17th Annual Joint Meeting

Chicago, Illinois, USA
13th–15th October, 2016
Welcome to the 17th annual Great Lakes GPCR Retreat. This annual conference continues to attract top-notch researchers focusing on G-protein coupled receptors (GPCRs), the membrane proteins that orchestrate most physiological processes in the human body and are popular drug targets. Our attendees are graduate students, post-doctoral trainees, principal investigators from the academia, scientists from the pharmaceutical industry, and others. Most often, speakers and attendees come from the regions in the United States and Canada surrounding the Great Lakes. However, the conference also provides a highly sought after opportunity to scientists from all over the United States and Canada as well as from Europe, Asia, and Australia to discuss the most recent advances in GPCR research. This diverse and enthusiastic group participates in workshops, lectures, poster presentations, and informal discussions to consider controversies, recent developments and emerging concepts related to GPCR biochemistry, biophysics, pharmacology, and drug development.

Historical Perspective

The first Great Lakes GPCR Retreat was held in London, Ontario, in October 1999. Next year, the GPCR Retreat leadership and the ‘Club des Récepteurs à Sept Domaines Trans-membranaires du Québec’ have reached the decision to hold a joint meeting. This arrangement has increased the attendance and quality of the conference, making it a highly regarded national and international event for dissemination of results, exchange of ideas, and development of research collaborations. Since its inception, the conference has taken place at various locations in the United States and Canada around the Great Lakes (Ontario, Quebec, Michigan, Ohio and New York), home to many investigators working on GPCRs.

G Protein-coupled Receptors

GPCRs are the largest family of eukaryotic integral membrane proteins. They respond to stimulation by extracellular ligands and transmit the environmental signals to the cell interior. The functions of GPCRs are diverse and include vision, smell and taste, behavioral and mood regulation, immune and inflammatory response, nervous system transmission, cell density sensing, cell proliferation and survival, and many others. GPCRs are important targets for pharmacological intervention in disease. The conditions where GPCR therapeutics are used include cancer, cardiac dysfunction, diabetes, central nervous system disorders, obesity, inflammation and pain. Currently, 28% of all therapeutic compounds target GPCRs. Much more will be developed in the near future.

Organizing Committee

Vadim Gaponenko, Chair (University of Illinois at Chicago)
Demet Arac (University of Chicago)
Matthias Majetschak (Loyola University Chicago)
Asrar Malik (University of Illinois at Chicago)
Adriano Marchese (Medical College of Wisconsin)
Richard Miller (Northwestern University)
Aminah Pradhan (University of Illinois at Chicago)
Mark Rasenick (University of Illinois at Chicago)
# Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sponsors &amp; Advertisements</td>
<td>4</td>
</tr>
<tr>
<td>Program</td>
<td>9</td>
</tr>
<tr>
<td>Poster Abstracts</td>
<td>15</td>
</tr>
<tr>
<td>Participants</td>
<td>66</td>
</tr>
</tbody>
</table>
Sponsors and Advertisements

We gratefully acknowledge all those who are passionate about GPCR research, including many academic departments and institutions, and industry partners who contributed to this conference. Without this generous support this meeting would not possible.

Our Sponsors include:

✓ University of Illinois at Chicago, Office for Vice Chancellor for Research
✓ University of Illinois at Chicago, College of Medicine
✓ University of Illinois at Chicago, Department of Biochemistry and Molecular Genetics
✓ University of Illinois at Chicago, Department of Pharmacology
✓ University of Illinois at Chicago, Department of Microbiology and Immunology
✓ University of Illinois at Chicago, Training Program in Neuroscience of Mental Health
✓ University of Illinois at Chicago, Department of Physiology and Biophysics
✓ Northwestern University, Department of Pharmacology
✓ University of Chicago, Department of Biochemistry and Molecular Biology
✓ University of Toronto Mississauga, Master of Biotechnology Program
✓ University of Toronto, Leslie Dan Faculty of Pharmacy
✓ Ottawa Hospital Research Institute
✓ Douglas Research Institute
✓ Case Western Reserve, Department of Pharmacology
✓ Michigan State University, Department of Pharmacology and Toxicology
✓ Medical College of Wisconsin, Department of Biochemistry
✓ Washington University in Saint Louis, Department of Developmental Biology
✓ GE Healthcare Life Sciences
✓ Trevena, INC.
✓ Fisher Scientific
✓ American Society for Pharmacology and Experimental Therapeutics (ASPET)
✓ The Rockefeller University, Journal of General Physiology
✓ American Association for the Advancement of Science, Science Signaling
It’s a hit. For sure.

**Introducing Biacore™ 8K,** the newest member of the Biacore family of products. Biacore 8K efficiently delivers binding data with the quality you expect while meeting tomorrow’s challenges in small molecule and biotherapeutic screening and characterization.

**Discover more, more efficiently.**
- Single solution for interaction analysis in both screening and characterization
- Screening of 2300 small molecule fragments in a day
- High-quality kinetic characterization of 64 interactions in 5 h
- 60 h unattended runtime with queueing abilities and rapid multi-run evaluations
- Confident interaction analysis of small molecules and complex targets such as GPCRs
- Accurate differentiation of high-affinity binders

Discover deeper insights more efficiently.

[gelifesciences.com/biacore8k](http://gelifesciences.com/biacore8k)

---

For local office contact information, visit gelifesciences.com/contact
29215287 AA 06/2016
Submit Your Best Research

The Journal of General Physiology publishes mechanistic and quantitative cellular and molecular physiology of the highest quality; provides a best in class author experience; and nurtures future generations of independent researchers. You are invited to submit your very best research on:

- Membrane protein physiology
- Protein structure and dynamics
- Lipid and membrane biophysics
- Cell mechanics and contractile systems
- Intracellular and intercellular signaling

You may submit your paper in ANY format. After submission, initial decisions are made within 4 days on whether the manuscripts will be sent for review, and post-review decisions are sent within 32 days.*

Share your work with the world — learn more at www.jgp.org today!

CONTACT US:
Sharona E. Gordon  Lesley C. Anson  Meighan E. Schreiber
Editor-in-Chief  Consulting Editor  Managing Editor
seg@uw.edu  lanson@rockefeller.edu  msschreiber@rockefeller.edu

*Average from January-August 2016.
Program

Wednesday, October 12th

18:00 Welcome Dinner
Location: 14th Floor, Western Stage Room

20:00 Registration
Location: 14th Floor Ballroom Foyer

Thursday, October 13th

7:00 Breakfast and Registration
Location: 14th Floor Ballroom Foyer

9:00 Welcoming Remarks by Organizers
Location: 14th Floor, Sauganash East Ballroom

Workshop “Computational Modeling of the Structure of G-protein Coupled Receptors”

9:15 Rocco Moretti, Vanderbilt University, USA
Principles of GPCR Protein Structure Modeling

10:30 Coffee Break
Location: 14th Floor Ballroom Foyer
10:45  Rocco Moretti, Vanderbilt University, USA
Setting-up and Running Modeling Computations

12:00  Lunch (on your own)

**Hyman Niznik Memorial Lecture**
Location: 14th Floor, Sauganash East Ballroom

13:00  Brigitte Kieffer, McGill University, Canada
GPCRs in Psychiatric Research

**SESSION 1: GPCRs in Pain and Addiction**
Location: 14th Floor, Sauganash East Ballroom

14:00  Michael Bruchas, Washington University, USA
Dissecting VTA Opioid Receptor Function in Reward and Aversion

14:30  Paul Belcher, GE Healthcare Life Sciences, USA
The Wonderland of Hit Finding: Screening and Characterization of Fragments against WT GPCR Targets Using SPR

15:00  John Traynor, University of Michigan, USA
Allosteric Modulation of the μ-Opioid Receptor: Mechanism and Implications
15:30  Paul Slesinger, Mt. Sinai University, USA
Mechanisms of Plasticity in GABA-B Receptor and GIRK Channel Signaling in the Reward Pathway

16:00  Amynah Pradhan, University of Illinois at Chicago, USA
Ligand Biased Signaling at the δ-Opioid Receptor

16:30  Coffee Break
Location: 14th Floor Ballroom Foyer

**SESSION 2: GPCRs in Cell Differentiation, Proliferation, and Survival**
Location: 14th Floor, Sauganash East Ballroom

16:45  Asrar Malik, University of Illinois at Chicago, USA
Role of Par-1 Signaling in Pluripotent Stem Cell Differentiation

17:15  Joshua Snyder, Duke University, USA
Driving Cell Fate by Pharmacological and Genetic Control of Lgr5 Trafficking

17:45  Nickolai Dulin, University of Chicago, USA
Targeting GPCR Signaling to Inhibit Myofibroblast Differentiation and Organ Fibrosis

18:15  Madan Babu, University of Cambridge, UK
Common Structural Principles of GPCR G protein Signaling

18:45  **Poster Setup**
Location: 14th Floor, Sauganash West Ballroom

19:00  **Poster Session 1**
**Even-numbered abstracts, for assignments see pages 66-68**

20:45  Dinner
Location: 15th Floor, Wolf Point Ballroom
Friday, October 14th

7:00  Breakfast and Registration
     Location: 14th Floor Ballroom Foyer

SESSION 3: Compartmentalized GPCR Signaling
     Location: 14th Floor, Sauganash East Ballroom

9:00  Mark Von Zastrow, University of California, San Francisco, USA
     Relationships between GPCR Signaling and Membrane Traffic
9:30  JoAnn Trejo, University of California, San Diego, USA
     Integration of Protease-activated Receptor 1 Inflammatory Signaling by Ubiquitin
10:00 Manojkumar Puthenveedu, Carnegie Melon University, USA
     Spatial Regulation of Opioid Receptor Trafficking and Signaling
10:30 Philip Wedegaertner, Thomas Jefferson University, USA
     G protein Regulation of Golgi Function
11:00 Christine Lavoie, Université de Sherbrooke, Canada
     The Role of Gαs Protein and Receptor Endosomal Sorting
11:30 Lunch (on your own)

SESSION 4: GPCRs in Mood and Anxiety – Van Tol Symposium
     Location: 14th Floor, Sauganash East Ballroom

13:00 Richard Neubig, Michigan State University, USA
     GPCRs in Epilepsy: GNAO1 as an Epilepsy Gene Driving Seizure and Movement Disorders
13:30 Martin Beaulieu, Université Laval, Canada
     Targeting arrestin-AKT Interface in Mood Disorders
14:00  Emily Jutkiewicz, University of Michigan, USA  
The Role of δ-Opioid Receptors in Mood Disorders
14:30  Mark Rasenick, University of Illinois at Chicago, USA  
G-protein Localization and the Biology of Depression
15:00  Paul Albert, Ottawa Hospital Research Institute, Canada  
In vivo Roles of Serotonin-1A Receptor Regulation in Depression and Anxiety Phenotypes
15:30  Coffee Break  
Location: 14th Floor Ballroom Foyer

**SESSION 5: Regulation of GPCR Function by Multimeric Assemblies**  
Location: 14th Floor, Sauganash East Ballroom
15:45  Vadim Cherezov, University of Southern California  
Structure and Function of Angiotesin Receptors
16:15  Sergi Ferre, National Institute on Drug Abuse, USA  
Allosterism within GPCR Oligomers: Back to Symmetry
16:45  Kirill Martemyanov, Scripps Florida, USA  
Fingerprinting Catalytic Activity of GPCRs in Living Cells
17:15  Jeffery Benovic, Thomas Jefferson University, USA  
Biasing β2-Adrenergic Receptor Signaling
17:45  Bryan Roth, University of North Carolina, USA  
Creating GPCR Therapeutics via Atomic-level Insights
18:15  Libin Ye, University of Toronto, Canada  
Understanding of GPCR Dynamics by Solution NMR and DEER Spectroscopy
18:30  Jace Jones-Tabah, McGill University, Canada  
Fiber-optic Imaging of FRET Biosensors for Recording GPCR Signaling *in vivo*
18:45  **Poster Setup**  
Location: 14th floor, Sauganash West Ballroom

19:00  **Poster Session 2**  
*Odd-numbered abstracts, for assignments see pages 66-68*

20:45  Dinner  
Location: 15th Floor, Wolf Point Ballroom

---

**Saturday, October 15th**

7:00  Breakfast and Registration  
Location: 14th Floor Ballroom Foyer

---

**SESSION 6: Adhesion GPCRs**  
Location: 14th Floor, Sauganash East Ballroom

9:30  Gregory Tall, University of Michigan, USA  
New Pharmacological Tools to Modulate Adhesion GPCR Tethered Peptide Agonism

10:00  Tobias Langenhan, University of Wurzburg, Germany  
Adhesion GPCRs in Mechanosensation

10:30  Kelly Monk, Washington University in Saint Louis, USA  
Adhesion GPCRs in Nervous System Development and Repair

11:00  Demet Arac, University of Chicago, USA  
Structural and Functional Basis of Adhesion GPCR Activation

11:30  **Poster Presentation Awards Ceremony, and Closing Remarks**
Protein Kinase D and Gβγ Mediate Protease-Biased Translocation of Protease-activated Receptor-2 from the Golgi Apparatus to the Plasma Membrane

Peishen Zhao\textsuperscript{1,2}, Luke Pattison\textsuperscript{1}, Dane Jensen\textsuperscript{1}, Daniel P. Poole\textsuperscript{1}, TinaMarie Lieu\textsuperscript{1,2}, Nigel.W. Bunnett\textsuperscript{1,2}

\textsuperscript{1}Monash Institute of Pharmaceutical Sciences, \textsuperscript{2}Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash University

Protease-activated receptor-2 (PAR\textsubscript{2}) mediates protease-evoked pain and inflammation. Proteases that cleave PAR\textsubscript{2} at different sites are biased agonists that evoke distinct patterns of receptor trafficking and signaling. The canonical agonist trypsin cleaves at R\textsubscript{36} S\textsubscript{37} to induce PAR\textsubscript{2} coupling to G\textalpha q and β- arrestins, followed by endocytosis and lysosomal degradation. The biased agonists cathepsin-S and elastase cleave at E\textsubscript{56} T\textsubscript{57} and A\textsubscript{66} S\textsubscript{67} V\textsubscript{68}, respectively, to induce PAR\textsubscript{2} coupling to G\textalpha s but not β-arrestins or endocytosis. Given the irreversible mechanism of proteolytic activation, sustained protease signaling requires mobilization of Golgi stores of PAR\textsubscript{2}, but the mechanism by which canonical and biased agonists induce this process is unknown. In HEK and KNRK cells, trypsin, cathepsin-S and elastase all stimulated PAR\textsubscript{2}-dependent phosphorylation (activation) of protein kinase D (PKD) in the Golgi apparatus, where PKD can regulate protein trafficking. These proteases stimulated translocation to the Golgi apparatus of Gβγ, a PKD activator, determined by bioluminescent resonance energy transfer analysis of the proximity of Gγ-Venus and Giantin-RLuc8 (Golgi protein). PAR\textsubscript{2} with a photoconvertible Kaede tag was expressed in KNRK cells to examine receptor translocation from the Golgi apparatus to the plasma membrane. Irradiation of the Golgi apparatus (405 nm) resulted in green-red photo-conversion of PAR\textsubscript{2}-Kaede. Trypsin, cathepsin S and elastase all induced depletion of photoconverted PAR\textsubscript{2}-Kaede from the Golgi apparatus, and repletion of photoconverted PAR\textsubscript{2}-Kaede at the plasma membrane. The PKD inhibitor CRT0066101 and the Gβγ inhibitor gallein prevented protease-evoked translocation of PAR\textsubscript{2}-Kaede from the Golgi apparatus to the plasma membrane. Incubation of HEK cells and rat dorsal root ganglia neurons with trypsin, cathepsin-S or elastase desensitized trypsin-stimulated Ca\textsuperscript{2+} signaling, consistent with PAR\textsubscript{2} cleavage and desensitization. Tryptsin-responsiveness gradually returned, indicating PAR\textsubscript{2} resensitization. CRT0066101 and gallein prevented resensitization of trypsin-, cathepsin-S- and elastase-stimulated HEK cells and neurons, consistent with a role for PKD and Gβγ in resensitization. Intraplantar administration of proteases to mice stimulated sustained (4 h) mechanical hyperalgesia, assessed by paw withdrawal responses to von Frey filaments. Oral administration of CRT0066101 attenuated hyperalgesia, consistent with a role for PKD mobilization of PAR\textsubscript{2} for sustained protease-evoked pain. Thus, canonical and biased proteases that activate of PAR\textsubscript{2} at the plasma membrane all stimulate Golgi translocation of Gβγ, which activates PKD. PKD controls Golgi to plasma membrane trafficking of PAR\textsubscript{2}, which repopulates the cell surface with intact receptors and allows sustained nociceptive signaling by extracellular proteases.
Unique profiles of catalytic activity on exhaustive sets of G protein substrates dictate GPCR action

Ikuko Masuho, Olga Ostrovskaya, Grant M. Kramer, Christopher D. Jones, Keqiang Xie, Kirill A. Martemyanov

1Department of Neuroscience, The Scripps Research Institute Florida, 2Harriet Wilkes Honors College, Florida Atlantic University

Individual G Protein-Coupled Receptors (GPCRs) exert diverse yet distinctive effects on cellular physiology, which is believed to originate from their differential coupling to an array of functionally various G proteins. However, comprehensive G protein coupling profiles in response to endogenous and synthetic ligands for most receptors are not known. Here, we developed a single-platform strategy for direct monitoring of G protein activation in living cells, allowing an innovative quantitative assessment of efficacy and activation rate across an exhaustive set of G proteins. Using this unique technology, we report that GPCRs activated by endogenous agonists exhibit characteristic, fingerprint-like, G protein-coupling profiles. All tested GPCRs couple to multiple G proteins and have multidimensional efficacy. Also, the measurement of activation rate as a readout of catalytic activity of GPCRs produces different profiles from efficacy, reflecting the hierarchical order of G protein selectivity. Finally, and perhaps most importantly, we present the evidence that synthetic drugs control catalytic activity of GPCR, functionally biasing the G protein selectivity of GPCRs. These data argue that our fingerprinting technology facilitate characterization of GPCRs and provide an intriguing new avenue for development of drugs leading therapeutically advantageous G protein signaling and sparing those contributing adverse side effects.

WNT stimulation dissociates a Frizzled 4 inactive state complex with Gα12/13

Elisa Arthofer, Belma Hot, Julian Petersen, Katerina Strakova, Stefan Jaeger, Manuel Grundmann, Evi Kostenis, J Silvio Gutkind, Gunnar Schulte

1Karolinska Institutet, Dept of Physiology & Pharm, Sweden, 2National Institutes of Health, Eunice Kennedy Shriver National Institute for Child Health and Human Development, 3Masaryk University, Institute for Experimental Biology, 4University of Bonn, Institute for Pharmaceutical Biology, 5University of California San Diego, Dept. of Pharmacology, Moores Cancer Center

Frizzleds are unconventional G protein-coupled receptors (GPCRs) that belong to the Class Frizzled. They are bound and activated by the WNT family of secreted lipoglycoproteins. To date, mechanisms of signal initiation and FZD-G protein coupling remain poorly understood. Previously, we showed that FZD6 assembles with Gαi1/Gαq, but not with Gαs, Gαo, and Gα12/13 and that these inactive-state complexes are dissociated by WNTs and regulated by the phosphoprotein Dishevelled (DVL). Here, we investigated the inactive state assembly of heterotrimeric G proteins with FZD6, a receptor important in retinal vascular development and frequently mutated in Norrie disease or familial exudative vitreoretinopathy (FEVR). Live cell imaging experiments employing fluorescence recovery after photobleaching (FRAP) show that human FZD4 assembles - in a DVL-independent manner - with Gα12/13 but not representatives of other heterotrimeric G protein subfamilies, such as Gαi1, Gαo, Gαs and
Gαq The FZD4-G protein complex dissociates upon stimulation with WNT-3A, WNT-5A, WNT-7A, and WNT-10B. In addition, WNT-induced dynamic mass redistribution (DMR) changes in untransfected and - even more so - in FZD4-GFP transfected cells depend on Gα12/13. Furthermore, expression of FZD4 and Gα12 or Gα13 in HEK293 cells induces WNT-dependent membrane recruitment of p115-RHOGEF, a direct target of Gα12/13 signaling, underlining the functionality of a FZD4-Gα12/13-RHO signaling axis. In summary, Gα12/13-mediated WNT/FZD4 signaling through p115-RHOGEF offers an intriguing and previously unappreciated mechanistic link of FZD4 signaling to cytoskeletal rearrangements and RHO signaling with implications for the regulation of angiogenesis during embryonic and tumor development.

(4) An atlas of G protein-coupled neurotransmitter and neuropeptide receptor expression patterns in C. elegans

Robert Fernandez1, Seongseop Kim1, Judy Pepper1, Erin Wang1, Michael R. Koelle1

1Yale University

About a dozen neurotransmitters and ~100 neuropeptides signal by activating G protein-coupled receptors (GPCRs) in the human brain. The small nematode C. elegans has neurotransmitters, neuropeptides, and neural GPCRs conserved in the human brain, and provides a system for studying how neural GPCR signaling is organized and utilized to help the nervous system carry out its functions. The ~5,000 synaptic connections between the 302 neurons of C. elegans have been completely mapped, and the individual neurons that express each neurotransmitter and most of the ~100 neuropeptide genes have been catalogued. We are adding an additional layer to this nervous system signaling map by creating an Atlas of which cells express each of C. elegans’ 27 GPCRs for small-molecule neurotransmitters and also each of its ~119 neuropeptide receptor homologs. For each neural GPCR, we are making a transgenic strain of C. elegans in which the GPCR gene is fused to the GFP coding sequences, so that the individual cells that express that GPCR are fluorescent and can be identified by confocal microscopy. Every cell in C. elegans is identifiable by position and morphology, and we are using a series of transgenes that label identified subsets of C. elegans neurons with red fluorescent proteins as markers to identify the GFP-expressing cells in our GPCR::GFP transgenic strains.

The type of results obtained from this Atlas are illustrated by our analysis of a single serotonin receptor, SER-4. This serotonin receptor is expressed in 30 neurons and 6 non-neuronal cells, which include vulval muscle cells and rectal epithelial cells. The SER-4 expressing cells are mostly synaptic to serotonin-releasing neurons, however there are cases where SER-4::GFP is expressed in cells that do not receive a direct synapse from these serotonin-releasing neurons. These SER-4 expressing cells may receive signals from serotonin released from distant neurons that diffuses through the extracellular space, a process termed “extrasynaptic signaling”. Some of these SER-4 expressing cells also receive direct synapses from neurons that release other biogenic amines similar in chemical structure to serotonin, suggesting that these lower affinity SER-4 ligands, rather than the highest affinity ligand serotonin, may activate SER-4 on these SER-4 expressing cells.

Completion of our Atlas, integrated with the synaptic wiring diagram and neurotransmitter maps, will provide a path to understand the functions of neurotransmitter/neuropeptide signaling through GPCRs.
in each individual neural circuit and every behavior carried out in *C. elegans*, and should generate insights that generalize beyond *C. elegans* into how neural GPCR signaling is organized. To demonstrate utility of this Atlas, we plan to identify all the neurotransmitter and neuropeptide GPCRs that act in the *C. elegans* egg-laying circuit and to characterize their biological roles in this circuit.

