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Meeting Review: Advances from the GPCR Retreat

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In London, Ontario, the 8th Annual Joint meeting of the Great Lakes GPCR Retreat and the Club des Récepteurs à Sept Domaines Transmembranaires (now known simply as the GPCR Retreat) was held September 27–29, 2007. This meeting gathers together a core group of investigators from Michigan, Ontario, and Québec and has steadily increased its attendance in both the eastern (Europe) and western (USA, Canada) directions. The highlight this year was a sneak preview of the β_2AR crystal structure provided by Brian Kobilka, but as can be seen below, many other cutting edge talks were heard as well.

Key Words: G protein-coupled receptors; Signaling; Protein trafficking; Protein agonism.

Novel GPCR Signaling Pathways

In addition to the classical signaling paradigms associated with the activation of G protein-coupled receptors (GPCRs), it has become widely appreciated that there are a number of novel pathways that are activated by and/or regulate the activity of either receptors or their G protein partners. Furthermore, novel roles for proteins classically involved in the regulation of GPCR activity, such as G protein-coupled receptor kinases (GRKs) and arrestins, continue to be discovered. In a keynote lecture dedicated to the memory of Dr. Hyman Niznik, Heidi Hamm described two novel signaling partners for G$\beta\gamma$ subunits, namely, the inaptly named receptor for activated C kinase, RACK1, and the SNARE complex (1). Dr Hamm's studies showed that RACK1 acts as a negative regulator of G$\beta\gamma$ signaling, because its binding site on G$\beta\gamma$ overlaps with

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those of phospholipase Cβ isoforms 2 and 3, adenylyl cyclase II, and PI3 kinase, but not other effectors such as G$\beta\gamma$-mediated activation of the ERK MAP kinase pathway or G$\beta\gamma$-mediated chemotaxis (2,3). By interfering with some pathways but not others, RACK1 appears to act as a switch in cells that tends to bias G$\beta\gamma$ signaling toward certain effectors, and this appears to underlie the ability of RACK1 to realign the signaling outcome of formyl-methionyl-leucyl-phenylalanine (fMLP) treatment of HL60 cells from a response-inducing cell polarization to one that stimulates chemotaxis. Dr. Hamm also outlined the intricate mechanism by which the activation of G$\beta\gamma$ by presynaptic Gi-coupled autoreceptors impedes presynaptic neurotransmitter release. Briefly, G$\beta\gamma$ inhibits the opening of voltage-gated calcium channels through its association with syntaxin and other proteins in the SNARE complex. G$\beta\gamma$ and synaptotagmin compete for binding to SNAP-25, syntaxin1A, and the SNARE complex. G$\beta\gamma$ binding therefore serves to impede SNARE-dependent exocytosis; however, this G$\beta\gamma$-dependent inhibition of neurotransmitter release can be overcome by increases in local calcium concentrations. Overall, the emerging picture is that G$\beta\gamma$ can tune the interaction of its partner proteins and vice versa.

Another relatively recent series of findings highlights receptor-independent modulation of G proteins complementing a parallel set of findings related to G protein-independent receptor signaling (4). Stephen Lanier's lab was first to demonstrate the existence and functional relevance of a group of proteins known as non-receptor activators of G protein signaling (AGS) and has identified 10 different AGS proteins to date (5,6). These are now known to work through a variety of mechanisms. Group I AGS proteins are guanine nucleotide exchange factors that promote receptor-independent G protein activation by facilitating GDP dissociation from, and thus GTP binding to, Gα subunits. Group II AGS proteins (also called GPR or GoLoco proteins), in contrast, *inhibit* GDP dissociation, but they may promote G$\beta\gamma$ signaling by altering the association between Gα and G$\beta\gamma$. Group III AGS proteins differ from the others in that they do not appear to bind appreciably to Gα, but rather they produce their effects by binding directly to G$\beta\gamma$. In his talk, Dr Lanier described a conditional AGS3 knockout mouse, and animals lacking this Group II AGS isoform exhibit reduced blood pressure and a loss of diurnal blood pressure variation. His results again highlight the need for a better understanding of receptor-independent G protein signaling.

