (10nM) (van der Westhuizen et. al., 2007) (n=6). Both HEK-RXFP3 and CHO-RXFP3 responded to H3 relaxin in a concentration-dependent manner (pEC50: 9.09+/-0.15 and 9.17+/-0.14, respectively) when stimulated for 5 min (n=3). Activation of ERK1/2 was completely blocked in the presence of pertussis toxin (100ng/ml, 18h) with response over 2-5 min 0.97+/-0.06 fold/basal (HEK-RXFP3) and 1.41+/-0.09 fold/basal (CHO-RXFP3), suggesting a prerequisite for Gi/o proteins (n=6; $P<0.001$). Transient transfection of pertussis toxin resistant G proteins (C351I mutation) revealed that ERK1/2 was activated via Gi2 and GoB in CHO-K1 cells and via Gi2, GoA and GoB in HEK293T cells (n=6). There was no ERK1/2 activation in either cell line with Gi1 or Gi3 proteins, We previously observed that the activation of ERK1/2 occurred by two pathways in these cells, one that required PKC and the other that required PI3K (van der Westhuizen et. al., 2007), and suggest that the differences observed in the activation of ERK1/2 in the two cell lines may arise through coupling to different isoforms of gi protiens.

Bathgate RA et. al. (2006) Pharmacol Rev 58:7-31
van der Westhuizen ET et. al. (2007) Mol Pharm EPub:PMID17351017

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Multi-use GPCR Cell Lines: One Cell Line, Many Assay Read-Outs

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In high-throughput screening, it is often advantageous to perform primary and follow-up screens with different assay formats to confirm hit identity. If the same cell line can be used to perform these screens, the data can be more easily cross-compared, as variability due to cell line specific differences is minimized. We show that our GeneBLAzer® validated cell lines can be used in multiple assay formats including: beta-lactamase reporter gene assays, Fluo-4 calcium assays, and radioligand binding assays. Data from a small compound library (LOPAC 1280™) antagonist screen of the 5HT1a-Ga15-NFAT-bla CHO-k1 cell line is shown for both a beta-lactamase and a Fluo-4 readout. Membrane preparations created from this 5HT1a cell line were then used to confirm some of the identified hits in a radio-ligand binding assay. We have further utilized Division-Arrest Technology to show that the 5HT1a cell line can also be effectively utilized in an assay-ready format which allows researchers to use cells as a reagent much like enzymes or membrane preparations.

Direct measurement of drug efficacies at G protein-coupled receptors

Jean-Pierre VILARDAGA

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Cell surface receptors known as G-protein coupled receptors (GPCRs) act as molecular switches to convert the binding of a large variety of extracellular chemical and sensory stimuli into cell signals. GPCRs regulate a multitude of physiological processes (e.g. heart beat), and have become principal targets by most of the clinical drugs. The fundamental property of drugs acting at receptors is the distinction of compounds that can either fully or partially stimulate (full or partial agonists) receptor-mediated signaling cascades, or reduce (inverse agonists) the receptors' basal activity. To understand the molecular basis for differences in the mode of action of these divers classes of compounds, we developed a fluorescent resonance energy transfer (FRET)-based method that allows the direct recording of ligand-induced conformational changes in GPCRs in real time in living cells.

First, we showed for the alpha2A-adrenergic receptor that inverse agonists differentiate themselves from agonists by triggering a very distinct conformational change of the receptor, and that the intrinsic efficacy of compounds directly correlated with their effects on the FRET signal. Next, we showed a direct association between beta1-adrenergic receptor polymorphisms and beta-blocker selectivity. Thus, our approach permits now a detailed investigation of drug efficacy at the level of the receptor, and to assess the effect of receptor polymorphisms, with respect to receptor conformation, in cell responses to clinical drugs.

Central G-protein regulation of cardiovascular and renal function in conscious Sprague-Dawley rats