(5) Characterization of a novel peptide nanoparticle biased antagonist of CCR3

Milica Grozdanovic¹, Lee K. Rousslang¹, Hazem Abdelkarim¹, Kimberly G. Laffey², Ben Hitchinson¹, Nadya Tarasova³, Vadim Gaponenko¹, Steven J. Ackerman¹

¹University of Illinois at Chicago (UIC), ²Mayo Clinic, Rochester, MN, ³National Cancer Institute at Frederick, Frederick, MD

Background: CCR3 is a highly promiscuous GPCR receptor, interacting with several inflammatory chemokines, including the high affinity agonists: eotaxin (CCL11), eotaxin-2 (CCL24), eotaxin-3 (CCL26), and RANTES (CCL5). CCR3 and its principal ligands play a prominent role in the pathogenesis of human allergic diseases including asthma, and this receptor has long been a target of drug discovery. Several small molecule antagonists have been developed to date; however, most of these inhibitors failed to enter phase II/III clinical trials. A possible underlying cause of this failure is the unbiased mode of inhibition that prevents receptor internalization and degradation, leading to drug tolerance. We sought to develop a novel peptide-based CCR3 antagonist (R321) with a biased mode of inhibition.

Methods: Self-assembly of the inhibitory R321 peptide into nanoparticles was analyzed by Dynamic Light Scattering (DLS). The inhibitory effect on CCR3 signaling was assessed by determining IC50 and IC90 values for eotaxin-induced CCR3-mediated chemotaxis of a CCR3+ eosinophil cell line, AML14.3D10-CCR3. Mechanism of inhibition was determined by assessing ERK 1/2 phosphorylation in CCR3+ cells by means of western blotting. Binding of R321 to reductively methylated CCR3 membrane preparations in the presence of CCL11 was analyzed by NMR.

Results: The R321 peptide, derived from the second transmembrane helix of CCR3, self-assembles into monodisperse nanoparticles (radius of 7.1 ± 0.7 nm, 7.2% polydispersity). IC50 and IC90 values for eotaxin induced CCR3-mediated chemotaxis were: 0.21± 0.4 µM and 1.75± 0.20 µM (CCL11); 0.14 ± 0.02 µM and 0.89 ± 0.12 µM (CCL24); and 0.10 ± 0.01 µM and 0.78 ± 0.10 µM (CCL26). Unlike two other tested small molecule inhibitors, the R321 peptide inhibited only the early phase of ERK1/2 activation (5 min post-stimulation) and not the late phase activation (30 min post-stimulation) associated with β-arrestin recruitment, receptor internalization and degradation. NMR spectra of reductively methylated CCR3 enriched membranes showed binding of R321 to CCR3 in the presence of CCL11. Even 50 µM R321 did not displace 1 µM CCL11, indicating a non-competitive allosteric mode of inhibition.

Conclusions: We evaluated the activity of a novel CCR3 self-assembling peptide antagonist with a potent inhibitory effect on CCR3-mediated chemotaxis of a CCR3+ eosinophil cell line and primary blood eosinophils. By demonstrating a selective inhibition of only the desired subset of the CCR3 signaling cascade, this novel biased antagonist may hold significant therapeutic promise by preventing receptor accumulation on the cell surface, thus eluding the development of drug tolerance.
Putative plant GPCR, GCR1, is an important feature of chemical defense and development in Arabidopsis thaliana

Katherine Warpeha¹, Alessia Para², DurreShahwar Muhammad¹, Nayfah Thnaibat¹, Ramis Memishi¹, Carlos Montero¹, Mike Naldrett³, Sophie Alvarez³

¹University of Illinois at Chicago, ²Northwestern University, ³University of Nebraska at Lincoln

There has been much interest in identifying novel G protein-coupled receptors (GPCR) and G-protein signaling pathways to increase the repertoire of drug targets in the study of disease and dysfunction. Plants have very few GPCR-type proteins, with perhaps only one GPCR, GCR1 (in the model plant Arabidopsis thaliana; At1g48270), with recent published structural bioinformatics data indicating that GCR1 may be the only homolog with the GPCR fold. Our laboratory has investigated the two signaling pathways that impact phenylpropanoids, both of which share GCR1 as a component of the pathway. Two effectors of this pathway are Pirin1 (PRN1; At3g59220) cleaves quercetin and is a transcriptional cofactor that impacts the cell cycle; and Arogenate Dehydratase3 (ADT3; At2g27820) is a phenylalanine biosynthesis enzyme which regulates the phenylpropanoids levels (including quercetin) in cells. In 4-6 d old seedlings we quantified responses of gcr1 mutants to abiotic signals, and compared responses and phenotypes. gcr1 mutant seedlings phenocopied gpa1 and adt3 for five abiotic stimuli. gcr1 mutants produce a unique chemical profile of phenylpropanoids. gcr1 mutants have an abnormal phenylpropanoid profile (compared to wt) which reveals dysregulation in early in the phenylpropanoid pathway, critical for chemical defense and proper formation of structures. gcr1 mutant cells of the epidermis possess some phenylpropanoids (compared to the adt3 mutant seedlings which are severely deficient in all phenylpropanoids), however, the phenylalanine levels are so low that normal cell division fails to occur. In young seedlings the G-protein pathway is critical to correct formation of developing cell lineages and the defense system of seedlings, upon which animals including humans depend for food and extractable chemicals from the phenylpropanoid pathway.

Single-molecule Analysis of the Supramolecular Organization of the M2 Muscarinic Receptor and the Gαi1 Protein

Claudiu Gradinaru¹, Dennis Fernandes¹, Rabindra Shivnaraine¹, James W. Wells¹

¹University of Toronto

G Protein Coupled Receptors (GPCRs) are one of the most important classes of membrane receptors owing to their role as drug targets. Family 1 GPCRs, such as the M₂ muscarinic cholinergic receptor, are activated upon binding of agonists which drives the coupling of the receptor and G protein. Within the activation process, the roles of oligomerization of the receptor and G proteins is unknown. In this study, we build on prior studies which has examined the oligomerization of the M₂ receptor alone, and characterize the state of oligomerization of the receptor when coupled to the Gα₁ protein and vice versa. Oligomeric sizes were measured by single-molecule photobleaching and the method was calibrated using homogenous populations of multimeric GFP controls. Measurements of the oligomeric states of the receptor and the G protein were taken both individually and when bound to each other. The M₂ receptor in the basal state, following activation by ligands, and after coupling with the G protein remains a tetramer throughout, whereas the oligomeric size of the G protein is dynamic. G proteins exist as hexamers in the basal state, couple as tetramers, and uncouple from the receptor
(i.e., fully activated) most likely as dimers. Photobleaching measurements on a constitutively active G protein mutant and on a peptide mimic of the wild-type receptor, as well as molecular dynamics simulations suggest that disassembly of G protein oligomers from the oligomeric M₂ receptor underlies their activation.

(8) The M₂ Muscarinic Receptor Signaling Complex Resolved by Single Molecule Tracking in Live Cells

Yuchong Li¹, Rabindra Shivnaraine², Huiqiao Ji², Fei Huang², Kevin Braeckmans³, James W. Wells², Claudiu Gradinaru²

¹University of Toronto Mississauga, ²University of Toronto, ³Ghent University

Many aspects of the cellular signaling pathways via G protein-coupled receptors (GPCRs) are not completely understood. In particular, three questions have been the focus of much attention and debate: the oligomeric status of the receptor, the coupling strength between the receptor and the G protein, and the regulation of the receptors in response to external stimuli. Here we examine those questions by 2D tracking the M₂ muscarinic receptors with Total Internal Reflection Fluorescence Microscopy (TIRFM) in the membrane of live cells.

M₂ Receptors and G proteins were genetically fused with fluorescent proteins (GFP and mCherry) and expressed in Chinese Hamster Ovary (CHO) cells. The distributions of oligomeric sizes and diffusion coefficients for both the receptors and the G proteins were extracted from single-particle tracking data obtained with TIRFM. Both of the receptors and G proteins were found to be localized in membrane micro-domains, showing multi-step photobleaching and exhibited a wide range of diffusion behaviors on a slower time scale than single protein controls. Corroborated with dual-color fluorescence correlation spectroscopy on the same samples, we propose that multiple oligomeric receptors and oligomeric G proteins sizes co-exist in close proximity inside a spatially confined signaling scaffold in the membrane of living cells. In addition, two-color simultaneous tracking of co-expressed receptors and G proteins shows preliminary evidence for receptor-G protein decoupling and receptor internalization upon induction with excessive agonist.

(9) Structure/function based analysis of Cytomegalovirus US28 vGPCR signaling and its effect on monocyte adhesion

Shu-En Wu¹, Emily Campbell¹, William Miller¹

¹University of Cincinnati

Human cytomegaloviruses (HCMV) encode four genes with significant homology to cellular G-protein coupled receptors. One of these genes, US28, has been demonstrated to exhibit high levels of agonist-independent signaling activity and to activate the Gaq family of G-proteins in lytically infected cells. The functional roles played by US28 in the viral life cycle are just beginning to be elucidated and it appears that US28 plays significant roles in viral latency, dissemination and horizontal transmission. Here, we show that US28 protein is strongly expressed in monocytes latently infected with HCMV and that it robustly triggers PLC-b signaling in the infected cells. Moreover, pharmacological and mutational analyses in THP-1 monocytes stably expressing US28 mutants confirms that this signaling is dependent on G-protein coupling, but independent of chemokine binding. Importantly, we demonstrate that this US28-promoted Gaq signaling is functionally important as it stimulates the adhesion of
monocytes to an endothelial monolayer. Based on the recent crystal structure of US28 from Garcia and colleagues, we have constructed additional mutants in the ionic-lock and ligand binding regions of the hydrophobic transmembrane domains, which provide significant insight into the mechanisms underlying constitutive US28 signaling. Specifically, mutations that restore ionic interactions between the DRY box at the distal end of TM3 and residues in TM6 abolish the constitutive signaling activity typically exhibited by US28. Our studies, which demonstrate that US28-stimulated Gaq signaling has profound effects on monocyte biology, suggest that US28-driven phenotypic changes in HCMV infected monocytes may play important roles in HCMV dissemination and/or pathogenesis. Finally, a molecular understanding of the structural features of US28 constitutive signaling may lead to the development of novel therapeutics that could be utilized to treat latent HCMV infections.

(10) Delta Opioid Receptor as a Target for Migraine – CGRP Co-expression and Inhibition of Medication Overuse Headache
Laura Segura¹, Alycia F. Tipton¹, Amynah A.A. Pradhan¹
¹University of Illinois at Chicago

Migraine is an extraordinarily common brain disorder for which therapeutic options continue to be limited. We have previously demonstrated that in preclinical animal models, delta opioid receptor agonists may be promising targets for the treatment of migraine. Delta agonists effectively inhibit cortical spreading depression, as well as nitroglycerin-induced hyperalgesia and negative affect. A better understanding of how delta opioid receptor modulates migraine mechanisms would encourage future development of this target. The neuropeptide, calcitonin gene related peptide (CGRP) plays a pivotal role in the induction and maintenance of migraine, primarily through the afferents projecting from the trigeminal ganglia. The aim of this study was to characterize the expression of delta opioid receptor in trigeminal ganglia, and on CGRP-expressing neurons specifically. To visualize the delta opioid receptor, we used knockin mice in which the endogenous receptor was replaced by a fluorescent tagged delta opioid receptor (DOR-eGFP). We observed a population of trigeminal ganglia which co-expressed CGRP with DOR-eGFP. This data suggests that delta agonists may produce their anti-migraine effects by directly modulating CGRP-expressing ganglia. As a further goal of this study we also tested delta agonists in a model of sumatriptan-induced medication overuse headache (MOH). In this case, C57BL/6 mice were treated chronically with sumatriptan for 11 days, which produced severe mechanical hypersensitivity. The delta agonist, SNC80, inhibited this hyperalgesia, and suggests that delta agonist could be an effective strategy for managing MOH. Together, this work provides further evidence that delta opioid receptors are promising targets for migraine treatment.

(11) Investigating Allosterically Induced Conformational Rearrangements in GPCRs: A Focus on the F Prostanoid-Angiotensin II Type 1 Receptor Heteromer
Rory Sleno¹, Dominic Devost¹, Darlaine Pétrin¹, Alice Zhang¹, Kyla Bourque¹, Terry Hébert¹
¹McGill University

G protein-coupled receptors (GPCRs) are highly dynamic proteins which transmit information after interacting with functionally different ligands that promote multiple, yet specific downstream
outputs. It is widely accepted that this complexity is achieved through induction of distinct conformations in the GPCR, whereby individual conformational states preferentially drive specific receptor signalling events. The available conformational capacity, and therefore possible receptor functions, can be further expanded when considering allosteric effects. Receptor signalling partners, such as receptor oligomerization, may provide novel conformational constraints generating new signalling modalities or protein life-cycle behaviours. Using GPCR conformation-sensitive biosensors, we investigated allosterically induced conformational changes in the recently reported F prostanoid (FP)/angiotensin II type 1 receptor (AT\textsubscript{1}R) heteromer (1). We identified asymmetrical conformational cross-talk between the partner receptors whereby ligand occupancy at the AT\textsubscript{1}R induced novel conformational changes in FP when compared to orthosteric ligand binding to FP. This allosteric communication is mediated through G\alpha\textsubscript{q} and may include involvement of proximal (phospholipase C) but not distal (protein kinase C) signalling partners. This transmission of information seems to be specific to AT\textsubscript{1}R as activation of G\alpha\textsubscript{q} by the oxytocin receptor (OTR) cannot recapitulate the same phenomenon. Finally, we demonstrate that β-arrestin-biased AT\textsubscript{1}R agonists can transmit a G\alpha\textsubscript{q} dependent signal to FP without activation of G\alpha\textsubscript{q} signalling.

The identification of partner induced GPCR conformations sets the stage for the explanation of novel allosteric effects when investigating multi-protein receptor complexes.


(12) Teaching an old drug new tricks: agonism, antagonism, selectivity and biased signaling of pilocarpine through M3 muscarinic receptor

Alexey Pronin\textsuperscript{1}, Qiang Wang\textsuperscript{1}, Vladlen Slepak\textsuperscript{1}

\textsuperscript{1}Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine.

Pilocarpine is a classic cholinergic receptor ligand used to treat glaucoma since 1876. Historical studies of its agonistic effect on eye pupil constriction and antagonism toward atropine led to development of the basic concept of a drug receptor ("receptive substance") in 1905. Since that time, a wealth of physiological and pharmacological studies have established that pilocarpine stimulates muscarinic family cholinergic GPCRs (M1R-M5R). The main effects of pilocarpine in the eye are mediated by the M3 receptor (M3R), a prototypical G\alpha\textsubscript{q}-coupled receptor. Here, we report several unexpected results pertaining to activation of M3R by pilocarpine. First, we show that pilocarpine fails to stimulate intracellular calcium release in HEK293 cells endogenously expressing the M3R. This outcome differs from other muscarinic agonists, such as carbachol. Moreover, we show that pilocarpine functions as a classical M3R antagonist by competitively inhibiting carbachol-stimulated intracellular calcium release. We corroborated these findings in mouse insulinoma Min6 cells, which also naturally express M3R. In contrast to other agonists, pilocarpine failed to stimulate insulin secretion or calcium responses, and inhibited insulinotropic effect of the full agonist oxotremorine-M. However, when we examined other signaling events downstream of M3R, we found that pilocarpine behaves like other muscarinic agonists by activating ERK1/2 kinase. This suggests that pilocarpine can act as a biased agonist capable of activating alternative downstream signaling pathways. Interestingly, when M3R was overexpressed in either HEK293 or CHO-K1 cells, pilocarpine now acted as a full agonist triggering intracellular calcium increase that was almost indistinguishable from responses stimulated by another muscarinic receptor, M1R. To study signaling events upstream of calcium release we employed a novel
fluorescent sensor for the signaling lipid phosphatidylinositol 4,5-bisphosphate (PIP2). As expected, stimulation of cells overexpressing either M1R or M3R with carbachol resulted in a robust reduction in PIP2. However, when cells were challenged with pilocarpine, PIP2 hydrolysis was observed only in cells overexpressing M1R, but not M3R. Furthermore, pilocarpine blocked PIP2 hydrolysis stimulated by carbachol in M3R-overexpressing cells. At the same time, the increase in intracellular calcium was still observed when pilocarpine was added together with calbachol. Taken together, our findings indicate pilocarpine can act as either agonist or antagonist of M3R, depending on the expression level of the receptor and the functional assay. These observations point to previously unappreciated selectivity and signaling bias of pilocarpine, a prototypical drug classified as either agonist or partial agonist. We speculate that this behavior might explain why pilocarpine has significantly fewer side effects compared to carbachol when used to treat xerostomia (dry mouth) or glaucoma.

(13) Sustained antidepressant treatment increases cAMP signaling by translocating $G_a$s from lipid rafts and increasing association with type 6 adenylyl cyclase (AC6): a process independent of monoamine transporters

**Jeffrey Schappi**$^1$, **Mark Rasenick**$^2$

$^1$UIC Physiology, $^2$UIC

Antidepressants of different chemical classes promote a sustained increase in cAMP, due, at least in part to the redistribution of $G_a$s from lipid rafts into non-raft membrane fractions. The net result of this redistribution is increased $G_a$s coupling with, and activation of, adenylyl cyclase (AC). This has been demonstrated, in both rats and cultured neural and glial cells by a number of techniques, including cell fractionation, functional assays, and imaging studies such as FRAP (Fluorescence Recovery After Photobleaching). Unlike animal models, C6 glioma lack monoamine reuptake transporters, suggesting that mechanisms of antidepressant response entails more than inhibition of neurotransmitter reuptake transporters or inhibition of monoamine breakdown. This is noteworthy, since inhibition of 5HT or NE uptake is quite rapid even though clinical effects of antidepressants require several weeks (and several days in the cellular models). Furthermore, while these neural and glial cells showed an “antidepressant response”, $G_a$ localization and cAMP production in kidney epithelial cells like COS1 and HEK293 were unchanged by antidepressant treatment. Similarly, membranes from liver and kidney of rats treated chronically with antidepressant did not show the same response as brain from those same animals. In this study we sought to determine whether cellular antidepressant response, with respect to increased cAMP signaling and $G_a$s localization, is dependent on the type of AC isoform expression. Cell lines “insensitive” to antidepressant treatment, such as HEK293, become responsive after transfection with AC6, showing translocation of $G_a$s from lipid rafts, both by cell fractionation and by FRAP. Likewise, knockdown of AC6 but not other adenylyl cyclase isoforms abolishes the antidepressant response in responsive cell lines such as C6 glioma. Thus, it is suggested that AC6 performs an anchoring function for $G_a$s outside of rafts, affixing the translocated $G_a$s into the non-raft domain and facilitating an antidepressant-induced increase in adenylyl cyclase activity.
(14) **Structural Basis for Regulation of GPR56/ADGRG1 by Its Alternatively Spliced Extracellular Domains**

**Gabriel Salzman**, Sarah Ackerman, Chen Ding, Akiko Koide, Katherine Leon, Rong Luo, Hannah Stoveken, Celia Fernandez, Gregory Tall, Xianhua Piao, Kelly Monk, Shohei Koide, Demet Arac-Ozkan

1University of Chicago, 2Washington University, 3Harvard Medical School, 4University of Rochester Medical Center, 5NYU Medical Center, 6Yale University

Characterized by their large and diverse extracellular regions (ECRs), adhesion G protein-coupled receptors (aGPCRs) play roles in both cell adhesion as well as conventional G protein signaling. To better understand these roles independently and in concert, we characterized the ECR of GPR56 (ADGRG1), an aGPCR involved in central nervous system (CNS) myelination, development of the cerebral cortex, and several types of cancer. Using biochemistry, protein engineering, structural biology and in vivo assays, we set out to identify the mechanisms underlying the physiological and pathophysiological functions of GPR56. First, we engineered a small protein, termed monobody (Mb), which binds tightly and specifically to the ECR of GPR56. We solved the crystal structure of the ECR of GPR56 bound to this Mb at 2.5Å resolution, revealing for the first time the domain architecture and atomic structure of a complete aGPCR ECR. The structure reveals two domains in the ECR of GPR56: a previously unidentified ‘PLL’ domain at the N-terminus and an unusually short, but autoproteolytically active GPCR-Autoproteolysis INducing (GAIN) domain. Using in vitro and in vivo functional approaches, we observed the effects of several structure-guided mutations and thereby identified a surface-exposed conserved patch on the PLL domain necessary for GPR56-mediated CNS myelination in zebrafish. Finally, we measured the effect of Mb binding on GPR56 signaling in vitro and observed a decrease in basal activity, classifying the Mb as an allosteric inverse-agonist. Our results suggest that the ECR of GPR56 has an essential role in regulating receptor function in an intricate and multifaceted manner.

(15) **In silico design of novel probes for the atypical opioid receptor MRGPRX2**

**Kate Lansu**, Joel Karpiak, Jing Liu, X.P. Huang, Wesley K. Kroeze, Jian Jin, Brian K. Shoichet, Bryan L. Roth

1University of North Carolina Chapel Hill, 2University of California, San Francisco, 3Icahn School of Medicine at Mount Sinai, New York

We sought to discover small molecule probes for the MRGPRX2 orphan GPCR, a primate-only receptor expressed in the dorsal root and trigeminal ganglia and mast cells. Briefly, we leveraged the results of an *in vitro* β-arrestin screen to develop structural models of MRGPRX2 and virtually screen against ~3.7 million small molecules to predict novel activating scaffolds. We validated our screening hits, predicted agonists, and the *in silico* MRGPRX2 model using mutagenesis and cell-based assays. We also found that opioid agonists from the *in vitro* screen and our selective ligand promoted intracellular calcium release and degranulation in the LAD2 human mast cells, where MRGPRX2 is endogenously expressed. siRNA knockdown of MRGPRX2 significantly reduced opioid-induced or selective probe-induced degranulation. We conclude that MRGPRX2 appears to be a unique atypical
opioid receptor that mediates opioid-induced degranulation in LAD2 human mast cells. Furthermore, our approach prompted the discovery of a pair of demonstrably selective probes for precisely interrogating MRGPRX2 function, which are now commercially available.

(16) **Missense mutations in Regulator of G protein Signaling 2 affect the protein function through multiple mechanisms**

**Hoa Phan**, **Benita Sjögren**, **Richard Neubig**

1Michigan State University

RGS2 attenuates Gαq/11 signaling which mediates the action of a majority of vasoconstrictors. The association between RGS2 deficiency and hypertension has been demonstrated by genetic knock-out mouse models and RGS2 mutations identified in Japanese hypertensive patients. Though biochemical functions of RGS2 are well characterized, the actual role of RGS2 in human hypertension is not well understood. Rare missense mutations in RGS2 have been identified through multiple exome sequencing projects, however, the functional significance of these mutations is unknown. The objective of this study is to examine the effect of human RGS2 missense mutations on how they differ from wild-type RGS2 in their ability to regulate GPCR-mediated signaling and respective mechanisms resulting in such differences. Wild-type and 16 RGS2 mutants were investigated by transient transfection in CHO cells. 9 RGS2 mutants had impaired inhibition of AT1R-mediated calcium release. Among these malfunctional mutants, the RGS2Q2L showed reduced protein expression, the RGS2D40Y and RGS2R44H showed reduced plasma membrane targeting. These functional deficits are currently being assessed at GAP activity toward Gαq.