Cristina Murga spoke about the direct regulation of MAP kinase signaling by the GPCR kinase GRK2. While best known for its ability to phosphorylate activated GPCRs, Dr Murga and her group have demonstrated that GRK2 is also able to phosphorylate p38 MAP kinase at a residue (Thr^{123}) distinct from the canonical site ($Thr^{180}GY^{182}$) known to be targeted by other classic upstream activators (7). This effect of GRK2 on p38 serves to decrease its binding to MKK6

and substrates and thus impair its activation and activity. This could be relevant in pathological situations, such as inflammation and cardiac dysfunction, where altered GRK2 levels have been found to correlate with p38 MAP kinase activation. Too little GRK2 correlates with excessive p38 activation seen in some inflammatory disorders such as arthritis and multiple sclerosis, whereas too much GRK2 parallels the reduced p38-mediated responses seen in hypertension or heart failure.

Kathryn DeFea was one of several speakers who described novel aspects of the complex roles played by β-arrestins in GPCR signaling. Both β-arrestin1 and β-arrestin2 appear to play key roles in orchestrating GPCR-stimulated chemotaxis and cell migration. Indeed, such responses, triggered by PAR2 protease-activated receptor signaling in numerous cell types, are impaired by the cellular knockdown of either arrestin isoform using siRNA or in knockout animals. Chemotaxis is an asymmetrical process that requires pseudopod extension, and these regions of the cell are found to be enriched in β-arrestins, where in response to PAR2 activation they increase the activity of the actin assembly protein cofilin by inhibiting LIM kinase activity and increasing activity of chronofin, a cofilin-specific phosphatase (8). This β-arrestin-dependent dephosphorylation of cofilin is independent of Gq-mediated increases in intracellular calcium that also occur in response to PAR2 signaling and thus provides another example of switching of receptor-mediated signals induced by arrestins.

Structural Features of GPCR Signaling Complexes

Our growing appreciation of the diversity of GPCR signaling in recent years is matched only by an appreciation of the organizational or architectural complexity of GPCR signaling complexes. A number of speakers at this year's GPCR retreat added richly to our appreciation of the temporal and architectural aspects of GPCR signaling, in terms of receptor trafficking, receptor interactions, and receptor structure.

Mark Rasenick discussed his research on Gsα and its interactions with GPCRs, lipid rafts, and the cytoskeleton. Using total internal reflectance fluorescence (TIRF) microscopy to observe the disappearance of this G protein from the plasma membrane, he demonstrated that Gsα internalization occurs via lipid rafts and follows a trajectory distinct from that of its effector adenylyl cyclase or its activating GPCR. Once inside the cell, the G protein appears to bind to microtubules and activate the intrinsic GTPase activity of tubulin, thereby leading to decreased microtubule mass and changes in cell shape. The theme that distinct signaling effects (often believed to be G protein-independent) follow changes in receptor localization has become well accepted in recent years. Less attention has been paid to either the trafficking itineraries or the signaling events that follow G protein or receptor internalization. The findings

presented by Dr. Rasenick may be important in explaining why clinically depressed patients tend to have increased levels of Gs in lipid rafts and suggest the possibility of new therapeutic strategies based on the G proteins themselves for this illness.

A number of recent studies have extended the notion of GPCR signaling complexes to include direct interactions between the receptor and different effector molecules [(9,10), reviewed in (11)]. Fang Liu described novel evidence of a physical and functional link between the D2 dopaminergic receptor and the dopamine transporter (DAT) and outlined how the disruption of this interaction may contribute to mental illness. Binding of DAT to the third intracellular loop of this receptor facilitates reuptake of dopamine from synapses after neurotransmission, and mice treated with a transactivating regulatory protein TAT-labeled peptide blocking this protein-protein interaction exhibited hyperlocomotor activity. In postmortem brain from schizophrenia patients, D2-DAT coupling was found to be decreased, which could underlie, at least in part, the hyperdopaminergia that occurs in this and other neuropsychiatric disorders.

Gerald Zamponi also discussed recent results from his laboratory, which show direct interactions between N-type voltage-gated calcium channels and nociceptin [opioid receptor-like (ORL-1)] receptors in dorsal root ganglia (DRG) neurons. He showed that these proteins initially cotraffic to the cell surface but then they can be internalized together in response to stimulation by agonist. It is interesting that he also demonstrated that the ORL-1/N-type channel complex was associated with μ-opioid receptors, probably via heterodimerization of the two receptor subtypes. These larger complexes also show cointernalization in response to the μ-opioid receptor agonist DAMGO, but only when both receptors are present. These observations may be critical in explaining how tolerance to opioids develops. In addition, these complexes might also extend to other receptors (such as D1 and D2 dopaminergic and $GABA_B$ receptors) in dorsal root ganglia neurons and other parts of the CNS.