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The role of G-protein signaling pathways involved in the central regulation of cardiovascular and renal function in vivo has not been determined. To investigate this question we examined the cardiovascular (CV) and renal responses produced by intracerebroventricular (i.c.v.) injection of the opioid-like peptide, nociceptin/Orphanin FQ (N/OFQ) in conscious rats following inhibition of Gάi/Gάo, Gάz orGάq transducer protein pathways. N/OFQ was used for these studies since central administration of this peptide produces marked CV depressor and diuretic responses in conscious rats via selective activation of the G-protein coupled N/OFQ peptide (NOP) receptor which signals through Gάi/Gάo, Gάz orGάq proteins. Methods: Prior to investigation rats received an i.c.v. injection of isotonic saline vehicle (5 µl, N=6), pertussis toxin (PTX, 1 µg, N=4, 48 hrs), a Gάz ODN (25 µg, N=6, 24-h) or a Gάq ODN (25 µg, N=3, 24-h), to inhibit Gάi/Gάo, Gάz or Gάq protein pathways, respectively. On the study day, N/OFQ (5.5 nmol/5 µl) or vehicle (5 µl) was injected i.c.v. into pre-treated conscious chronically instrumented rats. Mean arterial pressure (MAP) and heart rate (HR) were recorded and urine collected for 80-min (10-min periods). Results: Basal levels for CV and renal function were not different between treatment groups. In vehicle pretreated rats, i.c.v. N/OFQ significantly ($P<0.05$) reduced MAP (20-min; delta -16 ±5 mmHg) and HR (delta -82±14 bpm). In contrast, the hypotensive and bradycardic responses to N/OFQ were abolished in rats pretreated with PTX (20-min; delta -1±3 mmHg, delta 12±17 bpm) although urine output increased similarly between groups (50-min; vehicle, delta150±13 µl/min; PTX, delta 151±20 µl/min;). In Gάq ODN-pretreated rats, central N/OFQ also produced cardiovascular depressor and diuretic responses, but the duration of the hypotension and magnitude/duration of the diuresis were significantly greater than those produced by N/OFQ in vehicle-pretreated animals. In separate Gάz ODN pre-treated rats, i.c.v. N/OFQ also produced a reduction in MAP and HR, but the magnitude and duration of the diuresis was significantly blunted. Conclusion: I.c.v. PTX prevented the cardiovascular depressor, but not diuretic, responses to N/OFQ demonstrating that Gάi/Gάo activity is only required for mediating cardiovascular depressor function. Alternatively, there appears to be an interaction between Gάz (inhibit) and Gάq (augment) protein effects on central N/OFQ-evoked mechanisms controlling urine output.

Together, these findings demonstrate a clear and selective role of central G-proteins in the regulation of cardiovascular and renal function in conscious rats. DK43337, HL071212, P20 RR018766

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Detection of multiple H3 receptor affinity states utilizing [3H]A-349821, a novel, selective, non-imidazole histamine H3 receptor inverse agonist radioligand

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A-349821 is a selective histamine H3 receptor antagonist/inverse agonist. Herein, binding of the novel non-imidazole H3 receptor radioligand [3H]A-349821 to membranes expressing native or recombinant H3 receptors from rat or human sources was characterized and compared with the binding of the agonist [3H]N alpha-methylhistamine ([3H]NAMH). [3H]A-349821 bound with high affinity and specificity to an apparent single class of saturable sites and recognized human H3 receptors with 10-fold higher affinity compared to rat H3 receptors. [3H]A-349821 detected larger populations of receptors compared to [3H]NAMH. Displacement of [3H]A-349821 binding by H3 receptor antagonists/inverse agonists was monophasic, suggesting recognition of a single binding site, while that of H3 receptor agonists was biphasic, suggesting recognition of both high- and low-affinity H3 receptor sites. pKi values of high-affinity binding sites for H3 receptor competitors utilizing [3H]A-349821 were highly correlated with pKi values obtained with [3H]NAMH, consistent with labelling of H3 receptors by [3H]A-349821. Unlike assays utilizing [3H]NAMH, addition of GDP had no effect on saturation parameters measured with [3H]A-349821, while displacement of [3H]A-349821 binding by the H3 receptor agonist histamine was sensitive to GDP. In conclusion, [3H]A-349821 labels interconvertible high- and low affinity states of the H3 receptor, and displays improved selectivity over imidazole-containing H3 receptor antagonist radioligands. [3H]A-349821 competition studies showed significant differences in the proportions and potencies of high- and low-affinity sites across species, providing new information about the fundamental pharmacological nature of H3 receptors.

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A Ligand Based Structural Biology Approach for the Characterization of the Cannabinoid Receptor Binding Domains

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Functional heterologous over-expression and purification of large quantities of GPCRs is the key to more rapid progress in understanding their structure and function. Currently characterization of GPCR ligand binding domains as a major goal in the design of improved drug molecules is limited to computational prediction combined with mutagenesis experiments. In our “Ligand Based Structural Biology” approach we are using different classes of affinity labeled cannabinergic ligands to obtain structural information through the identification of a number of key amino acid residues associated with the binding of cannabinergic ligands. This goal involves a number of technically demanding experimental steps: (A) functional over-expression of CB2 cannabinoid receptors; (B) design and synthesis of high affinity and selective covalent ligands; (C) interaction of these ligands with CB2 receptor preparations; (D) purification of the ligand-receptor covalent complex; (E) identification of the amino acid residue(s) through MS analysis of the digested fragments. The precise knowledge of receptor-bound ligand structures can be fully accessed using molecular modeling based on our experimental data. Such information provides unique opportunities for drug discovery and substantially aids the development of tailor-made medicines.