Together, these studies will provide a molecular understanding of a number of human RGS2 mutant alleles in vitro. This will identify key candidate alleles for further study as contributors to human hypertension.

Supported by AHA predoctoral fellowship to H.P.

(17) **Investigating how allosteric nanobodies influence β2AR conformation**


1McGill University

Upon agonist stimulation, G protein-coupled receptors engage various downstream signalling cascades that regulate enzymes, ion channels, and a wide range of other target proteins. The human β2-adrenergic receptor (β2AR) is widely studied and is tightly integrated into an elaborate multi-component signalling network. Here, modulation of β2AR conformation has been studied using a panel of FlAsH BRET-based biosensors in combination with antibody-derived single domain proteins. Given their small size, solubility and ability to bind cryptic epitopes, nanobodies are ideal tools for receptor biology. As chaperones for crystallization one such nanobody resulted in the crystallization of the agonist bound form of the β2AR. NMR studies have also revealed an active state conformation of the β2AR while bound to a G protein mimic nanobody as these proteins are believed to stabilize different conformational states of receptors. Intramolecular BRET reports on receptor conformation in response to external stimuli. Here, we have used eight biosensors that have the FlAsH tag positioned on different
regions of the intracellular surface of the β2AR to probe how signal transduction partners govern receptor conformation and function. The wealth of our assay resides in the circumstance that not only is the β2AR localized to the membrane in living cells but the eight site specific tags will allow to complement NMR studies that only use single nucleotide labeling. This project utilizes FlAsH-walk within the β2AR to examine the connection between distinct receptor conformations stabilized by the different nanobodies and signalling outputs since the chosen nanobodies have distinct effects on G protein versus β-arrestin signalling. Our investigations will help in understanding how downstream effectors influence receptor function where such knowledge can help in the development of better drugs.

(18) Stabilization of human cone opsins for structural characterization

David Salom¹, Kota Katayama¹, Krzysztof Palczewski¹

¹Case Western Reserve University

Humans have two types of vision: dim-light vision mediated by rhodopsin (Rho) and color vision achieved by three cone pigments, each of which absorbs predominantly blue (λmax=425 nm), green (λmax=530 nm) or red (λmax=560 nm) light. Each cone pigment consists of a different opsin protein bound to a common chromophore molecule, 11-cis-retinal. Differential chromophore-protein interactions allow preferential absorption at a selected range of wavelengths. The mechanism of color tuning has attracted many researchers to try to answer the question “why are we able to distinguish colors?” In color tuning of visual pigments, three factors are mainly taken into account: (I) chromophore distortion, (II) electrostatic interaction between the protonated Schiff base and counterion, and (III) polarity around the b-ionone ring and polyene chain. Structural information is a prerequisite to understand the molecular basis of color vision. However, structural studies of cone pigments lag far behind those involving Rho, primarily because of difficulties in sample preparation, protein stabilization and crystallization. In fact, none of the cone pigments has yet been crystallized.

There are two major problems for crystallization of cone opsins: their instability in detergent micelles and their limited polar surface for protein-protein contacts. In an effort to facilitate structural characterization of cone opsins, we have undertaken several approaches to mitigate these problems.

(19) Understanding of GPCR dynamics and activation by solution NMR and DEER spectroscopy

Libin Ye¹, Ned Van Eps¹, Marco Zimmer¹, Oliver Ernst¹, Scott Prosser¹

¹University of Toronto

While X-ray crystallography and computational methods have greatly advanced our understanding of GPCR structural motifs and their role in function, our ability to delineate key functional states and details regarding the mechanism of activation of GPCRs is less understood. Pulsed EPR (DEER) spectroscopy provide the means to identify multiple states, and from distance measurements, connect these states to known structures identified by crystallography. NMR, and in particular fluorine (¹⁹F) NMR, provides quantitative information on topological properties, functional states, and their mutual exchange rates. This structural and dynamic perspective is key to advancing our understanding of activation mechanisms and the molecular underpinnings of GPCR-related pharmacology. Here, the A₂a adenosine receptor, a prototypical class A GPCR, was over-expressed and functionally purified
from *Pichia Pastoris*. Upon attaching an optimized $^{19}$F tag to a site on TM6 (V229C$_{6.31}$) through thiol conjugation, we find an ensemble of four states in equilibrium by deconvolution of spectra: (1) two inactive states in millisecond exchange, consistent with a formed (state $S_1$) and a broken (state $S_2$) salt bridge (known as ‘ionic lock’) between TM3 and TM6; and (2) two active states, $S_3$ and $S_3'$, as identified by binding of a G-protein-derived peptide. Consistently, preliminary DEER spectroscopy also depicted four states undergoing transition with a unique profile upon additional of different ligands. In contrast to a recent study of the $\beta_2$-adrenergic receptor, the present approach allowed identification of a second active state for A$_{2A}$R. Addition of inverse agonist (ZM241385) increases the population of the inactive states, while full agonists (UK432097 or NECA) stabilize the active state, $S_3'$, in a manner consistent with conformational selection. In contrast, partial agonist (LUF5834) and an allosteric modulator (HMA) exclusively increase the population of the $S_3$ state. Furthermore, while TM6 undergoes a gross outward shift upon activation, reflected by an up-field chemical shift of V229C$_{6.31}$, the rearrangement of helix packing detected through $^{19}$F NMR of A289C$_{7.54}$ on TM7 caused a clear downfield shift, consistent with recent X-ray crystal structures. Direct observation of ligand-dependent GPCR conformational equilibria and deduction of the underlying mechanisms of receptor activation will have wide reaching implications for our understanding of GPCR function in health and disease.

(20) Structural basis of intrinsic $\beta$-arrestin biased signaling at an atypical chemokine receptor by solution NMR spectroscopy

Andrew Kleist$^1$, Francis Peterson$^1$, Rob Tyler$^1$, Martin Gustavsson$^2$, Tracy Handel$^2$, Brian Volkman$^1$

$^1$Medical College of Wisconsin, $^2$University of California San Diego

Atypical chemokine receptors (ACKRs) are characterized by their inability to elicit G protein-mediated signaling events following interactions with their chemokine ligands, instead functioning exclusively through the cytosolic effector $\beta$-arrestin. The goal of this research is to define structural mechanisms underlying intrinsic receptor $\beta$-arrestin biased signaling at ACKR3 using solution Nuclear Magnetic Resonance (NMR) spectroscopy. We have developed a system that reports on unique ACKR3 conformations by site-specific labeling of cysteine, lysine, and methionine residues. Preliminary data suggest that ACKR3 exhibits unique conformational signatures when bound to a CXCL12 variant in the absence and presence of the FDA approved Plerixafor, among other ligands, establishing this system as a sensitive probe for ligand specific chemokine receptor conformations. Future efforts will use this system to manipulate ‘molecular switch’ residues in the receptor core to identify interactions responsible for $\beta$-arrestin recruitment to ACKR3.

(21) De Novo Epilepsy-Related Mutations in GNAO1 Exhibit both Gain and Loss-of-function Behavior

Huijie Feng$^1$, Benita Sjögren$^1$, Behirda Karaj$^1$, Vincent Shaw$^1$, Aysegul Gezer$^1$, Richard R. Neubig$^1$

$^1$Michigan State University

**Rationale:** The alpha subunit of G$_o$, Gα$_o$ (encoded by GNAO1) regulates neurotransmitter release and ion channel opening, which are important in the development and prevention of epileptic seizures. Although a link between mutations in Gα$_o$ protein and epilepsy has been identified, the underlying
mechanisms are not clear. In the current study, we assessed four de novo mutations in GNAO1 (G203R, I279N, D174G, ΔT191-F197) previously reported in children with epileptic encephalopathy (EIEE17) by testing their expression level and function to better understand the mechanisms of GNAO1-associated encephalopathy.

**Methods:** Wild type and mutant G\(\alpha\) were expressed in HEK293T cells using the pCI plasmid and their expression levels were tested by western blot/IR fluorescence detection. Function was tested by co-expression with the \(\alpha_{2a}\) adrenergic receptor and concentration response curves for inhibition of cAMP levels by the \(\alpha_2\) receptor agonist UK14,304 were assessed using the LANCE Ultra cAMP assay (Perkin Elmer, Waltham, MA). Results were compared to those for a known gain-of-function mutant (G184S) that we have shown causes an epilepsy phenotype in C57Bl/6J mice.

**Results:** The G\(\alpha\) G203R mutant expressed normally in HEK293T cells, but the I279N, D174G, and ΔT191-F197 mutants all had decreased protein expression. I279N, D174G and ΔT191-F197 exhibit different degrees of reduction in the maximal inhibition of cAMP levels (i.e. loss of function). Unexpectedly, for the G203R mutation, the EC\(_{50}\) for UK14,304 was markedly reduced (EC\(_{50}\) 12.1nM vs 31.5nM for WT G\(\alpha\)) while maximum inhibition of cAMP production was unchanged from WT G\(\alpha\) (102.1±1.0%). This gain-of-function behavior was similar to that of the G184S mutant (UK14,304 EC\(_{50}\) 11.7nM with a 107.7±0.9% maximal response comparing to WT G\(\alpha\)). Furthermore, the two complete loss-of-function mutants D174G and ΔT191-F197 did not exhibit dominant negative effects. A number of new de novo mutations have been published since and analysis of these mutant alleles is underway.

**Conclusions:** EIEE17 presents in infancy with early onset of intractable seizures, poor psychomotor development and possible brain abnormalities. Some patients may show involuntary movements. All GNAO1 mutants described have arisen de novo. Functional studies demonstrate that GNAO1 mutation associated epileptic encephalopathy result in both loss and gain of G\(\alpha\) function, suggesting a unique role of G\(\alpha\) modulation in the genesis of epileptic encephalopathy.

(22) Characterization of the internalization and compartmentalized signaling of the GLP-1R

**Madeleine M. Fletcher\(^1\), Michelle L. Halls\(^1\), Patrick M. Sexton\(^1\), Denise Wootten\(^1\)**

\(^1\)Monash Institute of Pharmaceutical Sciences, Monash University

**Introduction.** The glucagon-like peptide-1 receptor (GLP-1R) is a therapeutic target in the treatment of type 2 diabetes, neurodegenerative and cardiovascular diseases. Emerging evidence suggests that cells respond to GLP-1R activation, via compartmentalised signalling; the specific localisation of signalling mediators and receptors in discrete regions of the cell.

**Aims.** To explore the internalisation and trafficking of GLP-1R and its role in signaling upon activation by multiple peptide agonists.

**Methods.** Flp-In-Chinese Hamster Ovary cells were transiently transfected with GLP-1R and co-localisation of the receptor and cyclic adenosine monophosphate (cAMP) with subcellular compartments were assessed via bioluminescence and fluorescence resonance energy transfer, respectively. Global cellular cAMP accumulation and extracellular signal-related kinases 1/2
phosphorylation (pERK1/2) were measured through Perkin Elmer’s LANCE™ cAMP and AlphaScreen® SureFire®, respectively.

**Results.** Upon ligand binding, the GLP-1R internalizes rapidly and co-localizes with early, late and recycling endosomes. Internalization is inhibited by dominant-negative forms of dynamin and caveolin-1, and this inhibition of internalization reduces pERK1/2 and cAMP signaling. Different profiles of cAMP signaling were detected in plasma membrane (PM) and cytosolic compartments, with a more sustained cytosolic response. Glucagon-like peptide-1 was 10-fold more potent in producing cytosolic cAMP relative to PM cAMP despite being equipotent in global assays.

**Discussion.** Ligand stimulation promotes GLP-1R internalisation, via a dynamin and caveolae-mediated mechanism, and subsequently promotes trafficking through both degradative and recycling pathways. Inhibiting this process reduces cAMP and pERK1/2 levels, suggesting a potential role of signalling from an intracellular receptor. This is supported by the determination of distinct compartments of cAMP at the PM and in the cytosol.

*(23) New insight on experimental data using a streamlined, naive protocol for molecular dynamics analysis*

Laurent Bruneau Cossette\textsuperscript{1,2}, Jérôme Cabana\textsuperscript{1,2}, Philippe Sarret\textsuperscript{2}, Éric Marsault\textsuperscript{2}, Pierre Lavigne\textsuperscript{1}

\textsuperscript{1}Université de Sherbrooke, \textsuperscript{2}IPS - Université de Sherbrooke

Hidden Markov Models (HMMs) and time-lagged Independent Component Analysis (tICA) are simple, informative and accurate tools for the analysis or Molecular Dynamics (MD) data. They provide accurate information on both the thermodynamics (populations of states) and kinetics (timescales of state transitions) of proteins. However, they are relatively new: HMMs lack the analysis tools and validation that Markov State Models already have and tICAs, like PCAs, are hard to interpret.

The use of both techniques in a single set of tools, complemented with naive model selection criteria, macrostate analysis, residue contribution analysis and data display tools, provides new insight on experimental results on the structure of AT1-R, a GPCR. It has been found that AT1-R conformations, a few μs after ligand binding, are distributed in three macrostates, and that these macrostates could correspond to different signaling behaviors. The transitions between macrostates show major contributions from highly conserved residues, most likely involved in ligand binding and signaling. The information provided by this set of tools could be used to explore further the dynamics of AT1-R, as well as other GPCRs, in targeted structural and dynamics research (e.g. mutants, docking).

*(24) Evidence of a role for GPSM3 in cell division*

Drew Wallace\textsuperscript{1}, Alexey Pereverzev\textsuperscript{1}, Peter Chidiac\textsuperscript{1}

\textsuperscript{1}University of Western Ontario

Canonically, heterotrimeric G proteins are activated by G protein-coupled receptors (GPCRs) by promoting the exchange of GDP for GTP on the G\textsubscript{α} subunit. A group of receptor-independent activators of G protein signaling (AGS) proteins have also been identified, one of which is G protein signaling modulator 3 (GPSM3, also known as AGS4 and G18). GPSM3 acts as a guanine nucleotide
dissociation inhibitor (GDI) hindering the exchange of GDP for GTP and thereby promoting the inactive state of Gα and the active state of Gβγ. Other AGS proteins have been linked to cell division, participating in processes including generating spindle forces and spindle positioning and orientation. However, no findings presented in the literature thus far link GPSM3 to the process of cell division.

Vascular smooth muscle cells (VSMCs) derived from Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) and Human Embryonic Kidney 293 cells were used to study GPSM3’s role in cell division. GPSM3 transcript and protein levels in SHR-derived VSMCs both showed a decreasing trend during serum deprivation and an increasing trend during serum replacement. Additionally, SHR VSMCs divide at a much faster rate than WKY VSMCS, and GPSM3 transcript and proteins levels were found to be significantly higher in SHR versus WKY cells. HEK cells transfected with YFP-tagged GPSM3 divide faster than their YFP transfected counterparts, reaching confluence approximately 33% faster. Finally, dividing VSMCs subjected to immunofluorescent labeling show colocalization of β-tubulin and GPSM3 during metaphase, anaphase, and telophase. In summary, our findings suggest a possible role for GPSM3 in cell division, potentially contributing to this process via an interaction with the mitotic spindle.

(25) Aggregation Properties of Partial and Complete Misfolded Opsin Mutants

Megan Gragg1, Paul Park2

1Case Western Reserve University, 2Case Western Reserve University

Over 100 clinically identified rhodopsin mutations cause autosomal dominant retinitis pigmentosa (adRP), a progressive retinal degenerative disease initially affecting rod photoreceptor cells. A majority of rhodopsin mutations cause receptor misfolding and aggregation. Misfolded opsins cause adRP by uncertain mechanisms, and therefore, the misfolded opsin aggregates formed must be better understood to rationally design therapies for adRP. In order to investigate misfolded opsins, a complete misfolded mouse opsin mutant and wildtype (WT) mouse opsin was previously studied by Förster Resonance Energy Transfer (FRET) in live HEK293 cells. Our FRET-based approach differentiated between properly folded opsin oligomers formed by WT opsin and aggregates formed by misfolded mutant opsin.

In the current work, human WT and misfolded mutant opsin structure was examined by the same FRET approach in transfected HEK293 cells. We investigated a partial misfolded opsin mutant, P23H, and a complete misfolded opsin mutant, G188R. Similar to mouse opsin, human opsin predominantly formed oligomers, consistent with observations in native photoreceptor cell membranes from mouse and human retina. Human G188R and P23H opsin mutants both formed predominantly aggregates, suggesting misfolded opsins share similar molecular interactions to perhaps form similar quaternary structures.

The potential chaperoning effect of 9-cis-retinal on opsin folding was tested in cells expressing the P23H or G188R mutant opsins. 9-cis-retinal was added to cells expressing human opsin to form isorhodopsin. Isorhodopsin exhibited similar FRET properties as opsin, suggesting the formation of similar oligomers. In cells, 9-cis-retinal had different effects on the partial misfolded P23H opsin and the complete misfolded G188R opsin. 9-cis-retinal had no effect on G188R opsin; the mutant opsin formed predominantly aggregates even in the presence of chromophore. In contrast, the addition of 9-cis-
retinal to cells expressing P23H opsin resulted in more oligomers than aggregates, showing successful chaperoning of the partial misfolded opsin mutant. Thus, while the two misfolded opsin mutants appear to form similar aggregates, only the P23H opsin mutant can be chaperoned by 9-cis-retinal to form oligomers rather than aggregates. Since the partial misfolded mutant is resusable, different therapeutic strategies may be considered with patients harboring different types of misfolded opsin mutants.

(26) Discovery and Structure-Activity Relationship of a Bioactive Fragment of ELABELA that Modulates Vascular and Cardiac Functions

Alexandre Murza¹, Sainsily Xavier¹, David Coquerel¹, Jérôme Côté¹, Patricia Marx², Elie Besserer-Offroy¹, Jean-Michel Longpré¹, Olivier Lesur¹, Mannix Auger-Messier¹, Philippe Sarret¹, Éric Marsault³

¹Université de Sherbrooke, ²Bishop's University, ³IPS - Université de Sherbrooke

ELABELA (ELA) was recently discovered in 2013 as a novel endogenous ligand of the apelin receptor (APJ), a G protein-coupled receptor. ELA signaling was demonstrated to be crucial for normal heart and vasculature development during embryogenesis.¹ The structure-activity relationship (SAR) of ELA and its cardiovascular effects remain to be elucidated. Moreover, to explore the full therapeutic potential of ELA, it is important to decipher the key pharmacophores of this 32-mer peptide, with the additional goal to reduce its size. To discover a bioactive fragment of ELA, we examined the susceptibility of ELA to proteolytic cleavage in human plasma. Then, we performed an alanine scan on the active fragment to identify the key pharmacophores. ELA and shorter analogues were assessed in binding, signaling and receptor internalization assay. Left ventricular developed pressure using isolated-perfused rat hearts and in vivo hemodynamic and echocardiographic measurements were carried out on ELA and its potent bioactive fragment. We discovered a significantly smaller bioactive fragment consisting of the last 14 amino acids of native ELA which binds APJ, activates Gαi1 and β-arrestin-2 signaling pathways, and induces receptor internalization similarly to the parent peptide. The alanine scan revealed that the C-terminal moiety is the most critical for binding and signaling. This SAR sharply contrasts with apelin-13, in which key pharmacophores are mainly located at the N-terminal portion of the peptide. ELA and its fragment caused hypotensive effects and enhanced left ventricular contractility.² This study is the first to characterize the cardiovascular effects of ELA and its potent fragment. This represents a promising avenue for further exploitation of the therapeutic potential of ELA and the apelinergic system in cardiovascular diseases.


(27) Pharmacologically Studying the Interplay Between GPCR Internalization and Cellular Signaling Using a Novel Modulator of Receptor Trafficking

Jenna Giubilaro¹, Yoon Namkung¹, Stephane Laporte¹

¹McGill University

G-protein coupled receptors (GPCRs) are the largest family of cell surface receptors. Their dysfunction may lead to numerous diseases, making them a popular target for therapeutic drugs. One
target is receptor-mediated endocytosis, which inhibits upstream G-protein signaling and promotes alternate downstream β-arrestin-mediated signaling pathways. However, the mechanisms by which β-arrestins interact with other endocytic proteins and mediate receptor trafficking and signaling are not fully understood. Therefore, with the use of new BRET-based biosensors, a high-throughput screen was performed on a commercial library of compounds to identify new modulators of receptor trafficking that may be used to pharmacologically study how GPCR endocytosis and intracellular signaling are associated.

One of those small molecules, Traf 21, was selected as an endocytic inhibitor of angiotensin II type 1 receptor (AT1R) and is further characterized here. We have previously shown that Traf 21 prevents receptor internalization in HEK293 cells, but does not affect β-arrestin recruitment to receptors at the plasma membrane nor the formation of the b-arrestin2/AP-2 endocytic complex. Next, we determined that Traf 21 blocks GPCR and RTK activated ERK1/2 pathways. However, this MAPK inhibition is not due to Traf 21’s effects on G-protein signaling because Traf 21 does not prevent angiotensin II from activating Gaq, PLC or PKC. Preliminary data suggest Traf 21 is also an inhibitor of small GTPases.

Characterizing Traf 21 may contribute to the discovery of new pharmacological ways to target multiple steps of receptor endocytosis and signaling, which are involved in physiological processes that range from cardiovascular disease to cancer.

Acknowledgement: This work is supported by the CIHR and the RQRM.

(28) G protein-coupled receptor-mediated transcriptional effects in the development of cardiac hypertrophy

Ryan Martin¹, Jason Tanny¹, Terence E. Hébert¹

¹McGill University

Pathological cardiac hypertrophy is an adaptive response to an increased demand placed on the heart, with prolonged hypertrophic stress associated with poor cardiac function and eventual heart failure. The cardiac hypertrophic response, characterized by the increase in cardiomyocyte size, is elicited in response to neurohormones such as endothelin-1 and norepinephrine. Activation of their cognate G protein-coupled receptors activates a signalling cascade leading to the reactivation of the cardiac fetal gene program and stimulation of global RNA transcription. One mechanism implicated in the increase in global RNA transcription is the activation of positive transcription elongation factor b (P-TEFb), a dimer consisting of cyclin dependent kinase 9 and its cyclin partner, cyclin T. P-TEFb is required to release RNA polymerase II from a paused state in the transcription cycle by phosphorylating RNA polymerase II, DRB sensitivity inducing factor (DSIF) and negative elongation factor (NELF). The phosphorylation status of these transcription factors acts as a scaffold to recruit various proteins to the transcriptional machinery, such as histone modifying enzymes. The objective of our study is to investigate the role of factors recruited to the transcription machinery downstream of P-TEFb activity in the development of cardiac hypertrophy. Primary neonatal cardiomyocytes isolated from 1-3 day old Sprague-Dawley pups were used to model the hypertrophic response in vitro. Treatment of cardiomyocytes with endothelin-1 or phenylephrine stimulated an increase in cell surface area as analyzed by high-content microscopy. This hypertrophic response was blocked with the co-treatment the Cdk9 inhibitor iCdk9. Stimulation of global nascent RNA transcription measured by 5-ethynyl uridine
incorporation was observed in response to endothelin-1, with iCdk9 co-treatment blunting this effect. Inhibition of Cdk9 also prevented the increased transcription of genes that form the fetal gene program recapitulated during hypertrophy, such as atrial natriuretic peptide, in response to either hypertrophic agonist. We observed small global increases in histone modifications associated with actively transcribed genes, such as histone H3 lysine 79 methylation, in response to either agonist, with only monoubiquitinated histone H2B significantly decreasing in response to iCdk9 treatment. Although only a small global increase in histone H3K79 methylation was observed in response to either agonist, preliminary results show pre-treatment with an inhibitor of Dot1L, the histone H3K79 methyltransferase, blunted the hypertrophic response. Our study has demonstrated that P-TEFb activity is necessary for the cardiac hypertrophic response stimulated by G protein-coupled receptors. We have also shown that histone H3K79 methylation is required for the hypertrophic response.