Craig Doupnik described how GPCRs, G proteins, and RGS proteins come together to regulate the function of the Kir 3 family of G protein-activated inwardly rectifying potassium channels in both neurons and cardiomyocytes. Regardless of the particular channel subtype or activating receptor, the correct function of these channels appears to require the formation of a multiprotein complex containing all of the above constituents. The specificity and kinetic aspects of how such complexes physically undergo formation and dissociation appear to vary depending on the identity of the particular players involved, and this is reflected in the variability of channel gating properties from one GPCR/RGS protein combination to the next. RGS4 is directly and stably associated with Kir 3 channels while RGS3 interacts transiently with the channel complex via a collision-coupling mechanism (12). Thus, changes in the specific cellular content of RGS proteins and indeed other regulatory molecules may affect both the activation kinetics (through Kir 3-dependent, RGS-enhanced

GPCR GEF activity) and inactivation kinetics (through RGS GAP activity). Stable channel/RGS interactions may also play a role in channel assembly and targetting. Taken together, these studies suggests that cells may be able to fine-tune Kir 3 channel activity by altering RGS protein expression levels.

Brian Kobilka's highly anticipated talk, which closed the meeting, presented the highest resolution to date of the structure of the β_2-adrenergic receptor representing over 15 years of work from his lab. He presented two structures at resolutions of 3.4 and 2.4 Å, respectively. The first structure was that of the receptor stabilized with an antibody, obscuring a number of receptor features (13). The second structure was of a mutated receptor with the third intracellular loop replaced by T4 lysozyme, again made stable enough for high resolution studies (14,15). Although the observed structural features of this inactive conformation of this receptor are largely consistent with previous work on rhodopsin, some key differences were observed. For example, the ionic lock that maintains rhosdopsin in an inactive state was broken in the β_2AR even in the presence of an inverse agonist, highlighting the conformational flexibility of the β_2AR. In addition, the "lid" formed by the extracellular domains of rhodopsin that appears to cover the binding pocket was not found in the β_2AR. Rather, a more rigid structure, containing a helix formed by extracellular loop 2 in the β_2AR was consistent with a requirement in the β_2AR (but not rhodopsin) for physical entry and dissociation of activating ligands into and out of the binding pocket, respectively. He noted that structural changes in response to ligand binding depended less on the packing of the helices in the receptor rather than on individual interactions with specific amino acid side chains—observations that will bear on earlier structure-function studies performed by using site-directed mutagenesis. He also noted that the crystal packing of the second structure was dimeric in nature, although the meaning of this packing is unclear with respect to receptor oligomerization.

Physiological Models of GPCR Signaling

Although molecular studies have taught us a great deal about how GPCRs work and how they interact with signaling partners, in addition to providing us with great insights into the structures involved, it remains clear that a real understanding of their function requires a return to the physiological context in which they normally function. Furthermore, studying GPCR signaling in the context of the living animal also offers insight into their roles under pathological conditions as well.

Jurgen Wess discussed the role of the M3 muscarinic acetylcholine receptor in pancreatic β-cells. Studies with β-cell-specific M3 receptor mutant mice (conditional knockout mice and transgenic M3 receptor-overexpressing mice) demonstrated that β-cell M3 receptors facilitate glucose-dependent insulin release and are critical for the regulation of whole body glucose homeostasis (16).

He also described a neuron- and glial cell-specific knockout of the M3 receptor, which showed a dwarf-like phenotype, associated with a pronounced hypoplasia of the anterior pituitary gland. Studies with transgenic mice selectively expressing genetically engineered versions of the M3 receptor in pancreatic β-cells [so-called RASSLs or receptors activated solely by synthetic ligands; see (17,18)] indicated that acute activation of either Gq or Gs-mediated β-cell signaling pathways leads to enhanced stimulation of insulin release and greatly improved glucose tolerance.

John MacDonald described novel roles for pituitary adenylyl cyclase actiavting peptide (PACAP) receptors, coupled to both Gs and Gq, which were found to enhance the function of NMDA receptors in CA1 hippocampal neurons. The Gq effects preferentially involve the activation of Src, which targets NR2A subunit-containing NMDA receptors. The Gs pathway involves PKA phosphorylation and another Src-like kinase, Fyn, which targets NR2B subunit-containing receptors. He also described how D2, D3, and D4 dopamine receptors, which transactivate the platelet-derived growth factor (PDGF) receptor, lead to selective internalization of NR2B subunit-containing receptors. The specific populations of GPCRs at individual synapses can therefore provide a powerful bidirectional control on synaptic plasticity, which Dr. MacDonald termed metaplasticity, leading to either long-term potentiation or long-term depression.

Andrea Echkart described novel aspects of the regulation of GPCR signaling in hypertension, focusing in particular on the roles of the GPCR kinases GRK2 and GRK5. She described how the targeted overexpression of GRK2 in murine vascular smooth muscle cells correlated with increased systolic and diastolic blood pressures, as well as decreased dilatory responses to the β-adrenergic agonist isoproterenol in isolated aortic rings (19). The underlying cause of these changes appears to be increased Gq-mediated vasoconstriction in these transgenic animals. Use of a Gq-specific inhibitor peptide, which she has demonstrated to be of value in models of hypertension (20), attenuated the effects of GRK2 but not GRK5 overexpression. In contrast, similar phenotypic changes in an analogously targeted GRK5 overexpressing strain appeared to reflect a Gi- or Go-dependent mechanism, because the treatment of these animals with pertussis toxin reduced mean arterial pressure but had no effect on heart rate. It is interesting that she also noted that the β_1AR (known mainly for its role in the myocardium) also has important effects in the vasculature, probably via coupling to Gi.

Nathalie Vergnolle showed how protease-activated GPCRs (PARs 1–4) play a major role in transmission of pain in inflammatory responses (21). PAR-activating proteases have been shown to be up-regulated in inflammatory bowel disease. In both a rodent model as well as in patients with irritable bowel syndrome (IBS), serine proteases including trypsin and tryptase are increased in the intestinal lumen and appear to sensitize pain neurons via PAR2 and thus

are likely to account for allodynia and hyperalgesia in IBS (22). PARs represent an extremely interesting target for development of new drugs to treat inflammation.

GPCR Functions in Intracellular Compartments

In recent years, it has become clear that GPCRs are capable of signaling at locations distinct from the plasma membrane. Numerous studies have shown that the machinery associated with desensitization of cell surface signaling events and receptor internalization is intimately associated with the activation of a second wave of cellular signals. Stéphane Laporte discussed how signaling partners, recruited to activated GPCRs, may control the initial steps leading to internalization of receptors. β-arrestins recruited to GPCRs in turn can recruit Src kinases, thus leading to the phosphorylation of clathrin-coated pit proteins such as the adaptor protein AP-2 (23). Further recruitment of MAP kinase signaling components to these endocytosing receptors regulates the stability of GPCR/ β-arrestin complexes, which ultimately impacts on the intensity and duration of both cell surface and endosomal signaling events. Mark von Zastrow's talk centered on the kinetic regulation of GPCR trafficking through coated pits, and he raised the question of how receptors themselves might control this process. Using TIRF microscopy to image individual endocytic events at the cell surface, he showed that while β_2AR activation did not affect coated pit assembly, it could delay a late stage of coated pit maturation, specifically the scission of the pit through a PDZ-scaffolding-dependent mechanism, and thus retard endocytosis. Mutation of the C-terminal PDZ ligand on the receptor abrogated this effect and was associated with the accumulation of GPCRs in a subset of clathrin-coated pits. The significance of all this is not yet understood, but such a delay might perhaps allow the coordination of signaling events that in some cases can accompany receptor internalization. Henrik Dohlman referred to signaling in endosomes as "a parallel universe" to signaling initiated at the cell surface. He showed that VPS34 and VPS15, components of the phosphoinositide 3-kinase (PI-3K) complex, were both important in yeast endosomes as effectors for Gα-dependent signaling (24) and led to the recruitment of a MAP kinase signaling cascade to the endosome. He also showed that a cytosolic protein acts as a guanyl nucleotide exchange factor (GEF) for the Gpa1 but not Gpa1 isoform of Gα. It is interesting that activation was independent of the Ste2 receptor but could also amplify the plasma membrane-based responses to pheremone. In addition, activation by this GEF was independent of the Ste2 receptor; however, it could also serve to amplify plasma membrane-based, receptor-mediated responses to pheromone. Louis Luttrell further discussed the nature of signaling events related to endocytosis. In particular, he described experiments using embryonic fibroblast cells from transgenic mice deleted for β-arrestin1 and/or β-arrestin2 or agonists that

are biased for signaling dependent of β-arrestins [(25), more on the subject of ligand-directed signaling below]. These signaling events are usually believed to be G protein-independent because β-arrestin also silences G protein signaling. It is becoming clear that under a variety of conditions and in different cell types, β-arrestins differentially 1) control the duration of the primary, plasma membrane-delimited signaling pathways, 2) promote receptor internalization, and 3) elicit unique patterns of gene expression via activation of distinct pools of MAPK.