(29) A phosphoinositide-regulated Golgi checkpoint regulates surface delivery of delta opioid receptors in neurons

Daniel Shiwarski¹, Alycia Tipton², Melissa D. Giraldo³, Michael S. Gold³, Amynah A. Pradhan², Manojkumar A. Putheenveedu¹

¹Carnegie Mellon University, ²University of Illinois, Chicago, ³University of Pittsburgh School of Medicine

Many G protein-coupled receptors (GPCR) are internalized and degraded after activation. For these receptors, cells rely on the surface delivery of newly synthesized receptors for recovery of sensitivity. Whether and how GPCR biosynthetic delivery is regulated, and how this influences receptor physiology in vivo, are largely unknown. Here we describe a physiologically relevant neuronal signaling axis that regulates the surface delivery of the delta opioid receptor (DOR), a prototypic “single-use” GPCR, in peripheral sensory neurons. We define a phosphoinositide-regulated checkpoint that regulates DOR export from the Golgi and retains DOR in intracellular pools in neurons. Further, we provide a proof of concept that manipulating this checkpoint releases DOR from this storage pool, stimulates DOR surface delivery, and allows effective DOR-mediated antinociception in vivo. Our results provide an explanation for the low efficacy of DOR agonists in vivo, and identify a novel physiologically relevant exocytic regulatory system to control the surface levels of single-use GPCRs.

(30) Chronic blockade of metabotropic glutamate receptor 5 in APPswe/PS1dE9 and 3xTg-AD mouse models ameliorates Alzheimer’s disease pathogenesis

Alison Hamilton¹, Khaled S. Abd-Elrahman¹, Stephen S.G. Ferguson¹

¹University of Ottawa

Metabotropic glutamate receptor 5 (mGluR5) has been implicated in the pathogenesis of a number of neurodegenerative diseases, including Alzheimer’s disease (AD). Proposed to act as an extracellular scaffold for beta amyloid (Aβ), mGluR5 has been suggested to function as a receptor for the soluble oligomeric Aβ most closely linked to neuronal death and cognitive decline. The binding of Aβ has been shown to impair the lateral diffusion of mGluR5 and increase mGluR5 at the cell surface, leading to over activation of the receptor. This over activation of mGluR5 in the AD brain, has been implicated in: (1) the elevation of intracellular Ca²⁺, through the potentiation of NMDAR activity by mGluR5, (2) increased release of neurotoxic Aβ oligomers caused by increasing fragile X mental
retardation protein (FMRP) mediated translation of amyloid precursor protein (APP), and (3) alteration of autophagy. This presents the potential for mGluR5 to be a therapeutic target for the treatment of AD. CTEP is an orally bioavailable, highly selective negative allosteric modulator for mGluR5. We have recently published data showing that chronic treatment with CTEP improves learning and memory in APPswe/PS1dE9 and 3xtg-AD mice, as well as reducing both oligomeric and fibrillar Aβ. We now have data from both mouse models which shows that: Chronic CTEP treatment normalizes cell surface expression of mGluR5, as well as normalizing the expression of FMRP. GSK3β a protein which is overexpressed in AD, resulting in hyperphosphorylation of tau, is normalized. Finally we show that chronic treatment with CTEP normalizes p62, a marker for autophagy, which is commonly elevated in AD and other neurodegenerative diseases. Our data confirms a role for mGluR5 in the pathogenesis of AD, via alterations to signaling and trafficking of the receptor, that chronic blockade of mGluR5 ameliorates AD progression. Finally this data shows the potential for CTEP as a treatment for AD.

(31) Rational design of neurotensin 8-13 macrocyclic peptidic analogues

Marc Sousbie¹,²,³, Élie Besserer-Offroy¹,²,³, Jean-Michel Longpré¹,²,³, Philippe Sarret¹,²,³, Richard Leduc¹,²,³, Éric Marsault¹,²,³

¹Université de Sherbrooke, ²IPS - Université de Sherbrooke, ³Faculté de Médecine et des Sciences de la Santé, Département de Pharmacologie, Université de Sherbrooke

In the Central Nervous System, neurotensin (a tridecapeptide) activates the GPCRs NTS1 and NTS2, which leads to an antinociceptive effect (1). These receptors are therefore considered a good potential alternative target for the treatment of chronic pain. This condition is indeed broadly treated with morphine or similar opioids, despite their widely documented adverse effects (2).

The endogenous ligand of NTS1 and NTS2 being a peptide, it is not very usable as a pharmacological tool, let alone a drug. It has extremely poor bioavailability and half-life in vivo. In order to improve this, we decided to produce macrocyclic analogues of NT 8-13 (the six N-terminal amino acids of neurotensin, the shortest sequence to retain the full activity) (3). Macrocyclic peptides are indeed known to have various advantages (pharmacologically speaking) over their linear counterparts, mostly thanks to their restricted conformation. First, from a pharmacokinetics standpoint, peptidic macrocycles are usually more resistant to proteases, and therefore have an extended half-life. Moreover, the lower number of rotatable bonds and the higher propensity to form intramolecular H-bonds can improve absorption, even for relatively large molecules (4).

Second, from a pharmacodynamics standpoint, the fact that the macrocycle has less degrees of freedom compared to the linear ligand tempers the loss of entropy upon binding, thus facilitating it (provided that the macrocycle’s conformation is close to the one adopted by the ligand inside the binding pocket) (5). Most of our efforts so far have been focused on the design of macrocycles which imitate the receptor-bound neurotensin.

The availability of the rat NTS1 crystal with NT 8-13 as a ligand (6) appeared as an opportunity to use molecular modeling as a way to support the rational design of our compounds. We therefore produced a homology model of the human NTS1 and used it to dock our macrocycles and assess their ability to adopt the right conformation inside the binding pocket. Coupled to radioligand competition
binding experiments, this strategy enabled us to design and improve several series of peptidic macrocycles. This led to a macrocycle with a 40 nM affinity for NTS1 (compared to 1 nm for NT 8-13) with a reduced Polar Surface Area and extended half-life in rat plasma.

We believe that this first hit will open a door towards macrocycles which have much more drug-like properties than the endogenous peptide neurotensin.

References

1) P. Dobner, CMLS 2005
2) I. Kissin, Anestheisa and analgesia, 2010
4) J. G. Beck et al. JACS 2012

(32) Negative allosteric modulation of mGluR5 mitigates motor and cognitive impairments in Q175 mouse model of Huntington’s disease via activation of autophagy and clearance of huntingtin aggregates

Khaled Abd-Elrahman¹, Alison Hamilton¹, Shaunessy Hutchinson¹, Stephen Ferguson¹

¹University of Ottawa Brain and Mind institute

Introduction: Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder that causes progressive motor, cognitive and psychiatric impairments. A mutant form of huntingtin protein characterized by an expanded polyglutamine repeats and ability to form aggregates is known to be the underlying cause of HDⁱ. Despite the well-characterized etiology and ability of early genetic diagnosis, to date there is no disease modifying drug for HD patients. Evidence suggests that alterations in metabotropic glutamate receptor 5 (mGluR5) signaling contribute to the progression of excitotoxic damage associated with HD. Genetic deletion of mGluR5 ameliorated motor dysfunction and mutant huntingtin-dependent pathology in Q111 knock-in HD mouse model². Here, we tested whether acute and/or chronic blockade of mGluR5 using the orally bioavailable, selective mGluR5 negative allosteric modulator CTEP will improve motor and cognitive deficits in Q175 knock-in mouse model of HD.

Methods: Twelve-month-old wild type, heterozygous Q175 (Q175/+) and homozygous Q175 (Q175/Q175) mice were orally-treated with either vehicle or CTEP (2mg/Kg) every 48 hours for 12 weeks. All groups were assessed following 1 week (acute) and 12 weeks (chronic) of treatment for changes in grip strength, performance on accelerating rotarod, limb placement/coordination on ladder rung walking task, and recognition of novel object. At the end of the study animals were euthanized, brain slices and lysates were collected.

Results: Acute treatment with CTEP did not mitigate grip strength or performance on rotarod in Q175/+ or Q175/Q175 but significantly improved recognition scores of novel objects and % error in limb
placement on ladder rung task in Q175/+. Chronic CTEP treatment caused a significant improvement in: i) grip strength and recognition scores in both Q175/+ and Q175/Q175, and ii) latency to fall from rotarod and % error in limb placement on ladder walking task in Q175/Q175. CTEP inhibited ZBTB16-mediated proteasomal degradation of ATG14L, a critical regulator of autophagy, and reduced P62 levels in Q175/Q175 indicating activation of autophagy. This was paralleled by CTEP-induced clearance of mutant huntingtin aggregates in cortical and hippocampal brain slices of Q175/+ and Q175/Q175.

Conclusions: CTEP improves cognitive and motor dysfunction and enhances the clearance of mutant huntingtin aggregates via activation of autophagy in Q175 knock-in mice. Our findings provide further evidence for the crucial role of mGluR5 in HD and highlight the potential for clinically repurposing CTEP or its analogues to rectify HD progression.

References

Ribeiro FM et al. (2014). Expert Opin Ther Targets. 18:1293-1304


(33) Differential compartmentalization of cAMP is required for stimulation and inhibition of perinuclear phospholipase C, subsequent phosphatidylinositol 4-phosphate hydrolysis and cardiac hypertrophy – a role for the direct phosphorylation of PLCε by PKA

Craig Nash¹, Sundeep Malik¹, Alan Smrcka¹

¹University of Michigan

Recently, our laboratory determined that ET-1 and the EPAC-selective cAMP analogue, cpTOME, both induce PLCε-dependent hydrolysis of phosphatidylinositol 4-phosphate (PI4P) in the Golgi apparatus of neonatal rat ventricular myocytes (NRVMs), a key pathway for regulation of cardiac hypertrophy. EPAC, a GEF for Rap1, is scaffolded with PLCε at the nuclear envelope in cardiac myocytes through direct interactions with mAKAPβ, providing a mechanism for cpTOME-dependent local PLC activation and PI4P hydrolysis. Surprisingly, however; we find that Gs coupled receptors, including β-adrenergic receptors, do not stimulate PI4P hydrolysis at the Golgi. To further understand this process, we investigated the role of endogenous cAMP in the stimulation of PI4P hydrolysis. We found that Forksolin and selective inhibition of phosphodiesterase III or IV induce PI4P hydrolysis at the Golgi membrane. Conversely, treatment with the pan-PDE inhibitor IBMX does not cause PI4P hydrolysis, but a PKA or PKG inhibitor enables IBMX stimulation of PI4P hydrolysis. Preincubation of NRVMs with either a PKA or PKG activator abolishes PI4P hydrolysis in response to ET-1, cpTOME or Forskolin. These results indicate differential compartmentalization of at least two pools of cyclic nucleotides, one that regulates inhibitory PKA/PKG signals, and one that positively regulates EPAC-dependent of PI4P hydrolysis at the Golgi. Cilostamide and Rolipram, that selectively amplify the cAMP pool that stimulates PI4P hydrolysis, stimulate hypertrophy in NRVMs in a PLCε- and PI4P-dependent manner. To address the molecular mechanism involved in PKA-mediated inhibition of PI4P hydrolysis, we show that active PKA can directly act upon PLCε to inhibit both basal (in vitro) and βγ stimulated inositol phosphate production in COS-7 cells. These data taken together suggest a key role for the direct phosphorylation of PLCε by PKA (and potentially PKG) in the negative regulation of PLCε.
dependent PI4P hydrolysis, demonstrating a potential mechanism for PKA and PKG-dependent inhibition of cardiac hypertrophy. For Iso-dependent stimulation of cardiac hypertrophy, the balance between PKA dependent inhibition and EPAC-dependent activation of PLCε must be altered to favor PI4P hydrolysis and subsequent development of hypertrophy.

This work was supported by a grant from the NIGMS.

(34) The role of VCP in β2-adrenergic receptor maturation
Richard Wargachuk\textsuperscript{1}, Irina Glaznova\textsuperscript{1}, Darlaine Pétrin\textsuperscript{1}, Phan Trieu\textsuperscript{1}, Terence E. Hébert\textsuperscript{1}
\textsuperscript{1}McGill University

The β\textsubscript{2}-adrenergic receptor (β\textsubscript{2}AR) is a member of the G protein-coupled receptor (GPCR) family of proteins. GPCRs form signalling complexes with other receptors, G proteins and effector partners. The formation of these complexes is essential to ensure the appropriate response to extracellular signals. Cell-specific protein partners associated with the GPCR determine the response of the cell when β\textsubscript{2}AR is activated by adrenaline or other ligands. Many of the proteins that associate with GPCRs are first assembled in the endoplasmic reticulum. What controls how and when these associations are formed have yet to be determined but is likely to be of critical importance for understanding the nature of biased signalling in different cell types. A proteomic screen to identify proteins that associate with the β\textsubscript{2}AR identified many of components of the Endoplasmic-Reticulum-Associated Degradation (ERAD) quality control system, including the valosin-containing protein (VCP/p97). To better understand how the β\textsubscript{2}AR signaling complex is formed we have utilized a tetracycline induction system to control expression of the receptor in short pulses. The β\textsubscript{2}AR was tagged with a FLAG epitope and placed in an inducible vector stably expressed in HEK 293 cells where expression levels could be directly controlled. We have confirmed the interaction of VCP with moderately expressed levels of FLAG-β\textsubscript{2}AR, demonstrating that the interaction of FLAG-β\textsubscript{2}AR and VCP is not an artifact of overexpression of the β\textsubscript{2}AR. Using an siRNA-based approach, we knocked down VCP and noted that levels of FLAG-β\textsubscript{2}AR were increased compared to control cells. This increase in the level of FLAG-β\textsubscript{2}AR did not lead to an increase in the level of functional receptor observed at the cell surface. Similarly, inhibition of the proteasome lead to a dramatic increase in the abundance of TAP-β\textsubscript{2}AR, while cellular responses again remained unchanged. Taken together, our data suggests that VCP plays a key role in recognizing and removing misfolded β\textsubscript{2}AR receptors.

(35) Fiber-optic imaging of FRET biosensors for recording GPCR signalling in vivo
Jace Jones-Tabah\textsuperscript{1}, Faiza Benalioud\textsuperscript{1}, Paul B.S. Clarke\textsuperscript{1}, Terence E. Hébert\textsuperscript{1}
\textsuperscript{1}McGill University

In the central nervous system, G protein-coupled receptors (GPCRs) mediate many neuronal responses to neurotransmitters and neuromodulators, and are major drug targets for neuropsychiatric disease. Individual GPCRs signal via multiple downstream effectors, only some of which may mediate
therapeutic effects in vivo. Furthermore, the specific complement of signalling cascades engaged by a GPCR is determined by several factors, including the particular ligand, the cellular context, and the extracellular environment. Determining how specific GPCR signalling cascades produce biologic effects in the whole animal would advance our understanding of GPCR function in disease states. Such understanding would also facilitate the development of novel functionally selective ligands (i.e. those that only modulate a subset of pathways downstream of a given GPCR). To this end we aim to develop a method for imaging FRET based biosensors that report GPCR downstream signalling in real time in live animals. Here, we are focusing on genetically encoded FRET biosensors that can report second messenger production (cAMP, Ca^{2+}) and protein kinase activity (PKA, ERK1/2) with high spatial and temporal resolution. These biosensors can be expressed in vivo with cell-type specificity, and have negligible effects on endogenous signalling processes. Using stereotaxic injection of a lentiviral vector, we have expressed the red-shifted cAMP biosensor GEpac-mC in the rat striatum in a neuron-specific manner. Surgically implanted fiber-optic probes will be used to collect fluorescence emissions, and time-correlated single-photon counting will be used to quantify FRET by emission spectra and fluorescence lifetime. Flexible fiber-optic patch cords allow imaging to be performed in freely moving animals, allowing the simultaneous measurement of behavioral and signalling responses to pharmacological manipulation.

(36) Crosstalk between the delta-opioid receptors (DOR) and TRP channel subfamily A1 (TRPA1): A potential therapeutic target for chronic pain
MeeJung Ko¹, Richard M. van Rijn¹
¹Department of Medicinal Chemistry and Molecular Pharmacology, College of Pharmacy, Purdue University

Chronic pain is defined by complicated symptoms like burning, aching, or pins-and-needles-like sensation lasting longer than 12 weeks, and which currently affects 42 to 50 million people in the US (NIH 2011). Several studies have suggested that one subtype of non-selective calcium permeable channels, known as Transient Receptor Potential (TRP) channel subfamily A1 (TRPA1), mediates behavioral pain responses to thermal or chemical stimuli, and contributes to chronic pain. As such, direct inhibition of TRP channels was the initial strategy, but due to unwanted side effects from direct modulation of TRPA function such as thermal dysregulation, indirect modulation of TRP channels has become more preferred. Recent studies have suggested an indirect approach of reducing TRP channel subfamily V (TRPV) activity using opioid receptors by recruiting β-arrestin 2, an intracellular inhibitory protein, away from the TRPV channels. Here, we are currently investigating delta opioid receptors (DORs) that are commonly co-expressed with TRPA1 channels in sensory neurons. Our central hypothesis of the study is that the activation of DORs may modulate TRPA1-induced calcium signaling and consequent pain responses. Importantly, several DOR-agonists are available that vary in their ability to recruit β-arrestin 2, allowing us to explore underlying mechanisms of DOR-TRPA1 crosstalk at the level of calcium signaling and pain responses.

To better address the potential interaction and underlying mechanisms mediated by TRPA1 channels and DOR activation, we utilize in vitro cellular assays, and in vivo animal nociceptive tests. The in vitro cellular calcium assay demonstrates intracellular calcium influx caused by TRPA1-activation. We also perform the Von Frey filament test to measure the mechanical sensitivity in animals,
which provides information on the *in vivo* consequences of TRPA1-activation. Results from these experiments suggested that TRPA1-activation elevated intracellular calcium levels in cells and increased mechanical hypersensitivity in mice as well. Additional results from *in vitro* calcium assay indicate that a synthetic DOR agonist SNC80, which has been previously shown to strongly recruit β-arrestin 2, increases TRPA1-induced intracellular calcium level as demonstrated by *in vitro* cellular assays. However, an endogenous DOR agonist leucine-enkephalin, which weakly recruits β-arrestin 2, reduces TRPA1-activation. In brief, both of our *in vivo* and *in vitro* results were capable of demonstrating the significance of TRPA1 channels in pain responses and the potential therapeutic targets for chronic pain. Especially, our *in vitro* results with the endogenous DOR agonist implicate potential therapeutic applications of leucine-enkephalin that may include multiple pain-related disorders such as acute or chronic pain. For our future direction, we plan to move forward to prove modulation of nociception by DOR-TRPA1 crosstalk *in vivo* using intrathecal injections prior to TRPA1 activation, which will provide insights to development of novel strategies to reduce TRPA1 channel activity and chronic pain via DOR-targeted drugs.

(37) Identification of Novel Small Molecule Stabilizers of RGS4 Protein Levels

Benita Sjogren¹, Behirda Karaj¹, Richard R. Neubig¹

¹Michigan State University

Regulator of G protein Signaling 4 (RGS4) is a member of a large family of proteins that regulate signaling through G protein-coupled receptors (GPCRs) by accelerating GTPase activity on active Gα subunits. RGS4 has a very short protein half-life due to rapid proteasomal degradation and we propose that stabilization of RGS4 protein levels could be a beneficial therapeutic strategy in diseases associated with low RGS4 protein levels, such as breast cancer and, possibly, schizophrenia. Our previous studies successfully demonstrated *in vivo* effects of pharmacological enhancement of RGS2 protein levels as well as *in vivo* effects of pharmacological RGS4 inhibition. This demonstrates that modulating protein levels and/or function of an RGS protein is a valid approach in drug discovery.

To identify small molecule enhancers of RGS4 protein levels (and thereby function) we developed a cell based high-throughput β-galactosidase assay and screened a diversity collection of 100,000 compounds for hits that would increase RGS4 protein. The hits identified were confirmed in dose-response with fresh powder and validated hits were further evaluated for their effects on endogenous RGS4 protein in SH-SY5Y cells, as well as their effects on RGS4 mRNA levels and protein half-life.

We identified two structurally distinct series of compounds that stabilize RGS4 protein levels in recombinant as well as endogenous cell systems. Further development of these compounds are under investigation and they could serve as novel leads for drug development and/or pharmacological tools to study RGS4 protein function. Ongoing studies are aimed towards investigating the mechanism of action of the identified compounds as well as the effects of pharmacological enhancement of RGS4 protein *in vitro* and *in vivo*. 
Dopamine receptor/sodium channel protein complex, a target for antiepileptic mood stabilizers?

Gohar Fakhfouri¹, Thomas Del’Guidice¹, Annie Barbeau¹, Mohamed Chahine¹, Jean-Martin Beaulieu¹

¹Universite Laval

Despite their widespread prescription for the management of bipolar disorder, the antiepileptic drugs lamotrigine and valproate have yet unclear underpinning mechanisms for their mood stabilizing effects. This hampers the research for the development of newer and safer treatments. Voltage gated sodium channels (Nav) represent a major target of these mood stabilizers. We have previously shown that lithium inhibits a β-arrestin mediated modality of dopamine D2 receptor (D2R) signalling. We also demonstrated recently that chronic administration of lamotrigine and valproate regulates Akt/GSK3β signaling and that the D2R is required for the exertion of such regulation. Since dopamine receptors have been shown to interact with different type of ion channels, we investigated a possible interaction of D2R/Nav and further explored how lamotrigine can affect dopamine-mediated behaviors and signalling.

Our findings demonstrate that acute treatment with lamotrigine inhibited the amphetamine-induced hyperactivity, while the antiepileptic Nav blocker phenytoin, which is devoid of mood stabilizing properties, had no effect. Such effect of lamotrigine was lost in D2RKO mice. Selective stimulation of D1R or D2R revealed that lamotrigine antagonises only the second (post-synaptic) phase of D2R-induced locomotor activity while it had no impact on D1R-dependent locomotion.

Acute lamotrigine, but not phenytoin, also inhibits the β arrestin-2 mediated Akt/GSK-3β signalling modality of D2R, showing that lamotrigine acts as a functional antagonist of D2R. At the molecular level, Nav is expressed at close proximity and forms a complex with D2R through C-terminal of the channel. Intriguingly, such interaction does not affect the Nav properties and fails to influence D2R mediated cAMP reduction. The research is ongoing to unravel the functional consequence of Nav/D2R interaction.