Signaling from internalizing and endosomal receptors has almost become a classic GPCR paradigm in the last several years. Bruce Allen discussed a more recent stream of studies indicating that GPCRs and their associated signaling coterie may be targeted to other intracellular locations as well (26). Specifically, he discussed recent work from his lab showing that functional endothelin and β-adrenergic receptors were located on the nuclear membrane (27,28). It is interesting that these receptors can modulate the initiation of transcription in a PTX-sensitive manner, specifically the transcription of ribosomal RNA species. It was also possible to switch receptor signaling from an activation of transcription to an inhibition by inhibiting PKB, suggesting coupling to multiple signaling pathways. Further studies will tease out the molecular details and address the physiological relevance of nuclear GPCR signaling.

Ligand-Directed Signaling Events

A recurrent theme throughout the meeting was the pleiotropic nature of GPCR signaling. Recent experiments suggest that GPCR signaling pathways can be functionally compartmentalized so that different agonists acting at the same receptor may have different potencies depending on which effector pathway is being assayed. Terry Kenakin's talk focused on the theoretical aspects of these notions and how pharmacological assays are the key to detecting and understanding these effects. He also discussed the practical importance (and challenge) of designing selective pharmacological agents to modulate clinically useful signaling events while concurrently limiting detrimental effects that occur through the same receptor. Dr. Kenakin outlined an exciting theoretical example of this concept, wherein an allosteric ligand for the CCR5 chemokine receptor was discovered on the basis of its ability to act as a "pharmacological policeman" blocking the ability of the receptor to permit mediate HIV entry into cells without inhibiting the physiological ability of the receptor to bind its endogenous ligand, the chemokine CCL3L1. The recently discovered heterogeneity of the relative potency of a series of CCR5-based allosteric HIV-1 entry inhibitors for HIV entry vs. CCL3L1-mediated receptor internalization shows the potential for this effect. This approach could hold great promise for the development of improved receptor-based therapeutics. Michel Bouvier described some elegant molecular approaches to the study of ligand-directed

signaling (29). In the first series of experiments, his laboratory screened a panel of β-adrenergic ligands for effects on two distinct signaling pathways, the ERK MAP kinase pathway and the classic effector for the βAR, adenylyl cyclase. Using a cartesian representation of efficacy for each drug vs. each pathway, it was clear that some ligands had similar efficacies for both effectors but others had a more complicated profile in that in some cases inverse agonists for one signaling arc were agonists for the other and the signaling partners involved were different. This picture became even more complicated when two very similar receptors, the β_1AR and the β_2AR were compared (30). In a final series of experiments, he described the development of bioluminescent resonance energy transfer (BRET)-based biosensors for differential efficacy, rooted in our knowledge of the structure of G proteins and how different ligands for a receptor might induce different responses to a series of well-placed reporters for conformation between receptor and G protein (31). These measures highlight what he called the pluridimensinal character of efficacy and will inform our notions of drug discovery and development in the coming years. Terry Hébert discussed a possible mechanistic basis for pleiotropic receptor responses to ligand. It is highly likely that distinct, stable receptor-based signaling complexes are formed during receptor biosynthesis and contain G proteins, effectors, and possibly other regulatory molecules as well. Recent work from his lab has shown that G proteins play a central role in assembling GPCR signaling complexes in addition to their more generally appreciated role in cellular signaling. Both GPCRs and effector molecules interact with G$\beta\gamma$ subunits in the ER prior to assembly with Gα (32,33). It has become clear that assembly of the G$\beta\gamma$ complex is an event regulated by other proteins as well [see (34) for review]. DRiP78, an ER-resident molecular chaperone belonging to the J domain family of proteins, plays a role in the assembly of the G$\beta\gamma$ complex by associating with nascent Gγ and a Gβ-specific chaperone, phosducin-like protein 1 (35). Targeting GPCR signaling complex-specific chaperones with peptidomimetic or small molecules may eventually be converted into novel therapeutic strategies based on the notion that stimulus-directed signaling requires particular protein complexes.

In all, the world of GPCRs clearly still holds some surprises, many of which may be reported at the next GPCR retreat in 2008.

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