Biases in PDZ Protein Regulation of GPCR Trafficking and Signaling

Henry A. Dunn¹, Stephen Ferguson¹

¹Department of Cellular & Molecular Medicine, Faculty of Medicine, University of Ottawa

Corticotropin-releasing factor receptor 1 (CRFR1) and serotonin 2A receptor (5-HT2AR) have been targeted as important G protein-coupled receptors (GPCRs) for the generation of new pharmacological treatment strategies for mental illnesses. Interestingly, both of these receptors contain class I PDZ-binding motifs on their distal carboxyl termini that are required for CRFR1-mediated enhancement of 5-HT2AR signaling: a mechanism that may underlie stress-induced anxiety and depression. The structurally homologous PDZ proteins PSD-95 and SAP97 have both been implicated in schizophrenia, and previous studies have demonstrated PSD-95 to be required for hallucinogenic and atypical anti-psychotic action via 5-HT2AR activity. Therefore, understanding PDZ protein regulation of CRFR1, 5-HT2AR, and the crosstalk therein may provide important insights for the creation of next-generation treatments for mental illness. In the current studies, we observed both redundancies and specific biases for SAP97 and PSD-95 regulation of CRFR1 and 5-HT2AR. Both
SAP97 and PSD-95 are important for promoting membrane stability of CRFR1 and 5-HT2AR which may be a mechanism of antagonizing β-arrestin2 recruitment. There is no apparent effect of these proteins on Gs-coupled signaling via CRFR1, however both SAP97 and PSD-95 promote Gq-coupled inositol phosphate accumulation via 5-HT2AR. Notably, endogenous SAP97 was integral for both CRF- and 5-HT-mediated ERK1/2 phosphorylation; however PSD-95 appears to have no effect despite extensive structural homology. Neither SAP97 nor PSD-95 appear to be exclusively responsible for CRFR1-mediated enhancement of 5-HT2AR signaling. Therefore, we begin to tease out the biases of PDZ protein regulation of GPCR trafficking and signaling.

(40) Novel Small Molecule Activators of Adhesion G Protein-Coupled Receptors

Hannah Stoveken1, Alan Smrcka1,2, Gregory Tall1

1University of Michigan, 2University of Rochester High Throughput Screening Core

Adhesion GPCRs (aGPCRs) are a subfamily of GPCRs with known roles in immune cell function, organ and tissue development, and cancer progression. They possess a unique underlying pharmacology whereby aGPCRs constitutively cleave themselves within a conserved, extracellular GPCR Autoproteolysis Inducing (GAIN) domain to generate a concealed tethered agonist. Following self-cleavage, the bipartite receptor traffics to the plasma membrane as one non-covalently bound unit with the newly generated tethered agonist sequestered in the hydrophobic core of the GAIN domain. We hypothesize that receptor activation occurs first, by binding of aGPCR extracellular domains (ECDs) to anchored extracellular matrix (ECM) proteins or cell surface ligands presented by neighboring cells. Cell movement-mediated shear force then dissociate the ECDs to expose the previously hidden tethered agonist. By utilizing prepared insect cell membranes expressing the aGPCR GPR56/ADGRG1, we reconstituted the receptor with purified, recombinant G proteins and directly demonstrated that operative dissociation of the ECD from the 7-transmembrane domain (7TM) augmented G protein 13 activation kinetics. aGPCR 7TMs expressed without the ECDs are also capable of activating G proteins. Known aGPCR ligands, principally ECM proteins like collagens and laminins, are challenging to manipulate in vitro and do not model authentic receptor pharmacology as typical soluble GPCR ligands. We hypothesized that aGPCR small molecules could be identified to meet the need for aGPCR synthetic modulators and for use as leads in drug development for aGPCR-directed disease. Using the G13-coupled aGPCR GPR56 and our delineation of the tethered agonist activation mechanism, we developed a Serum Response Element (SRE)-luciferase gene reporter assay for high throughput screening to identify aGPCR activators that functionally replicate signaling of the tethered agonist-bound receptor. A 2,000 compound library (Spectrum Collection) composed of known drugs and natural products was screened for activators of a GPR56 7TM receptor with a compromised tethered agonist. From these screens, we identified two related classes of natural compounds that were capable of activating a subset of aGPCRs but did not activate two non-aGPCRs tested. The most potent compound identified was the gedunin-derivative, 3α-acetoxydihydrodeoxygedunin, which activated the low-activity GPR56 A386M receptor with an EC50 of ~5µM. By performing structure-activity relationship analysis, we narrowed down the functional groups that were essential for receptor activation. The aGPCR activators identified and characterized in our studies will serve as invaluable research tools to understand this unique-class of GPCRs.
Gαi-GTP inhibits integrin dependent cell adhesion

Jesi Lee Anne To¹, Alan V Smrcka¹

¹University of Michigan

Chemokine receptors are G-protein coupled receptors (GPCR) that primarily couple to the Gi/o family of G-proteins. Their activation triggers dissociation of the heterotrimeric G-protein, Gα and Gβγ dimer, both of which can initiate downstream signal transduction pathways critical to chemokine function. The role of Gβγ in immunology has been very well studied and characterized. In comparison, the role of Gαi in chemokine signaling has not been well understood. This lack of understanding is, in part, because perturbations that inhibit Gαi signaling also inactivate signaling of the obligate Gβγ dimer. Our lab recently published evidence that Gαi has a direct role in cell migration of neutrophils. These studies revealed that Gαi regulates cell polarization through a cAMP-dependent mechanism, and adhesion in a cAMP-independent mechanism. Radil has been shown to interact with Rap1 and Gβγ and plays an integral role in adhesion. To elucidate the cAMP-independent role of Gαi in adhesion, we performed epistasis analysis by overexpressing either the constitutively active Rap1a(G12V) or its downstream effector Radil in the presence or absence of constitutively active Gαi1(Q204L) in neutrophil-like HL60 cells or in HT-1080 fibrosarcoma cells. HL60 cells expressing Rap1a(G12V) or Radil have an elongated phenotype due to enhanced uropod adhesion as they attempt to migrate on fibronectin. Surprisingly, this elongated phenotype by Rap1a(G12V) and Radil is reversed by Gαi1(Q204L), but not by Gαi1 expression, suggesting that Gαi1-GTP regulates adhesion at the level of, or downstream, of Radil. In HT-1080 cells, Rap1a(G12V) and Radil cause an increase in cell spreading and adhesion to fibronectin coated surface which is reversed by Gαi1(Q204L). In contrast, Gαi1(Q204L) did not inhibit RIAM dependent increase in cell adhesion. This data indicate that adhesion regulation by Gαi-GTP occurs downstream of Rap1a and Radil, but is upstream of components such as integrins, talin, and FAK that are regulated by both Radil and RIAM. These data identify a novel role of Gαi-GTP in regulation of cell adhesion. Cell migration involves cycles of adhesion and de-adhesion as the cell moves forward. We propose that cycling of Gβγ and Gαi-GTP activation is important in dynamic regulation of this process.

Structure Based Drug Discovery with stabilised class B GPCRs

John A. Christopher¹, Stephen P. Andrews¹, Asma H. Baig¹, Alastair J. H. Brown¹, Sue H. Brown¹, Kirstie A. Bennett¹, Andrea Bortolato¹, Robert K. Y. Cheng¹, Miles Congreve¹, Robert M. Cooke¹, Andrew S. Doré¹, James C. Errey¹, Cédric Fiez-Vandal¹, Ali Jazayeri¹, James Kean¹, Markus Koglin¹, Daniel Lamb¹, Alistair O’Brien¹, Krzysztof Okrasa¹, Jayesh C. Patel¹, Nathan J. Robertson¹, Maria Serrano-Vega¹, Stacey M. Southall¹, Ben Tehan¹, Iryna Teobald¹, Giselle R. Wiggin¹, Fiona H. Marshall¹

¹Heptares Therapeutics

G protein-coupled receptors (GPCRs) are a valuable family of drug targets but a significant number with compelling pre-clinical or clinical validation remain highly challenging for drug discovery. A key issue is their instability when solubilised away from the membrane, which results in them being
difficult to crystallise and solve their X-ray structures. Heptares uses its proprietary StaR® technology to thermostabilise GPCRs by mutagenesis into a chosen conformational state. The purified proteins can then be used for biophysical screening techniques and crystallisation to yield X-ray structures with multiple ligands. Using the StaR® approach, Heptares has solved structures of multiple Class B GPCRs, leading to the identification of novel, allosteric, binding sites. Details of the allosteric binding sites of the CRF1 and glucagon receptors, insights into their druggability and implications for wider Class B targets will be presented.

(43) Investigate the mu-delta opioid receptor heteromer interface using a multipronged approach

Doungkamol Alongkronrusmee¹, Hamed Tabatabaei Ghomi¹, Shiqi Tang¹, Markus A. Lill¹, Richard M. Van Rijn¹

¹Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette 47907 IN, United States

Mu opioid receptor (MOR) agonists have been a mainstay of pain therapy for thousands of years. However, their prolonged use for chronic pain can produce several side effects. Previous studies have suggested that MORs can physically interact with delta opioid receptors (DORs) at the transmembrane domains five and six (TM5-6) interface to form so-called MOR-DOR heteromers. MOR-DOR heteromers appear to display unique functions relative to MOR monomer/homomers and may contribute to adverse effects of long-term opioid therapy. The disruption of heteromeric complexes may therefore be useful to lessen the side effects of MOR agonists. Yet, there is still a lack of tools that target MOR-DOR heteromers for investigating their physiological relevance. To aid in the development of such tools, it is important to understand the MOR-DOR interaction, particularly determine which amino acids in the heteromer interface are important in the heteromer formation.

Based on the putative heteromer interface elucidated by crystal structures of the MOR and DOR, I constructed nearly thirty mutants spanning TM5-6 of the DOR. Three interface mutants located in the intracellular loop 3 (IL3) which connects TM5 with TM6 were also generated and used as positive controls since they have been established to be crucial for heteromer interface stability. Using a novel heteromer-selective calcium signaling assay we had developed by fusing a chimeric Gqi4 protein to a truncated DOR, I demonstrated that 22 out of 29 mutants, except single mutations at 209, 222, 239 or 288 or double mutations at 241+288 or 242+288 or at IL3, failed to inhibit the MOR-DOR heteromer function. These interface sites were then validated using a bimolecular fluorescence complementation (BiFC) assay for which I fused the MOR and DOR to complementary fragments of a fluorescent protein. The BiFC data verified the results for mutations at TM5-6, except amino acid positions 209, 222, 239 or 288 or at IL3, not to be disruptive.

In order to control false-negative results in screening assays for the heteromer formation, the mutants were N-terminally fused with a FLAG epitope tag of which receptor expression was determined by flow cytometry. I have not observed any TM5-6 mutants that have the membrane expression similar to the wild type significantly disrupt the heteromer interface, except mutations at IL3. The false negatives including single mutations at 209, 222, 239 or 288 or double mutations at 241+288 or 242+288 have the membrane expression decreased to about 60% of the control, suggesting that a lack of heteromer formation of these mutants may be related to their low membrane expression.

43
Using a multipronged approach, the results thus far reveal that TM5-6, but not the IL3, do not involve in the heteromer formation. The crystal structures of the MOR and DOR describing the oligomeric arrangement of TM5-6 are very hypothetical as they are based on artificial crystals, and only of MOR-MOR proteins, with a predicted MOR-DOR interaction. Eventually, these studies will move us closer to our goal to develop protein-protein interaction inhibitors at IL3 that can prevent adverse effects of chronic opioid therapy.

(44) Crack-cocaine inhalation induces behavioral and molecular alterations in dopaminergic and endocannabinoid systems in the prefrontal cortex and striatum of mice.

Lorena B Areal¹², Alice L Herlinger³, Cristina Martins e Silva³, Stephen Ferguson¹, Rita G W Pires³

¹University of Ottawa, ²Federal University of Minas Gerais, Brazil, ³Federal University of Espirito Santo, Brazil

Crack-cocaine addiction represents a public health problem worldwide, especially in developing countries. However, few studies have focused on neurobiological mechanisms underlying the severe addiction produced by this drug, which seems to differ from powder cocaine in many aspects. This study investigated behavioral, biochemical and molecular changes in mice inhaling crack-cocaine, focusing on dopaminergic and endocannabinoid systems in the prefrontal cortex and striatum. Mice from crack group were submitted to 2 inhalation sessions/day during 11 days, while control group was placed in an identical apparatus and condition, without the crack smoke. In the behavioral aspect, open field test performed after each inhalation session revealed that mice from crack group exhibited hyperlocomotion and a peculiar jumping behavior, which increased with repeated drug treatment. Additionally, mice submitted to crack exposure showed impaired working memory and decreased social interaction. GC-MS analysis of the blood collected right after the last inhalation session revealed higher levels of anhydroecgonine methyl ester (AEME), a specific metabolite of cocaine pyrolysis, than cocaine itself. In the prefrontal cortex, crack induced increased gene expression of dopaminergic system components, namely dopamine (DA) receptors D1R, D2R and D3R, and L-DOPA-synthesis enzyme, tyrosine hydroxylase (TH). Despite the D2R transcription activation in response to crack, total D2 protein expression was not altered. However, crack inhalation resulted in increased D2S/D2L protein expression ratio, which is noteworthy a common finding in the prefrontal cortex of schizophrenic subjects. Interestingly, we found that DA, DOPAC and HVA, content were significantly decreased in the prefrontal cortex of these mice. Thus, it is likely that D2S could be reducing DA synthesis, since D2S activation leads to inhibition of TH phosphorylation and, therefore, its activity. In the other hand, gene expression analysis of the striatum revealed a downregulation of D1R and TH. Another important component of the reward circuitry is the endocannabinoid system, which modulates dopamine activity through the activation of CB1 receptors. Therefore, gene expression of components of this system was also assessed and most genes, including CB1R and endocannabinoids degradation enzymes, were downregulated in the prefrontal cortex after crack exposure. These changes may also be involved in the decreased dopamine levels observed in the prefrontal cortex of crack group. Conversely, in the striatum, mRNA levels of anadamide synthesis and degradation enzymes (N-PLD and FAAH, respectively) were increased in mice exposed to crack, reinforcing that the molecular alterations promoted by crack-cocaine are region-specific. Our data reveal behavioral and molecular changes related to dopamine and endocannabinoid neurotransmission promoted by crack-cocaine exposure.
Considering the significant high levels of AEME and that most of the findings have not been described in cocaine hydrochloride models we suggest that the observed alterations are crack-cocaine specific and may also be mediated by AEME.

(45) Investigation of Interspecies Variations in Signaling Bias at the Delta Opioid Receptor

Robert Cassell¹, Kendall Mores¹, Richard van Rijn¹

¹Department of Medicinal Chemistry and Molecular Pharmacology, College of Pharmacy, Purdue University

As a putative target for the treatment of alcoholism and comorbid anxiety and depression, the delta opioid receptor (DOR) has shown great promise in animal models that has thus far failed to translate effectively in to the clinic. A possible explanation for the discrepancy in efficacy observed between human and animal models is the presence of additional potential phosphorylation sites at the C-terminal region of the human DOR relative to rodent receptors. Increased phosphorylation of a G protein-coupled receptor’s intracellular regions promotes recruitment of the signaling protein β-arrestin. It has been shown for opioid receptors that β-arrestin-induced signaling is associated with adverse effects. For example, our laboratory has recently shown a strong positive correlation between the β-arrestin 2 recruitment efficacy of DOR agonists and alcohol self-administration in mice, highlighting the negative consequences associated with opioid drugs that recruit β-arrestin 2. We hypothesize that the human DOR displays a native bias towards the adverse β-arrestin recruitment pathway because of the unique presence of additional putative phosphorylation sites. In order to test our hypothesis, we have begun to characterize a selection of DOR ligands chosen for diversity in signaling bias for binding, cAMP inhibition and β-arrestin recruitment by separately utilizing the mouse, rat and human receptors expressed within cultured cells. We predict that G-protein/β-arrestin bias for these agonists will be shifted towards β-arrestin signaling on human DORs. Such an outcome would suggest that development of DOR drugs should be either performed on human DORs or measures taken to account for the decrease in signaling bias when testing is performed on rodent DORs.

(46) Investigating RGS Inhibitor Mechanism by Hydrogen/Deuterium Exchange

Vincent Shaw¹, Tony Schilmiller¹, Harish Vashisth², Richard Neubig¹

¹Michigan State University, ²University of New Hampshire

Regulator of G-protein Signaling (RGS) proteins play a key regulatory role in G-protein coupled receptor (GPCR) signaling. RGS proteins accelerate the hydrolysis of GTP bound to an active G protein’s alpha subunit, thus terminating signaling.

A series of compounds, the thiadiazolidinones, inhibit RGS proteins by covalent modification of cysteine residues. Thiadiazolidinones are most potent against RGS4, followed by RGS19 and RGS8. Our hypothesis is that the basis for this specificity difference is variation in flexibility between RGS isoforms, causing differential transient exposure of otherwise buried cysteines.

Hydrogen/Deuterium Exchange, or HDX, is a tool for gauging protein dynamics that takes advantage of spontaneous exchange between solvent deuterium and amide hydrogens of the protein
backbone. Highly dynamic or solvent-exposed protein regions will exchange more rapidly. These regions can be identified by digesting the protein with pepsin and quantifying each fragment's mass increase by mass spectrometry.

RGS4 has greater deuterium exchange than RGS8 in helix 4, which contains a cysteine shared by RGS4, RGS8, and RGS19. This indicates greater solvent exposure of helix 4, which may allow better access by thidaizolidinones in RGS4 than in RGS8. RGS19 has higher deuterium exchange than RGS4 or RGS8 across helices 4-7. One would expect this to lead to increased access by thidaizolidinones, but RGS19 is less potently inhibited than RGS4. This may be because RGS19 has fewer cysteines than RGS4 or RGS8. Finally, all three RGS proteins show high deuterium exchange in helix 6 relative to other regions. Pronounced movement of helix 6 may provide a route by which thidaizolidinones can access buried cysteine residues on helices 4 and 7.

(47)  Targeting Rho GTPases in Drug Resistant Melanoma

Sean Misek¹, Kathleen Gallo¹, Richard Neubig¹

¹Michigan State University

Much of the recent focus of melanoma targeted therapy has been on the ERK pathway, which is aberrantly activated in approximately 90% of melanoma tumors (over half of which express BRAF_{V600E}). Current targeted therapies such as vemurafenib (BRAF_{V600E} inhibitor), or a combination therapy using dabrafenib (BRAF_{V600E} inhibitor) and low dose trametinib (MEK inhibitor) shows profound initial effects in a majority of BRAF_{V600E} expressing tumors. However, these responses are often short-lived and resistances typically develops within months. Resistance to these targeted therapies can arise from multiple mechanisms, including activation of pro-survival signaling pathways parallel to the ERK pathway. The goal of this work is to identify pharmacologically targetable resistance mechanisms so that effective combination therapies can be developed.

Activating mutations in multiple GPCRs (such as CYSLTR2) have been recently discovered in human melanoma tumors. Furthermore, activating mutations in GNAQ/GNA11 have been identified in over 80% of uveal melanoma tumors, and act as primary oncogenes in these tumors by activating small Rho GTPases (such as RhoA). Despite the clear role of the RhoA subfamily of Rho GTPases (RhoA/B/C) as melanoma oncogenes, their role in drug resistance is not well understood. It is challenging to develop small molecule inhibitors which directly target the activity of small Rho GTPases, so an alternative approach is to inhibit downstream pathways. Through modulation of the actin cytoskeleton Rho can induce gene transcription through multiple transcriptional co-activators including Myocardin-Related Transcription Factor (MRTF) and Yes-Associated Protein 1 (YAP).

My bioinformatics analysis demonstrates that MRTF-A gene expression is correlated with poor overall survival in a large cohort of cutaneous melanoma patients. Furthermore, expression of a set of 216 MRTF target genes is enriched in dabrafenib/trametinib resistant cutaneous melanoma tumors compared to matched pre-treatment tumors, suggesting that MRTF activation may be involved in drug resistance. Based upon these results I hypothesized that small Rho GTPases may promote resistance to MAPK pathway targeted therapies through activation of MRTF/YAP.

To test this hypothesis I generated vemurafenib resistant melanoma cells through chronic exposure to vemurafenib. This vemurafenib-resistant cell population is enriched for actin stress fiber
positive cells, and these cells have increased Myosin Light Chain 2 (MLC2) phosphorylation, suggesting that there is increased Rho activation. Furthermore, these drug resistant cells are more sensitive to pharmacological inhibition of MRTF activity. These preliminary data suggest that vemurafenib resistant melanoma cells may be re-wired to depend on the Rho-induced gene transcription for their survival, and that a combination therapy simultaneously targeting these two pathways may be an effective treatment strategy for BRAF inhibitor-resistant melanomas.

(48) Conformational complexity of class C G-protein-coupled receptors
Reza Vafabakhsh¹, Chris Habrian², Josh Levitz², Ehud Isacoff²
¹Northwestern University, ²University of California, Berkeley

G protein-coupled receptors (GPCRs) constitute the largest family of membrane receptors in eukaryotes and due to their broad involvement in physiological processes they have become the largest family of drug targets in biology. Metabotropic glutamate receptors (mGluRs) are dimeric class C GPCRs that are involved in the regulation of neurotransmitter release and modulation of neuronal excitability and serve as drug targets for neurological disorders. Although recent crystal structures have provided insight into their over architecture, these structures only provide a snapshot into protein's conformational profile and lack kinetic information. I developed a novel approach which allowed me, for the first time, to visualize the activation process of group-C G protein coupled receptors (GPCR) at the single molecule level (Nature, 2015). I found that 1) the ligand binding domains in metabotropic glutamate receptors interconvert between three conformations: resting, activated and a short-lived intermediate state and I quantified the kinetics of transitions between these states; 2) I showed that orthosteric agonists induce transitions between these conformational states with efficacy determined by occupancy of the active conformation; 3) In spite of 70% sequence identity with mGluR2, mGluR3 displays basal dynamics, which are Ca2+ dependent and lead to basal protein activation; 4) Heterodimeric receptors show novel conformational properties. My results revealed the hidden complexity of the activation process of mGluRs.

49 - Structural and functional studies of latrophilin-family adhesion G-protein coupled receptors
Yue Lu¹, Olha Nazarko¹, Richard Sando², Gabriel Salzman¹, Amanuel Kibrom¹, Katherine Leon¹, Thomas Südhof², Demet Araç¹
¹University of Chicago, ²Stanford University

Adhesion G-protein coupled receptors (aGPCRs) contain uniquely large, autoproteolyzed extracellular regions that are involved in cell adhesion and may couple cellular adhesion to receptor signaling. The latrophilin (Lphn) family of aGPCRs has important roles in brain development, such as mediating interactions with fibronectin leucine-rich repeat transmembrane proteins (FLRTs) in order to form synaptic junctions. There is growing interest in Lphns as promising drug targets because mutations in Lphns are associated with neurological disorders and cancers. To aid in this effort, we used structural and functional approaches to characterize Lphn ligand-binding and signaling. We present the crystal structure of an extracellular fragment of Lphn3 in complex with FLRT3 and a model of the Lphn3/FLRT3/Uncoordinated-5 trimeric complex, which provide insight into the role of cell-
adhesion proteins in synapse function. In addition, we developed a signaling assay to potentially identify residues in the transmembrane domain of Lphn1 that are important for receptor activity.

(50) Proteinase-activated receptor 4 mediates cell shape changes by a Gq/11, Gi independent, RhoA dependent mechanism
Christie Vanderboor\textsuperscript{1}, Pierre Thibeault\textsuperscript{2}, Rithwik Ramachandran\textsuperscript{2}

\textsuperscript{1}Western University, \textsuperscript{2}University of Western Ontario

Proteinase-activated receptor 4 (PAR4) is one of four members of the PAR family of GPCRs (PAR1-4). These receptors are activated upon enzymatic cleavage to reveal a tethered ligand. PAR4 is cleaved by trypsin and thrombin and is a significant stimulus for platelet aggregation. We observed cell shape changes and membrane blebbing upon activation of PAR4. We demonstrate by pharmacological inhibition that these cell shape changes are G\textsubscript{q,11} and G\textsubscript{i} independent. Use a of CRISPR/Cas9 generated RhoA knock out cell line, shows that PAR4 mediated cell shape change is RhoA dependent. Furthermore, we demonstrate that PAR4 is expressed in rat vascular smooth muscle cells (VSMC) and that PAR4 mediated cell shape changes occur in VSMC in a ROCK dependent manner.

(51) Regulation of the Orexin Receptor 1 by MRAP2
Alix A. Rouault\textsuperscript{1}, Abigail A. Lee\textsuperscript{1}, Julien Sebag\textsuperscript{1}

\textsuperscript{1}University of Iowa

The Melanocortin Receptor Accessory Protein 2 (MRAP2) is a GPCR accessory protein that has proven to be a major regulator of energy homeostasis through the regulation of trafficking and / or signaling of several GPCRs that control food intake and energy expenditure. The loss of MRAP2 causes severe obesity in both rodents and humans. Whereas MRAP proteins were originally thought to exclusively interact with melanocortin receptors, including MC2R and MC4R, the importance of MRAP2 actions on the Prokineticin Receptor 1 has recently been demonstrated, thus establishing that MRAPs can regulate non-melanocortin GPCRs. In pursuit of understanding the reaches of MRAP2, this study assessed the role of MRAP2 in the regulation of the Orexin receptor 1(OX1R). OX1R is a Gaq coupled receptor predominately located in the brain, including the hypothalamus. While the role of the orexin system is more widely accepted as a regulator of arousal and wakefulness, it has also been associated with feeding and appetite. Here we show that MRAP2 is a potent inhibitor of the OX1R. Indeed, we found that MRAP2 significantly decreases the surface density of OX1R leading to a decrease in ligand binding and receptor signaling. This study has identified a role for MRAP2 in the modulation of yet another non-melanocortin receptor thus further arguing that MRAP2 may regulate numerous GPCRs involved in the control of energy homeostasis.

(52) Requirement of MRAP2 for ghrelin-mediated hunger sensing
Dollada Srisai\textsuperscript{1}, Julien Sebag\textsuperscript{1}

\textsuperscript{1}University of Iowa

Ghrelin is the only known circulating orexigenic hormone. It is primarily secreted by the stomach and acts at its receptor, GHSR1a, in the hypothalamus to signal hunger and promote food intake. For
this reasons, inhibition of ghrelin signaling is a promising strategy for the treatment of obesity. In this study we identify an obligatory partner of GHSR1a and a potential new target for the treatment of obesity. Indeed, we show that the melanocortin receptor accessory protein 2 (MRAP2) interacts with GHSR1a and is essential for ghrelin-mediated activation of GHSR1a both in-vitro and in-vivo. We demonstrate that, in the absence of MRAP2, ghrelin fails to activate AGRP neurons in the hypothalamus and does not promote food intake. Our results suggest that targeting the MRAP2/GHSR1a complex represents a new strategy for the inhibition of central ghrelin actions and for the development of new treatments for obesity.

(53) HDAC6 inhibitors show a cellular antidepressant signature, translocating activated Gαs from lipid-rafts

Harinder Singh¹, Jeffrey Schappi², Mark Rasenick³

¹University of Illinois at Chicago (UIC), ²UIC Physiology, ³UIC

Presently available antidepressant therapies for treating major depressive disorder (MDD) meet with variable therapeutic success. Histone deacetylase-6 (HDAC-6) enzymes involved in deacetylation of α-tubulin are overexpressed in mood disorders. HDAC6 knockout mice mimic traditional antidepressant treatments. Nonetheless, a possible role for HDAC6 inhibitors in the treatment of depression remains elusive. Previously we have shown that sustained treatment of rats or glioma cells with several classes of antidepressants translocates Gαs from lipid rafts toward increased association with adenyl cyclase in a non-raft plasma membrane domain. Concomitant with this is a sustained increase in cAMP production. While Gαs interacts directly with tubulin to modify microtubule dynamics, tubulin also acts as an anchor for Gαs in lipid rafts. Since HDAC-6 inhibitors potentiate α-tubulin acetylation, we hypothesize that acetylation of α-tubulin disrupts tubulin-Gαs anchoring, rendering Gαs free to activate AC. To test this, C6 Glioma (C6) cells were treated with HDAC-6 inhibitor, tubastatin-A. The acetylation status of α-tubulin and localization of Gαs subunit in/out of lipid-raft membrane domains were studied. Chronic treatment with tubastatin-A not only increased acetylation of α-tubulin but also moved Gαs out of lipid-rafts, without changing total Gαs. However, traditional antidepressants, escitalopram (SSRI) and imipramine (TCA), as shown in our previous studies only showed Gαs translocation out of rafts and no changes in acetylation status of α-tubulin. Fluorescence Recovery After Photobleaching (FRAP) on C6 cells stably expressing GFP-Gαs, was conducted and cells pretreated with tubastatin-A showed an "antidepressant signature" similar to that of with escitalopram. Finally, two indicators of downstream cAMP signaling were examined. cAMP response element binding protein phosphorylation (CREB) and expression of brain derived neurotrophic factor (BDNF) were both elevated by tubastatin-A, similar to that seen following treatment with SSRI and TCA. These findings suggest HDAC6 inhibitors show a cellular profile resembling antidepressants. Therefore, compounds that decrease tubulin-Gαs complexes by increasing acetylation of α-tubulin may show promise for antidepressant action.

(54) A tail of GPR56 - quest for adhesion GPCR desensitization

Lei Xu¹, Adil Khan¹

¹University of Rochester

Adhesion G-protein coupled receptors are the second most abundant class of G-protein coupled receptors (GPCRs) and are believed to have functions in both cell signaling and cell adhesion. Conventional GPCRs are phosphorylated at their C-terminal tail and internalized via arrestins in order to be desensitized to prevent constitutive signaling through them following activation by a ligand. It is not known whether desensitization of adhesion GPCRs is regulated by a similar mechanism. We use a
model adhesion GPCR, GPR56 (ADGRG1) to determine whether adhesion GPCRs undergo desensitization in a similar fashion. Our preliminary analysis suggests that potential phosphorylation residues in the C-terminal tail of GPR56 may be important for the desensitization process and that this process could be mediated via arrestins.

The cytoplasmic tail of GPR56 contains a series of serine residues which could be the phosphorylation sites to mediate desensitization of GPR56. To investigate this possibility, we deleted ten amino acids at the 3' end of GPR56 (GPR56 3'Δ), containing six serine residues, and tracked its internalization by introducing a fluorogen activated peptide (FAP) tag in the receptor. An otherwise non-fluorescent fluorogen is added to the cells expressing FAP-GPR56, which binds to FAP and starts to emit fluorescence. We used this technology to observe the internalization of FAP-GPR56 and FAP-GPR56 3'Δ under a microscope. We found that FAP-GPR563'Δ mutant primarily stayed on the cell surface and did not internalized as efficiently as its intact counterpart suggesting that the C-terminal residues might be important for receptor internalization. We then investigated the involvement of arrestins in this process. We knocked down β-Arrrestin 1 in melanoma cells expressing FAP-GPR56 and tracked its internalization. We found that arrestin knockdown hindered the internalization of GPR56 suggesting that arrestin plays a role in desensitization of GPR56 and that this could occur via phosphorylation of GPR56 C-terminal residues.

Since GPR56 3'Δ mutant did not internalize efficiently and stayed on cell surface for longer time, we predicted that it will signal more in comparison to GPR56. To confirm this we used serum response element (SRE) reporter assay. GPR56 is known to activate G-protein α12/13 that initiates a signaling cascade which eventually leads to activation of SRE. A luciferase gene was fused downstream of SRE such that when transfected in cells, enhanced activation of SRE will result in increased luciferase expression. We co-transfected the SRE-Luciferase construct with GPR56 and GPR563'Δ separately in HEK293T cells and assayed for luciferase activity. Our data showed that there is a 2-fold increase in SRE activation through GPR56 3'Δ over GPR56 further suggesting that the C-terminal residues mediate GPR56 desensitization.

(55) Endogenous nuclear β-adrenergic receptors oppose signalling coming from the cell surface

Nicolas Audet¹, Naëla Jamnemode², Terry Hébert¹

¹McGill University, ²Université Joseph Fourier

Until recently, G protein-coupled receptors (GPCRs) were believed to be functional only on the cell surface. However, a number of GPCRs have been demonstrated to be targeted to the nuclear membrane, including some β-adrenergic receptor (βAR) subtypes. We have shown that βARs are targeted to the nuclear membrane of rat adult cardiomyocytes where they are functional with respect to cellular signalling. Since several clinically used βAR ligands can easily cross the cell membrane, monitoring the signalling properties of the different populations of βARs is crucial to understand the mechanisms of these drugs and improve their efficacies. However, the use of primary adult cardiomyocytes or isolated nuclei makes it difficult to study the molecular pharmacology of nuclear βAR in a manner that combines ease of screening and a relevant cell model. Thus, we developed cellular models of the AC16 cardiomyocyte cell line expressing nuclear FRET EPAC cAMP, nuclear and cytoplasmic FRET EKAR MAPK ERK1/2 biosensors. We hypothesized that nuclear signalling of βAR is
distinct from that of cell membrane βARs. Using high content FRET microscopy and automated image analysis, we show that isoproterenol (100 nM) increases nuclear cAMP levels and decreases nuclear MAPK ERK1/2 activity with all the sensors. To assess nuclear βAR signalling, we blocked cell surface βARs with the non-permeable antagonist CGP12177A (100 nM). The antagonist blocked isoproterenol-mediated signalling in the cytoplasm. However, when cell surface receptors were blocked, isoproterenol decreased cAMP levels in the nucleus and this decrease could not be blocked by pertussis toxin (100 ng/ml; 16h). Moreover, this decrease in cAMP level was correlated with an increase in MAPK ERK1/2 activity in the nucleus. The increase observed when we blocked cell surface receptors remains unchanged. These results suggest that isoproterenol targets a nuclear population of βARs that signals directly in the nucleus to balance signalling derived from the cell membrane. We hope these experiments will help understand the nature and scope of nuclear signalling controlled by these distinct pools of receptors and, ultimately, to translate these results into development of improved treatment for heart disease.

(56)  Asymmetrical β-Arrestin-Dependent Signalling by the AT1R/FP Dimer
Dany Fillion¹, Terry Hébert¹
¹McGill University

Initially identified as functional monomers, G protein-coupled receptors (GPCRs) can also form dimers that can be viewed as distinct signalling hubs for cellular signal integration. We previously found that the angiotensin II (AngII) type 1 receptor (AT₁R), and the prostaglandin F2α (PGF₂α) receptor (FP), both important in the control of smooth muscle contractility, form a functional heterodimeric complex in both HEK293 and vascular smooth muscle cells. Moreover, we found that the signalling output of a given receptor within this dimer was allosterically regulated by ligand occupancy of the other receptor partner. For example, symmetrical regulation (i.e., only one of the two receptors modulated the other) was observed for receptor-mediated Gq signalling while asymmetrical regulation (i.e., both receptors modulated each other) was observed for MAPK activation. In addition to the canonical G protein coupling pathways, GPCRs recruit and engage β-arrestin-dependent pathways as well. We noted that AngII induced a rapid and robust recruitment of βarr1&2 to AT₁R and, to a lesser extent, the dimer, as expected since AT₁R is a strong recruiter of βarr1&2. However, PGF₂α did not induce such recruitment to FP alone, but did when the AT₁R was present in a dimer. Surprisingly, βarr1&2 were found to be recruited to the AT₁R partner of the dimer, as it was the case for the AngII-induced recruitment. Thus, we hypothesized that both AngII- and PGF₂α-induced activation of the AT₁R/FP dimer, or their monomers taken separately, signal by stabilizing distinct conformations of βarr2. Using multiple BRET-based sensors, we aim to (1) assess the recruitment profile kinetics of both βarr1&2 to the dimer when stimulated by either AngII or PGF₂α; (2) tease out the role of G proteins in such recruitment; (3) and establishing the conformational signature of βarr2 in response to either ligand. FP-mediated recruitment of βarr1&2 to the dimer is asymmetric with respect to AT₁R and results in a unique βarr2 interaction profile. G₃q, G₁₁₁, G₁₂ and G₁₃ was found to be critical for PGF₂α-induced βarr1&2 recruitment to the dimer since their combined absence totally abrogated the response, and their separate expression was sufficient to partially restore it. Despite being recruited to the same AT₁R molecule, βarr2 adopted also two distinct conformations, as reported by the sensors. AngII promoted a similar but greater conformational change in βarr2 compared to PGF₂α in general, although some FP-specific responses
were noted. Taken together, our data shed light on a new mechanism whereby PGF\textsubscript{2α} specifically recruits and signal through βarr1&2 only in the context of AT\textsubscript{1}R/FP heterodimer, suggesting that this may be a new allosteric signalling entity that can be considered as a novel therapeutic avenue.

(57) Rho/MRTF-pathway Inhibition Sensitizes NRAS Mutant Melanomas to Trametinib

Kathryn Appleton\textsuperscript{1}, Thomas S. Dexheimer\textsuperscript{1}, Richard R. Neubig\textsuperscript{1}

\textsuperscript{1}Michigan State University

Melanoma is the most deadly form of skin cancer, and its annual incidence is increasing due to sun exposure and other risk factors. The primary focus of melanoma targeted therapy has been the Ras/MEK/ERK pathway, which is aberrantly activated in approximately 80% of human cutaneous melanomas (~55% BRAF\textsuperscript{V600} mutations and 25% NRAS mutations). NRAS mutant melanomas are currently treated with chemotherapy, emerging immunotherapies and MEK inhibitors. MEK inhibitors are given as monotherapies, but are limited by toxicity and often result in the emergence of mechanisms leading to resistance. Evidence implicates the role of Rho-activated gene transcription in melanoma metastasis mediated by the nuclear localization of the transcriptional coactivator, myocardin-related transcription factor (MRTF). Here, we highlight a role for Rho and MRTF signaling and its reversal by pharmacologic inhibition in the potentiation of trametinib (MEK inhibitor) in NRAS mutant melanomas. Combination of trametinib with the Rho/MRTF-pathway inhibitor, CCG-22740, cooperatively causes reduced cell viability in NRAS mutant melanomas in vitro. Sensitivity to this treatment combination strongly correlates with expression of the MRTF target gene CYR61. MRTF nuclear localization is also observed in the sensitive NRAS mutant melanoma cell lines tested. Furthermore, combination of the Rho/MRTF-pathway inhibitor with trametinib reduces clonogenicity in the aggressive NRAS mutant melanoma cell line SK-Mel-147. These findings highlight the potential of concurrently targeting the Rho/MRTF pathway and the ERK pathway in NRAS mutant melanomas.

(58) Photocyclic behavior of rhodopsin induced by a novel isomerization mechanism

Sahil Gulati\textsuperscript{1,2}, Beata Jastrzebska\textsuperscript{1}, Kota Katayama\textsuperscript{1}, Philip D. Kiser\textsuperscript{1,3}, Phoebe L. Stewart\textsuperscript{1,2}, Krzysztof Palczewski\textsuperscript{1,2}

\textsuperscript{1}Department of Pharmacology, School of Medicine, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, USA, \textsuperscript{2}Cleveland Center for Membrane and Structural Biology, Case Western Reserve University, 1819E 101st Street, Cleveland, OH 44106, USA, \textsuperscript{3}Research Service, Louis Stokes Cleveland VA Medical Center, Cleveland, OH 44106, USA

Vertebrate rhodopsin (Rh) contains 11-cis-retinal as a chromophore to convert light energy into visual signals. On absorption of light, 11-cis-retinal is isomerized to all-trans-retinal, constituting a one way reaction that activates transducin (Gt) followed by chromophore release. Here we report that bovine Rh, regenerated instead with a 6-carbon ring retinal chromophore featuring a C11=C12 double bond locked in its cis conformation (Rh6mr), employs a novel retinal isomerization mechanism by converting 11-cis to 11,13-dicis configuration for prolonged Gt activation. Time-dependent UV-Vis spectroscopy, HPLC and molecular mechanics (MM) analyses revealed a thermal re-isomerization of the 11,13-dicis to the 11-cis configuration on a slow time scale, which enables Rh6mr to function in a
photocyclic manner similar to that of microbial Rh(s). With this photocyclic behavior, Rh6mr repeatedly recruits and activates Gt in response to light stimuli. The susceptibility of Rh6mr to hydroxylamine in the dark strongly indicates a relatively open Rh6mr cytoplasmic side as compared to Rh. Based on these comprehensive structure-function studies, we unveil a unique mechanism of Rh activation by 11-cis to 11,13-dicis isomerization.

(59) Proteinase Activated Receptor 4 (PAR4) Internalization- Role for C-terminal tail motifs and Beta-arrestin recruitment

Pierre Thibeault¹, Christie Vanderboor², Rithwik Ramachandran¹

¹University of Western Ontario, ²Western University

Proteinase-activated receptor 4 (PAR4) is one of four GPCRs in the PAR receptor family (PAR1-PAR4). These receptors are unique in being activated by proteolytic cleavage of the N-terminus by enzymes such as thrombin, trypsin and Cathepsin-G. As a result, PARs are irreversibly activated. We hypothesize that this irreversible activation engages internalization and trafficking pathways that are distinct from canonical GPCR trafficking pathways.

In our survey of PAR4-effector protein interactions we have identified interactions with the multifunctional scaffold proteins beta-arrestin 1 and 2. In order to understand the molecular basis of PAR4 interaction with beta-arrestins we made a number of receptor mutations. We find that alanine substitution of the c-terminal YXXL motif (PAR4-Y322-L326A) decreases beta-arrestin recruitment to PAR4. Further we identify a C-terminal 8 amino acid motif (RAGLFQRS) that completely abolishes beta-arrestin recruitment to PAR4 and does not internalize.

In order to confirm a role for beta-arrestins in PAR4 internalization, we generated a beta-arrestin1/2 knockout cell line on the HEK293 background using CRISPR/Cas9 targeting. We find that in the absence of beta-arrestin1/2 activated PAR4 does not internalize.

We conclude that PAR4 recruits beta-arrestin through receptor C-terminal tail interactions and beta-arrestin interaction is important for PAR4 internalization through a clathrin and dynamin dependent pathway.

(60) Orthosteric and Allosteric Activation Mechanisms of the Serotonin 5-HT2B receptor

John McCorvy¹, Sheng Wang¹, Daniel Wacker¹, Bemnat Agegnehu¹, Anat Levit², Brian K. Shoichet², Bryan L. Roth¹,³

¹Department of Pharmacology, ²National Institute of Mental Health Psychoactive Drug Screening Program, University of North Carolina, Chapel Hill NC, ³Department of Pharmaceutical Chemistry, University of California, San Francisco, CA, ³Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC

To understand the molecular basis of ligand recognition and activation of the 5-HT₂B receptor via the orthosteric site, we solved the 5-HT₂B structure in complex with the β-arrestin biased agonist methyl ergonovine. Methyl ergonovine is the active metabolite of methysergide, an antimigraine medication that exhibits 5-HT₂B antagonism. Based on the methyl ergonovine 5-HT₂B crystal structure,
we hypothesized that methysergide’s N(1)-methyl exerts antagonism due to steric clash with residue Ala5.46 located in TM5 in the orthosteric site. To alleviate this steric clash, we mutated Ala5.46 to glycine to make space for methysergide’s N(1)-methyl in the orthosteric site, which recovered methysergide’s agonist efficacy and selectively converted methysergide into a β-arrestin biased agonist similar to methyl ergonovine. To confirm methysergide’s binding pose, we crystallized methysergide in the 5-HT2B A5.46G mutant receptor confirming that methysergide’s N(1)-methyl occupies the space at residue 5.46 to confer activation. To explore the influence of the extended binding region on biased signaling, we solved the 5-HT2B structure in complex with lysergic acid diethylamide (LSD) and lisuride, a 5-HT2B β-arrestin biased agonist and antagonist, respectively. Comparison of these two structures indicates that LSD’s diethylamide contacts Leu7.35 in TM7, whereas lisuride’s diethyl moiety remains near TM3 and away from TM7. Mutation of Leu7.35 to phenylalanine selectively recovered lisuride’s agonist efficacy, but completely abolished β-arrestin recruitment leading to a Gq-biased 5-HT2B receptor. Targeting of the extended binding pocket of the 5-HT2B receptor by in silico docking campaigns has led to arrestin-biased positive allosteric modulators.

(61) Role of G alpha s in ketamine’s antidepressant action

Nathan Wray1, Jeffrey Schappi2, Mark Rasenick3

1University of Illinois at Chicago, 2UIC Physiology, 3UIC

Previous studies have demonstrated that all extant classes of antidepressants increase coupling between the G protein, Gαs and adenylyl cyclase, resulting in persistent cAMP elevation. This effect requires sustained drug treatment and is observed after 3 days in cultured neural or glial cells or 3 weeks in rats. This is apparently due to Gαs being released from constraints of a lipid raft environment to cholesterol-poor regions of the plasma membrane. Consistent with this, both peripheral tissue and postmortem brain from depressed human subjects show a greater proportion of Gαs in lipid rafts. While most antidepressants require several weeks for a clinical effect, ketamine appears to alleviate depression within hours, only to relapse after several days. In an effort to determine whether ketamine showed an antidepressant biosignature similar to other antidepressants, studies in cultured cells were initiated. It was hypothesized that ketamine would have an effect similar to antidepressants but along a shorter time course. C6 cells were treated with 1uM ketamine, harvested and lipid raft fractions prepared and the amount of Gαs was assessed by Western blot. C6 cells with a stable fluorescent Gαs fusion protein (GFP-Gαs) was treated similarly with ketamine and the mobility of GFP-Gαs was determined by Fluorescence Recovery after Photobleaching (FRAP). Brief ketamine treatment evokes a biochemical hallmark (translocation of Gαs) seen after prolonged treatment with several species of drugs with established antidepressant activity. This is not mimicked by other NMDA antagonists, suggesting an additional site for ketamine action. The ketamine induced Gαs translocation allows increased functional coupling of Gαs and adenylyl cyclase to increase intracellular cyclic adenosine monophosphate (cAMP). Furthermore, increased intracellular cAMP mediates phosphorylation of cAMP response element-binding protein (CREB), which increases BDNF levels. BDNF production was indeed dependent on cAMP as the cAMP antagonist Rp-cAMPS attenuated BDNF expression levels. Furthermore, BDNF expression was also seen in primary astrocytes after ketamine treatment and attenuated by Rp-cAMPS. These results reveal a novel antidepressant mechanism mediated by acute ketamine treatment in glial cells that may contribute to ketamine’s antidepressant effect. Furthermore, the translocation of GFP-Gαs produced by ketamine and all tested compounds with antidepressant
activity (but not mood-stabilizers, antipsychotics or anxiolytics) might serve as a useful platform for identifying compounds with potential antidepressant activity and for predicting clinical response.

(62) Characterizing biased signalling and trafficking of three non-synonymous single nucleotide polymorphisms found in the angiotensin II type 1 receptor

Sahil Kumar¹,², Yoon Namkung¹, Stephane Laporte¹,³,⁴

¹Glen Site, Research Institute - McGill University Health Centre, Montréal, Québec, ²Division of Experimental Medicine, Faculty of Medicine, McGill University, Montréal, Québec, ³Department of Medicine, Faculty of Medicine, McGill University, Montréal, Québec, ⁴Department of Pharmacology & Therapeutics, Faculty of Medicine, McGill University, Montréal, Québec

Hypertension and its risks can be partially ascribed to the endogenous hormone angiotensin II (Ang II), which is critical to regulate blood volume and vascular resistance through the angiotensin II type 1 receptor’s (AT₁R). Twelve naturally-occurring variations in the AT₁R coding sequence, also known as single nucleotide polymorphisms (SNPs), have been reported in the NCBI short genetic variations database to date. The findings of the A1166C polymorphism of the AT₁R published in 1994 by Bonnardeaux et al. generated a tremendous amount of interest in investigating the possible link of SNPs with disease. In this line of evidence, it undetermined whether a SNP can direct downstream cellular responses linked to disease by way of creating a specific, stabilized conformation of the receptor. For the AT₁ receptor, non-synonymous single nucleotide polymorphisms in the coding region of the protein could disrupt these inter- and intramolecular interactions required to stabilize the same active conformation as the wild-type receptor. We hypothesize that by affecting the receptor’s ability to engage certain G proteins or β-arrestins, this can direct the signalling of the receptor through a specific therapeutic pathway, while mitigating the role of undesired responses, much like how biased ligands are being developed for this purpose.

Herein, we characterized three of the twelve SNPs of the human angiotensin II type 1 receptor. To assess how signalling effectors engaged with the receptors, we used a panel of bioluminescence resonance energy transfer (BRET)-based biosensors to determine the relative activity of the receptor to engage its cognate G proteins (Gα₁₂, Gα₁₃, Gα₁₄, Gα₁₅); β-arrestin-2 recruitment and trafficking; and receptor internalization and recycling upon Ang II stimulation.

Results show that one particular SNP led to a significantly marked biased decrease in Gα₁₂ coupling upon ligand stimulation, whereas it retained robust activity to the other G proteins. Furthermore, upon agonist stimulation, this SNP is able to recruit β-arrestin-2 to the plasma membrane, however, fails to remain associated with β-arrestin-2 in early endosomes. Although, this polymorphic receptor was still shown to internalize into early endosomes via immunofluorescence staining. With regards to recycling, this polymorphic receptor was shown to have a greater association with Rab4 and Rab11 when compared to the stimulated wild-type AT₁R over time, and recovery to the plasma membrane was accelerated for this SNP-containing receptor.

We show this interplay between G proteins and β-arrestins can now be modulated by naturally-occurring mutations of the receptor and this mosaicism of behaviours can potentially impact responses. Understanding the links between the signalling signatures and the physiological or pathophysiological
responses may help identify and developing new biased ligands – and perhaps better targeted therapeutics.

(63) PDZ Protein Regulation of β-arrestin Recruitment and Receptor Trafficking
Sarah Gupta\textsuperscript{1,2}, Henry Dunn\textsuperscript{2}, Stephen Ferguson\textsuperscript{2}
\textsuperscript{1}Western University, \textsuperscript{2}University of Ottawa

β-arrestins are versatile adaptor proteins that play a vital role in regulation of G protein coupled receptor (GPCR) trafficking and signalling properties. PDZ proteins have previously been shown to modulate β-arrestin2 recruitment and receptor internalization of many GPCRs. This includes Corticotropin-Releasing Factor Receptor 1 (CRFR1), a receptor expressed widely in the brain whose antagonists have been shown to demonstrate both anxiolytic- and antidepressant-like effects. Previous studies have demonstrated that PDZ protein PSD-95 antagonizes CRFR1 internalization by preventing β-arrestin interactions required for endocytosis of the receptor. Therefore, further characterizing the interplay between β-arrestins and PDZ proteins may aid in determining a potential mechanism for PDZ protein regulation of GPCR trafficking. Through co-immunoprecipitation experiments done in HEK293 cells, our findings suggest that PDZ proteins PSD-95, MAGI1, and PDZK1 complex with β-arrestin2 by interacting via the PDZ domains. Using a proteomic approach, several potential residues within β-arrestin2 were determined that may mediate these interactions. Mutational analyses reveal that the β-arrestin2 A175F mutant causes a decrease in interaction with PSD-95. Additionally, this mutant form of β-arrestin2 shows decreased CRF-stimulated recruitment to CRFR1 over time and a reduced maximal response for CRF-stimulated β-arrestin translocation. Overall, the results of these studies suggest that PDZ proteins may interact with β-Arrestin2 to play a critical role in regulation of β-arrestin2 recruitment and trafficking properties for GPCRs such as CRFR1. A further understanding of how PDZ proteins regulate GPCRs could contribute to the design and development of new pharmacological treatments and prevention strategies for a multitude of human mental illnesses.

(64) Novel Chemical Probes for the Delineation of GPCR Functional States by Fluorine NMR
Alexander Orazietti\textsuperscript{1}, Advait Hasabnis\textsuperscript{2}, Leizl Polinario\textsuperscript{3}, Jerome Gould\textsuperscript{3}, Scott Prosser\textsuperscript{1,2,3}
\textsuperscript{1}University of Toronto; Dept. of Biochemistry, \textsuperscript{2}University of Toronto; Dept. of Chemistry, \textsuperscript{3}University of Toronto at Mississauga; Dept of Chemistry and Physical Sciences

The mechanistic process of GPCR activation has been heavily informed by the use of X-ray crystallography, pulsed EPR spectroscopy, and computational modelling. These techniques have provided great insight into the relevant structural motifs and conformational changes which occur upon ligand binding but struggle to characterise their dynamics or to describe the full ensemble of functional states. NMR, and specifically \textsuperscript{19}F NMR, excels in the elucidation of conformational ensembles and the quantitative description of protein functional dynamics. It is especially useful in the delineation of protein functional states and is sensitive to changes in these states under myriad conditions including drug binding or allosteric modulators. Fluorine nuclei are scarce in biological systems and are also
exquisitely sensitive to changes in environment due to Van der Waals and electrostatic interactions exhibiting a much wider range of chemical shifts, relative to proton nuclei. Thiol-conjugated $^{19}$F NMR has been useful in bridging structural GPCR information from crystallographic techniques with dynamic state information to identify conformational ensembles in apo and other ligand bound conditions. This project describes the identification of key chemical moieties exhibiting greater sensitivity to changes in microenvironment and specifically solvent exposure. Current generation thiol-specific probes are sensitive to changes in microenvironment but are limited in their use at higher magnetic field strengths due to greater broadening of spectral linewidths and significant peak overlap. To assess the suitability for $^{19}$F NMR experiments on GPCRs, novel fluorinated chemical probes have been tested against current generation fluorinated probes across a range of solvent environmental conditions simulating changes in solvent exposure due to protein conformational change. The novel probes display a significantly greater sensitivity, via improved chemical shift difference, to changes in microenvironment. Greater sensitivity is observed specifically with respect to changes in solvent polarity and pH. Thiol-specificity is demonstrated for the novel fluorinated chemical probes via conjugation with glutathione. Further development of these novel thiol-specific fluorinated probes represents a significant advancement of $^{19}$F NMR as a spectroscopic technique for biophysical protein characterization and specifically necessary for its widespread application in the field of GPCR structural characterization.

(65) Loss of RGS control at Gao: effect on nociceptin receptor-mediated behavior

**Nick Senese¹, John Traynor¹**

¹University of Michigan

The nociceptin receptor (NOPr) is a typical Gi/o coupled G-protein coupled receptor (GPCR) with expression throughout the central nervous system. Activation of central NOPr has two well-known behavioral effects in rodents: an anxiolytic action and a reduction in nociceptive threshold. New strategies that can dissociate these effects (for example providing an anxiolytic effect without affecting nociceptive threshold) could therefore prove useful. Here we utilize a mouse model with a modified Gao knock-in protein that is not affected by the family of proteins known as the Regulators of G-protein Signaling (RGS) proteins. RGS proteins have GTPase accelerating activity that promotes inactivation of the GTP-bound Gα subunit. Therefore any GPCR which normally signals through Gao, including NOPr, should have increased downstream signaling in these mice. Using this mouse model we tested the hypothesis that selectively promoting signaling downstream of Gao will affect some NOPr related behaviors but not others. We employed two behavioral paradigms: the von Frey test to assess changes in nociceptive threshold, and the elevated plus maze (EPM) to examine anxiolytic effects. The RGSi Gao knock-in mice showed a baseline hyperalgesic phenotype that was reversed by administration of the NOPr antagonist J-113397, consistent with increased NOPr activity. In contrast, these animals did not show a difference from wild type controls when tested on the EPM. Compared to their wild-type littermates the RGSi Gao knock-in mice had no changes in the overall expression of NOPr, or the potency of nociceptin (NOPr’s endogenous ligand) to activate the receptor.

(66) Characterization of Novel Small Molecule Ligands for the Chemokine Receptor CXCR4
Brittany Hopkins$^{1,3}$, Rama K Mishra$^{2,3}$, Gary Schiltz$^{2,3}$, Richard J Miller$^{1,3}$

$^1$Department of Pharmacology, $^2$Center for Molecular Innovation and Drug Discovery, $^3$Northwestern University

Chemokines are traditionally known for their roles in leukocyte migration, however, a growing body of evidence has demonstrated that chemokines and their cognate G-protein coupled receptors (GPCRs) are implicated in a variety of processes. Of particular interest to us is the chemokine, stromal-derived factor (SDF-1) or CXCL12. The signaling between SDF-1 and its GPCR, CXCR4, have been implicated in embryonic development, cancer metastasis, neuropathic pain, and WHIM syndrome; CXCR4 is also known to be a co-receptor for HIV-1 entry into immune cells. Given the importance of the SDF-1/CXCR4 signaling axis in these diverse biological processes, we formed a collaboration with Northwestern's Center for Molecular Innovation and Drug Discovery to generate a series of small molecule ligands for the CXCR4 receptor. In order to pharmacologically characterize these potential novel ligands we have utilized a variety of techniques including: monitoring Ca$^{2+}$ mobilization via Fura-2AM imaging, competition based radioligand binding assays, and assessments of beta-arrestin signaling. These approaches led to the successful characterization a novel class of small molecule CXCR4 agonists. Structure activity studies of these molecules are currently under way. Characterizing these novel small molecule ligands for CXCR4 will provide us with powerful pharmacological probes to better understand the physiological roles of the SDF-1/CXCR4 signaling axis.

(67) The Use of CXCR4 Biased Antagonists Avoids the Development of Drug Tolerance

Ben Hitchinson$^1$, Jonathan Eby$^2$, Xianlong Gao$^2$, Joshua J. Ziarek$^3$, Francois Guite-Vinet$^4$, Nikolaus Heveker$^4$, Matthias Majetschak$^2$, Brian F. Volkman$^3$, Nadya I. Tarasova$^5$, Vadim Gaponenko$^1$

$^1$University of Illinois at Chicago, $^2$Loyola University Chicago Stritch School of Medicine, $^3$Medical College of Wisconsin, $^4$University of Montréal, $^5$National Cancer Institute

Close to 50% of all pharmaceutical agents target G protein-coupled receptors (GPCRs). In many cases, patients develop tolerance to these drugs and the problem of drug tolerance is not fully addressed by the pharmaceutical industry. We therefore asked whether it is possible to avoid tolerance to receptor inhibition using functional selectivity. To investigate, we used the GPCR CXCR4 and its ligand CXCL12 as an example. This chemokine-receptor pair regulates cell migration and stem cell homing to the bone marrow, but also promotes cancer metastasis. Therapeutic targeting of CXCR4 relies on a single unbiased antagonist, AMD3100 that inhibits all receptor signalling. However, tolerance to AMD3100 develops rapidly because receptors accumulate on the surface of the cell due to inhibition of β-arrestin induced endocytosis/desensitisation. Chemokines can then bind to the overexpressed receptors and induce a response even in the presence of the drug. To explore the use of a functionally selective drug in avoiding tolerance, we first demonstrate that a peptide derived from the second transmembrane helix of CXCR4 acts as a biased antagonist. The peptide, X4-2-6, potently and selectively inhibits the function of G-proteins through an allosteric mechanism but permits β-arrestin recruitment and CXCR4 endocytosis. Secondly, X4-2-6 but not AMD3100 prevents receptor accumulation on the cell surface. The peptide retains its ability to inhibit chemotaxis after prolonged exposure, while the unbiased antagonist does not. We then use a small molecule biased antagonist to
demonstrate the same effect. Thus, we have identified biased antagonists of CXCR4 that overcomes the problem of drug tolerance associated with AMD3100. This demonstrates the feasibility of using functionally selective drugs as anti-GPCR therapies to circumvent tolerance.

(68) Using CRISPR/Cas9 technology to examine the role of KCTD5 in Gbeta/gamma signalling

Jennifer Sung¹, Dominic Devost¹, Rhiannon Campden¹, Terry Hébert¹

¹McGill University

Gbeta/gamma subunits are an integral component of G protein-coupled receptor (GPCR) signalling, but many of the processes they regulated remain poorly understood. Recent studies have demonstrated Gbeta/gamma subunits function in distinct subcellular organelles such as the nucleus. One area with therapeutic potential in modulating Gbeta/gamma signalling in disease is the control of Gbeta/gamma subunit degradation. Due to their established roles in cell adhesion and migration, Gbeta/gamma subunits are of interest in cancer suppression studies. For example, preclinical data has indicated that inhibition of Gbeta/gamma signalling in murine models of cancer slows tumour growth. Following a proteomic screen which identified Potassium Channel Tetramerization Domain Containing 5 (KCTD5) as a Gbeta/gamma interacting protein, preliminary experiments suggest that it is involved in targeting Gbeta/gamma for degradation. We hypothesize that KCTD5 reduces Gbeta/gamma signalling capacity by targeting it to the proteasome and our objective is to investigate KCTD5 effects in altering Gbeta/gamma signalling. Toward that end, we generated a HEK 293 KCTD5 knockout cell line using CRISPR/Cas9 technology, and our specific aims include analysing how the loss of KCTD5 affects cell viability and migration as a prelude to identification of potential new cancer therapeutic targets.

(69) Parmodulins as biased ligands of Protease-Activated Receptor 1 (PAR1)

Disha Gandhi¹, Karen De Ceunynck², Christian G. Peters², Ricardo Rosas¹, Robert Flaumenhaft², Chris Dockendorff¹

¹Marquette University, Dept. of Chemistry, ²Harvard Medical School

Recent advances in the understanding of G-protein coupled receptor (GPCR) pharmacology have demonstrated that certain ligands can selectively engage different GPCR-mediated signaling pathways. This phenomenon, called biased signaling, has been demonstrated with protease-activated receptors (PARs), a unique subset of GPCRs with profound effects on diverse phenomena such as platelet activation, inflammation, and cancer cell metastasis. We previously discovered a class of small molecules, called parmodulins, that act to selectively inhibit platelet granule secretion via PAR1-activated Gq. Presented here is an update of our efforts to 1) characterize the promising cytoprotective effects of these compounds; 2) understand their mode of action; and 3) identify analogs with improved properties using medicinal chemistry.

(70) New BRET biosensor to monitor β2-Adrenergic Receptor conformational changes from the inside of the cell

Louis-Philippe Picard¹, Schönegge A. M.¹, Lohse M. J., Bouvier M.¹

¹Université de Montréal
The classical model of GPCR activation is based on an equilibrium between two state of the receptor, the active and inactive conformation. However, different studies have been shown that different ligands can have different signaling profiles through the same receptor. This introduces the concept of bias signaling or functional selectivity. In this model, the receptor has multiple conformation leading to different signaling potential. To understand the structural changes leading to the activation of specific effector, we have developed an BRET based conformational biosensor of the β2-adrenergic receptor. Using the NLuc as donor between residues 251 and 252 of the third intracellular loop and the YFP as acceptor at the c-termini part of a truncated form of the receptor at residue 369, we successfully monitor conformational changes in the receptor using multiple ligands that we could correlate with signaling pathways using other BRET based biosensors for the production of cAMP, the activation of Gs and the recruitment of β-arrestin in multiplex in HEK293T cells.

(71) Functional characterization of natural variants of melatonin type 1 receptor

Alan Hegron1,2,3,4,5, Bianca Plouffe5, Amélie Bonnefond6, Christian Le Gouill5, Philippe Froguel6, Ralf Jockers1,2,3, Michel Bouvier4,5

1Inserm, U1016, Institut Cochin, Paris, France, 2CNRS UMR 8104, Paris, France, 3Univ. Paris Descartes, Sorbonne Paris Cité, Paris, France, 4Department of Biochemistry and Molecular Medicine, University of Montreal, 5Institute for Research in Immunology and Cancer, University of Montréal, 6CNRS UMR8199, Lille, France

Melatonin is a circulating neurohormone mainly released from the pineal gland in a seasonal and circadian manner regulating many physiological functions, such as glycemia homeostasis. Indeed, melatonin type 1 receptor (MT1R) is expressed in the islets of Langerhans and has been reported to modulate insulin and glucagon secretion from β-cells and α-cells, respectively. Alternatively, MT1R expression in pancreas of Type 2 diabetes (T2D) mouse model is different than normoglycemic mice. Although, the signalling mechanisms underlying relationship between melatonin and insulin are complex and controversial. Recently, a genetic variant (rs2119882) found on MT1R promoter has been associated to higher risks to develop T2D. Consequently, possible variants on the MT1R coding region modifying receptor signalling could also modulate incidence to develop T2D.

To answer this question, we recently performed a genome-wide association studies with both normoglycemic individuals and T2D patients and 13 synonymous polymorphisms of MT1R were identified. Our aim is to functionally characterize these mutant receptors to determine if some mutations contribute to T2D development and to pinpoint the impaired signalling pathways associated with higher risks to develop this disease. Using enzyme-linked immunosorbent assay (ELISA), we first confirmed that all the 13 mutants are expressed at the cell surface. Then, we used a BRET-based assay to screen all the panel of G proteins activated by wild-type and mutant MT1R. Our results show that while MT1R activates many G proteins, G protein activation is globally impaired for some mutants while biased G protein activation is revealed for other mutants. Further investigations will be performed by monitoring downstream events, but altogether, these G protein signatures already allow identification of key residues involved in MT1R signal transduction process but also residues having a selective role in a given subset of G proteins only. The complete signalling signatures will also help us to understand in a better way the relationship between melatonin, insulin and glucagon, but also to possibly identify new genetic markers for higher risks to develop T2D.
Lesson from 40 SNPs found in melatonin type 2 receptor

Bianca Plouffe, Angeliki Karamitri, Amélie Bonnnefond, Jonathan M. Gallion, Mathilde Boisset, Mickaël Canouil, Christian Le Gouill, Philippe Froguel, Olivier Lichtarge, Ralf Jockers, Michel Bouvier

1Institute for Research in Immunology and Cancer, Université de Montreal, Montreal, Canada, 2Institut Cochin INSERM U1016 CNRS UMR 8104, Université Paris Descartes, Paris, France, 3Université de Lille, CNRS UMR 8199 - EGID, Institut Pasteur de Lille, Lille, France, 4Baylor College of Medicine, Molecular and Human Genetics, Houston, Texas, USA, 5Department of Genomics of Common Disease, Imperial College of London, London, UK

Type 2 Diabetes (T2D) is a multifactorial disease characterized by insulin resistance. Environmental, behavioural, but also genetic factors contribute to T2D development. Among genetic factors, melatonin type 2 receptor (MT2R) raised our attention as MT2R is expressed in human pancreatic b cells and reported to inhibit insulin secretion. A recent genome-wide association studies revealed 40 synonymous polymorphisms in this receptor. While some mutations are found exclusively in patients suffering from T2D, others are found in both T2D and normal subjects or in normal subjects only. To verify if defective MT2R signaling can increase risks to develop T2D, we generated a «signaling signature» of these 40 mutant receptors. Our results show that all the MT2R mutants exhibit normal cell surface expression and only 4 show loss of melatonin binding. As MT2R is reported to be coupled to Gai/o proteins, we used a bioluminescence resonance energy transfer (BRET)-based assay to identify the Gai/o isoforms activated by this receptor. Our results pointed out that MT2R activates Gai1 and Gaz isoforms, so we monitored both basal and melatonin-mediated Gαi1 and Gαz activation by the MT2R mutants. We also monitored basal and melatonin-mediated β-arrestin recruitment, melatonin-induced cAMP reduction and ERK phosphorylation. By plotting all these data on radial graphs, we generated signaling signature of each mutant receptor. Using non-negative matrix factorization (NMF), we grouped together mutations having similar signaling profile and obtained 5 different types (clusters) of signatures. The 1st cluster is composed of mutations behaving like wild-type MT2R. The 2nd and 3rd clusters contain mutations with biased signaling, (loss of agonist-mediated β-arrestin recruitment or predominant loss of agonist-mediated Gai1 over Gaz). Mutations within the 4th and the 5th clusters exhibit severe or moderate global impaired signaling. Interestingly, there is a clear difference in distribution of mutations leading to biased signaling and mutations impairing the general signal transduction process. Our results also suggest that at least for MT2R, ability of Gai1 to be activated is more sensitive to receptor mutations than Gaz and highlight the crucial role of the 3rd intracellular loop (ICL3) of MT2R in β-arrestin recruitment. Statistical analysis of our data suggests that impairment of G protein and β-arrestin pathways, but not ERK activation increase the risks to develop T2D. As our signalling signatures generated a considerable amount of functional data, we used them to challenge the Evolutionary Action (EA), a computational approach to predict the magnitude of impact a mutation will have on function. As our results show a linear correlation between EA scores and our functional data, this demonstrates EA robustness to predict mutation outcomes. Altogether, our signaling signatures allowed us to identify residues in ICL3 involved in MT2R-mediated β-arrestin recruitment and residues differently regulating Gai1 and Gaz activation by MT2R, to identify new genetic markers increasing risks to develop T2D and to demonstrate the robustness of EA to predict mutation outcomes.
Altered metabolism in RGS2 KO mice

Katherine Lee¹, Robert Gros¹, Peter Chidiac¹

¹University of Western Ontario

RGS2 is known to inhibit Gq- and Gs-mediated GPCR signalling. We have previously reported that RGS2 knock out mice (rgs2-/-) exhibit a lean phenotype compared to their wildtype (WT) counterparts, with significantly reduced fat deposits, leptin levels, and serum lipids. The objective of this study is to further elucidate the role of RGS2 in metabolism. CLAMS metabolic cages were used to measure various indices in 1 month-, 3 month-, and 12 month-old rgs2-/- and WT male Bl/6 mice. Animals were acclimatised in metabolic cages for 24 hours (12-hour light and dark cycles), followed by another 24 hours of metabolic recordings.

At both 3 months and 12 months, rgs2-/- mice displayed increased rates of respiratory intake (vO₂) and expiratory output (vCO₂), particularly during the dark cycle, suggesting increased metabolic activity in the knockouts. Furthermore, calculated respiratory exchange ratios (RER) suggest an increase in carbohydrate rather than fat or protein metabolism in rgs2-/- mice of all three age groups compared to WT. Hyperphagia, as well as increased water intake, were also observed in the knockouts at 3 and 12 months. Even with significantly increased food and water intake, 3 and 12 month-old rgs2-/- mice maintained lower body weights compared to WT mice, and displayed comparable ambulatory activity.

In addition, a 16-hour fast, followed by metabolic cage measurements, was performed in 3-month old male mice to determine possible alterations in metabolism following changes in food intake. Fasting typically results in energy conservation, which can be demonstrated via decreased vO₂ and vCO₂ levels. Compared to WT mice, we observed a significantly delayed temporal response in the decrease of vO₂ and vCO₂ activity in rgs2-/- mice. Furthermore, heat levels (kcal/hr) followed a similar pattern, suggesting a clear, internal metabolic phenotype. Similar to our ad libitum results, there was no discernable difference between genotypes for ambulatory activity, and rgs2-/- mice remained hyperphagic following the reintroduction of food post-fast. Furthermore, microarray analysis of skeletal tissue identified significant differentially expressed genes between WT and KO mice that may be involved in the observed metabolic phenotype.

The lack of changes in overall activity between genotypes in both ad libitum and fasted mice indicate that weight loss in rgs2-/- mice is due to internal energy metabolism rather than overt physical activity. These results suggest that RGS2 may play an important role in the regulation of body metabolism.

Regulation of β2-adrenergic receptor post-endocytic trafficking

Elena Shuvaeva¹, Shanna Bowman¹, Rachel Vistein¹, Manojkumar Puthenveedu¹

¹Carnegie Mellon University

The post-endocytic recycling of the β2-adrenergic receptor (β2AR), a prototypical GPCR, is subject to regulation through phosphorylation of serines in its C-terminus by protein kinase A. This
phosphorylation restricts the receptor to specialized actin-associated endosomal tubules, slowing the resensitization of the cell to signal. This has consequences for both surface and endosomal β2AR signaling. We are interested in identifying the proteins blocking β2AR from constitutive recycling through non-specialized endosomal tubules. Stable isotope labeling with amino acids in cell culture (SILAC) followed by mass spectrometry were carried out, identifying β2AR binding partners, including the six subunits that make up the mammalian CORVET (class C core vacuole/endosome tethering) complex. While the CORVET complex is well-studied in S. cerevisiae, where it is involved in endosomal fusion in early endosomes, little is known of its function in mammalian cells. We want to determine what role the CORVET complex plays in regulation of β2AR recycling. Initial fixed cell two-color immunofluorescence assays showed that knockdown of VPS8 (a specific subunit of the CORVET complex) increases β2AR levels at the cell surface after washout and antagonist addition, suggesting that the CORVET complex is decreasing recycling of the receptor. However, co-immunoprecipitation has indicated that VPS8 interacts more strongly with C-terminal serine mutant β2AR, which cannot be phosphorylated by PKA and is not restricted to recycling through the actin-associated endosomal tubules. This result suggests a different mechanism for the CORVET complex’s role in β2AR regulation.

Additionally, we are interested in the role of early endosomal protein endofin in β2AR regulation; fixed cell two-color IF assays have shown that knockdown of this protein decreases receptor recycling, suggesting a potential role in recruiting the receptor to the actin-associated tubules.

(75) **Sequence specific control of endocytosis by GPCRs regulates receptor signaling.**

Zara Weinberg¹, AS Zajac¹, DJ Shiwarshi¹, T Phan¹, MA Puthenveedu¹

¹Carnegie Mellon University

G-Protein Coupled Receptors are now readily accepted to signal via a variety of intracellular pathways mediated primarily by heterotrimeric g-proteins and the effector arrestin. The role that receptor trafficking plays in controlling receptor signaling has only recently been appreciated. The present work extends previous findings from our lab that the B2-adrenergic receptor (B2AR) and the mu-opioid receptor (MOR) can control the rate at which they are endocytosed after ligand binding. With the mu-opioid receptor, we show here that its endocytic rate is directly correlated to magnitude of arrestin signaling, and that manually increasing endocytic dwell time with the use of dynamin inhibitors is sufficient to increase signaling. Furthermore, we use B2AR to explore the molecular mechanism through which this endocytic delay may be mediated. Overall, the present work shows that modulation of endocytic rate by C-terminal sequences of specific GPCRs is a mechanism through which their signaling magnitude can be controlled.

(76) **Allosteric Regulation of the Prostaglandin F2α (PGF2α) Receptor Signaling: a Promising New Avenue for Preterm Labor Prevention**

Ljiljana Nikolajev¹, Carine Bourguet⁴, William D. Lubell⁴, Stéphane A. Laporte¹,²,³

¹Department of Pharmacology and Therapeutics, McGill University, ²Department of Medicine, McGill University, ³Department of Anatomy and Cell Biology, McGill University, ⁴Department of Chemistry, Université de Montréal
G protein-coupled receptors (GPCR) represent the largest and most diverse family of cell-surface receptors; as such they represent important targets for drug design. The prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$) receptor (FP) is a GPCR that plays a crucial role in parturition as it is a mediator of uterine cell contraction. PGF$_{2\alpha}$ stimulation of FP is responsible for Ca$^{2+}$ mobilization and MAPK activation through coupling to Ga$_q$, but it also mediates the activation of the small GTPase Rho by coupling to Ga$_{12/13}$. Altogether, these signaling cascades lead the cellular contractility. In collaboration with other labs, we developed a new modulator of FP, PDC113.824 (PDC) which was shown to prevent myometrial cells contraction by selectively inhibiting PGF$_{2\alpha}$-mediated Rho/Rock signaling cascade via Ga$_{12}$, while enhancing ERK1/2 and PKC signaling via Ga$_{aq}$. As such, this new molecule has great pharmacological potential for pre-term labor prevention as well as for primary dysmenorrhea treatment, a condition for which PDC’s parental compound, THG, successfully completed phase II in clinical trial.

We first aim is to elucidate more thoroughly the pathways and effects activated by FP-mediated signaling. Then we ought to use these new knowledge to better understand how PDC and its other derivatives are modulating FP response in order to prevent contractions.

Using a panel of biosensors and using bioluminescence-resonance energy transfer (BRET) to look at the ability of the receptor to engage with G proteins (Ga$_q$, Ga$_{12}$, Ga$_{13}$); and to activate downstream effectors such as PKC or Rho. Results show preferential coupling of the receptor with Ga$_{aq}$. Interestingly, we demonstrate that by using an inhibitor of Ga$_{aq}$ (UBO-QIC), coupling of both Ga$_{12}$ and Ga$_{13}$ to the receptor are significantly increased. Testing our allosteric modulator onto these biological events, we confirm that PDC is a potentiator of PKC activation. The molecule however does not seem to affect G protein coupling following PGF$_{2\alpha}$ stimulation.

Ultimately, the study’s aim is to better understand how the FP is signaling and how the allosteric molecules that we developed interact with and re-direct the signalling of our receptor. Understanding such mechanism should help in the development of a new drug that could be used to treat women undergoing preterm labor, in which FP participates.

(77) Engineering an Orthogonal Chemokine-GPCR Interface

**Michael Wedemeyer**$^1$, Andrew Kleist$^1$, Monica Thomas$^1$, Francis Peterson$^1$, Brian Volkman$^1$

$^1$Medical College of Wisconsin

Chemotactic cytokines (Chemokines) are small, soluble proteins that elicit cell migration responses during development, immune surveillance, inflammation, and cancer. Chemokines bind to G protein-coupled receptors to provoke downstream signaling responses, including chemotaxis along an established chemokine gradient towards the source of chemokine release.

Recent structural information has led to the development of the two-site two-step model for chemokine:GPCR interaction. This model states that aspects of the chemokine:GPCR interface such as binding specificity, receptor activation, and differential signaling are mainly driven by specific areas of association deemed site 1 and site 2. The goal of this project is to engineer a chemokine:GPCR pair that are binding partners solely with each other by modularizing the interaction and designing novel receptors that are exclusively activated by novel chemokines.
Development of a Protein-protein Interaction Inhibition Assay Using Biacore™ 8K
Anna Moberg¹, Johan Öhman¹, Tomoya Mitani², Satoru Nagatoishi³

¹GE Healthcare Bio-Sciences AB, SE-75184 Uppsala, Sweden, ²GE Healthcare Life Sciences, Tokyo, Japan, ³School of Engineering, The University of Tokyo, Tokyo 108-9639, Japan

Small molecule inhibition of protein-protein interaction (PPI) is a hot research area in drug discovery and the number of interesting drug targets are increasing by the day. However, PPI's comprise a lot of challenges, such as identifying how a specific protein-protein interaction should be inhibited. The interaction area is usually large and potential inhibitors tend to be larger and more lipophilic than the average fragment. Thus, there are often problems with specificity and interactions are generally weak. Consequently, PPI research depend largely on technically advanced instrumentation and intuitive and dedicated software to allow for efficient screening and confident selection of candidates.

Here we present a study to identify suitable candidates for inhibition of the interaction between Intrinsic Disordered Protein (IDP) X and protein Y. Early interaction data showed that the preparation of IDP X was impure. Purification was performed using size-exclusion chromatography on ÄKTA™ pure chromatography system. Biacore 8K was used to efficiently identify the active fraction and to optimize assay conditions, followed by the setup and run of a PPI screening experiment in less than one day.

Probing GPCR conformation in cell by chemical crosslinking and mass spectrometry
Bill Huang¹, Ji-Won Lee¹, Hee-Yong Kim¹

¹National Institutes of Health

The orphan GPR110 (ADGRF1) belongs to the adhesion GPCR class with distinctively long N-terminus. We have recently demonstrated that GPR110 is a functional receptor for synaptamide, an endogenous metabolite of docosahexaenoic acid (DHA) essential for proper brain development. The binding of synaptamide to GPR110 mediates neurogenesis, neuritogenesis and synaptogenesis in developing neurons in a cAMP dependent manner. In an effort to understand the molecular basis of the activation of GPR110, we probed GPR110 conformation in living cells using chemical crosslinking and mass spectrometry. HEK cells overexpressing HA-GPR110 were incubated with disuccinimidyl suberate (DSS, a lysine-specific crosslinker). The DSS-modified HA-GPR110 were pulled-down and subjected to SDS-PAGE, tryptic digestion, and nanoLC-ESI-MS/MS. The MS-based approach identified 17 crosslinked lysine pairs in the N-terminal domain of GPR110. Among them, K29-K38, K187-K240, K240-K254, K398-442, K398-K438, K398-K427, K427-K442, K427-K438, and K438-K442, resulted from through-space crosslinking between two peptide segments, while K31-K32, K38-39, K70-73, K151-K157, K235-K240, K438-K442, K427-K432, and K432-438, were cross-linked within a single peptide segment (loop-links). In addition, a through-space crosslinking between the intracellular loop III (IL3) and the C-terminus (K783-K852), and 4 loop-links (K864-K873, K852-K860, K860-864, and K875-K878) in the C-terminal region were identified. The data indicated that the alpha carbon distance between each crosslinked lysine pair is within ~24 Å, the maximum crosslinking length of DSS. Our results represent the first experimental data for the three-dimensional structure of GPR110 in living cells. This strategy should be useful in probing the conformational change of this receptor at different activation stages.
<table>
<thead>
<tr>
<th>Name</th>
<th>Email</th>
<th>Affiliation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Khaled</td>
<td><a href="mailto:kshabel@ucalgary.ca">kshabel@ucalgary.ca</a></td>
<td>University of Ottawa Brain and Mind Institute</td>
</tr>
<tr>
<td>Lauren</td>
<td><a href="mailto:laibee@luc.edu">laibee@luc.edu</a></td>
<td>Loyola University</td>
</tr>
<tr>
<td>Paul</td>
<td><a href="mailto:palbert@uottawa.ca">palbert@uottawa.ca</a></td>
<td>University of Ottawa</td>
</tr>
<tr>
<td>Mudassir</td>
<td><a href="mailto:mail@mcw.edu">mail@mcw.edu</a></td>
<td>Medical College of Wisconsin</td>
</tr>
<tr>
<td>Doungkamol</td>
<td><a href="mailto:dalongkr@purdue.edu">dalongkr@purdue.edu</a></td>
<td>Purdue University</td>
</tr>
<tr>
<td>Kathryn</td>
<td><a href="mailto:appel10@msu.edu">appel10@msu.edu</a></td>
<td>Michigan State University</td>
</tr>
<tr>
<td>Demet</td>
<td><a href="mailto:arac@uchicago.edu">arac@uchicago.edu</a></td>
<td>University of Chicago</td>
</tr>
<tr>
<td>Elisa</td>
<td><a href="mailto:elisa.artherof@nih.gov">elisa.artherof@nih.gov</a></td>
<td>NIH/Karolinska Institute</td>
</tr>
<tr>
<td>Nicolas</td>
<td><a href="mailto:nicolas.audet2@mail.mcgill.ca">nicolas.audet2@mail.mcgill.ca</a></td>
<td>McGill University</td>
</tr>
<tr>
<td>Lorena B.</td>
<td><a href="mailto:lorenabianchinae@gmail.com">lorenabianchinae@gmail.com</a></td>
<td>University of Ottawa</td>
</tr>
<tr>
<td>M. Madan</td>
<td><a href="mailto:madanm@mrc-imb.cam.ac.uk">madanm@mrc-imb.cam.ac.uk</a></td>
<td>University of Cambridge</td>
</tr>
<tr>
<td>Sumit</td>
<td><a href="mailto:sbandeka@umich.edu">sbandeka@umich.edu</a></td>
<td>University of Michigan</td>
</tr>
<tr>
<td>Avik</td>
<td><a href="mailto:abanerje@uic.edu">abanerje@uic.edu</a></td>
<td>University of Illinois</td>
</tr>
<tr>
<td>Martin</td>
<td><a href="mailto:martin.beaulieu@crulrg.ulaval.ca">martin.beaulieu@crulrg.ulaval.ca</a></td>
<td>Université Laval</td>
</tr>
<tr>
<td>Paul</td>
<td><a href="mailto:brian.j.bender@vandebilt.edu">brian.j.bender@vandebilt.edu</a></td>
<td>Vanderbilt University</td>
</tr>
<tr>
<td>Jeffrey</td>
<td><a href="mailto:jeffrey.benovic@jefferson.edu">jeffrey.benovic@jefferson.edu</a></td>
<td>Thomas Jefferson University</td>
</tr>
<tr>
<td>Kyla</td>
<td><a href="mailto:kyla.bourque@mail.mcgill.ca">kyla.bourque@mail.mcgill.ca</a></td>
<td>McGill University</td>
</tr>
<tr>
<td>Richard</td>
<td><a href="mailto:rich.breyer@vandebilt.edu">rich.breyer@vandebilt.edu</a></td>
<td>Vanderbilt University Medical Center</td>
</tr>
<tr>
<td>Michael</td>
<td><a href="mailto:bruchas@morpeus.wustl.edu">bruchas@morpeus.wustl.edu</a></td>
<td>Washington University</td>
</tr>
<tr>
<td>Laurent</td>
<td><a href="mailto:laurent.burenau.cotesse@uSherbrooke.ca">laurent.burenau.cotesse@uSherbrooke.ca</a></td>
<td>Université de Sherbrooke</td>
</tr>
<tr>
<td>Jimmie</td>
<td><a href="mailto:jbuhrmaster@luc.edu">jbuhrmaster@luc.edu</a></td>
<td>Medical College of Wisconsin</td>
</tr>
<tr>
<td>Emily</td>
<td><a href="mailto:campbey@mail.uc.edu">campbey@mail.uc.edu</a></td>
<td>University of Cincinnati</td>
</tr>
<tr>
<td>Richard</td>
<td><a href="mailto:rcarr@trevena.com">rcarr@trevena.com</a></td>
<td>Trevena, Inc.</td>
</tr>
<tr>
<td>Robert</td>
<td><a href="mailto:rcassell@purdue.edu">rcassell@purdue.edu</a></td>
<td>Purdue University</td>
</tr>
<tr>
<td>You-Hong</td>
<td><a href="mailto:yocheng@luc.edu">yocheng@luc.edu</a></td>
<td>Loyola University</td>
</tr>
<tr>
<td>Vadim</td>
<td><a href="mailto:cherezov@usc.edu">cherezov@usc.edu</a></td>
<td>University of Southern California</td>
</tr>
<tr>
<td>Peter</td>
<td><a href="mailto:Peter.Chidiac@schulich.uwo.ca">Peter.Chidiac@schulich.uwo.ca</a></td>
<td>University of Western Ontario</td>
</tr>
<tr>
<td>Tiffany</td>
<td><a href="mailto:tiffany.ding27@gmail.com">tiffany.ding27@gmail.com</a></td>
<td>Northwestern University</td>
</tr>
<tr>
<td>Chris</td>
<td><a href="mailto:christopher.dockendorff@marquette.edu">christopher.dockendorff@marquette.edu</a></td>
<td>Marquette University</td>
</tr>
<tr>
<td>Nickolai</td>
<td><a href="mailto:ndulin@medicine.bsd.uchicago.edu">ndulin@medicine.bsd.uchicago.edu</a></td>
<td>University of Chicago</td>
</tr>
<tr>
<td>Mark</td>
<td><a href="mailto:Mark_Dumont@urmc.rochester.edu">Mark_Dumont@urmc.rochester.edu</a></td>
<td>University of Rochester</td>
</tr>
<tr>
<td>Henry</td>
<td><a href="mailto:henry.dunn@live.com">henry.dunn@live.com</a></td>
<td>University of Ottawa</td>
</tr>
<tr>
<td>Jonathan</td>
<td><a href="mailto:jebyl@luc.edu">jebyl@luc.edu</a></td>
<td>Loyola University</td>
</tr>
<tr>
<td>Elizabeth</td>
<td><a href="mailto:eenglishl@mcw.edu">eenglishl@mcw.edu</a></td>
<td>Medical College of Wisconsin</td>
</tr>
<tr>
<td>Gohar</td>
<td>gohar.fakhouri@<a href="mailto:1@ulaval.ca">1@ulaval.ca</a></td>
<td>Université Laval</td>
</tr>
<tr>
<td>Huijie</td>
<td><a href="mailto:cuimiraclefeng@gmail.com">cuimiraclefeng@gmail.com</a></td>
<td>Michigan State University</td>
</tr>
<tr>
<td>Stephen</td>
<td><a href="mailto:sferguso@uottawa.ca">sferguso@uottawa.ca</a></td>
<td>University of Ottawa</td>
</tr>
<tr>
<td>Robert</td>
<td><a href="mailto:robert.fernandezanugo@yale.edu">robert.fernandezanugo@yale.edu</a></td>
<td>Yale University</td>
</tr>
<tr>
<td>Celia</td>
<td><a href="mailto:cferandez@uchicago.edu">cferandez@uchicago.edu</a></td>
<td>University of Chicago</td>
</tr>
<tr>
<td>Sergei</td>
<td><a href="mailto:sferre@ina.nida.nih.gov">sferre@ina.nida.nih.gov</a></td>
<td>National Institute on Drug Abuse</td>
</tr>
<tr>
<td>Cedric</td>
<td><a href="mailto:cedric.fiez-vandal@heptaeas.com">cedric.fiez-vandal@heptaeas.com</a></td>
<td>Heptaeas Therapeutics</td>
</tr>
<tr>
<td>Dany</td>
<td><a href="mailto:dany.fillion@mcmgill.ca">dany.fillion@mcmgill.ca</a></td>
<td>McGill University</td>
</tr>
<tr>
<td>Madeleine</td>
<td><a href="mailto:madeleine.fletcher@monshaeas.com">madeleine.fletcher@monshaeas.com</a></td>
<td>Monash University</td>
</tr>
<tr>
<td>John</td>
<td><a href="mailto:jfoley@aas.org">jfoley@aas.org</a></td>
<td>Science Signaling</td>
</tr>
<tr>
<td>Disha</td>
<td><a href="mailto:disha.gandi@marquette.edu">disha.gandi@marquette.edu</a></td>
<td>Marquette University</td>
</tr>
<tr>
<td>Xianlong</td>
<td><a href="mailto:xigao@luc.edu">xigao@luc.edu</a></td>
<td>Loyola University</td>
</tr>
<tr>
<td>Vadim</td>
<td><a href="mailto:vadimg@uiuc.edu">vadimg@uiuc.edu</a></td>
<td>University of Illinois at Chicago</td>
</tr>
<tr>
<td>Annette</td>
<td><a href="mailto:agilchrist@midwestern.edu">agilchrist@midwestern.edu</a></td>
<td>Midwestern University</td>
</tr>
<tr>
<td>Jenna</td>
<td><a href="mailto:jenna.giubilaro@mcgill.ca">jenna.giubilaro@mcgill.ca</a></td>
<td>McGill University</td>
</tr>
<tr>
<td>Claudiu</td>
<td><a href="mailto:claudio@utoronto.ca">claudio@utoronto.ca</a></td>
<td>University of Toronto</td>
</tr>
<tr>
<td>Megan</td>
<td><a href="mailto:meg120@case.edu">meg120@case.edu</a></td>
<td>Case Western Reserve University</td>
</tr>
<tr>
<td>Eric</td>
<td><a href="mailto:eric.greve@marquette.edu">eric.greve@marquette.edu</a></td>
<td>Marquette University</td>
</tr>
<tr>
<td>Milica</td>
<td><a href="mailto:milicag@uic.edu">milicag@uic.edu</a></td>
<td>University of Illinois at Chicago</td>
</tr>
<tr>
<td>Sahil</td>
<td><a href="mailto:sxg556@case.edu">sxg556@case.edu</a></td>
<td>Case Western Reserve University</td>
</tr>
<tr>
<td>Sarah</td>
<td><a href="mailto:sgupt2@uwo.ca">sgupt2@uwo.ca</a></td>
<td>Western University</td>
</tr>
<tr>
<td>Sandra</td>
<td><a href="mailto:Sandra.Hackelberg@northwestern.edu">Sandra.Hackelberg@northwestern.edu</a></td>
<td>Northwestern University</td>
</tr>
<tr>
<td>Alison</td>
<td><a href="mailto:ahamilt6@uottawa.ca">ahamilt6@uottawa.ca</a></td>
<td>University of Ottawa</td>
</tr>
</tbody>
</table>

University of Ottawa
Northwestern University
Western University
University of Chicago
University of Illinois at Chicago
Purdue University
Michigan State University
University of Michigan
University of Illinois