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Pierre André Lafon (IGF, France)
Cécile Derieux (Leipzig University, Germany)
Robert Quast (CBS, France)

**Sponsors of the meeting:**

# Program of the meeting

## *Day 1 – September 30th*

18h00 ***Keynote Lecture*** **– Brian Kobilka** (Stanford Univ. School of Medicine, CA, USA)
"Challenges in drug discovery for G protein coupled receptors"

19h00 **Poster Session I & Dinner**

## *Day 2 - October 1st*

09h00 **Welcome from the iGPCRnet**

**Session I – GPCR signalling and spatial organisation** (Chairs: Julie Sanchez (UK) & Xavier Iturrioz (FR))

09h10 "Probing the functional selectivity of GPCR signaling: Potential for drug discovery"
Michel Bouvier (Montreal Univ., Canada)

09h35 "The viral-encoded US28 chemokine receptor contributing to glioblastoma constitutively activates Gq from endosomes: does signalling location matter?"
Bianca Plouffe (UK)

09h45 PDEs define a cAMP signaling nanoarchitecture in intact cells
Andreas Bock (Germany)

09h55 Gi/o and Gq/11 proteins cooperate in asymmetric GPCR dimers to activate ERK signaling
Erika Cecon (France)

10h05 "Molecular origins and principles governing adrenaline efficacy and potency in the human β2-adrenergic receptor"
Franziska Heydenreich (UK)

10h15 *Q&A*

10h30 *Coffee Break*

**Session II – GPCR function dynamics and modulation** (Chairs: Cécile Dérieux (DE) & Bernard Masri (FR))

10h50 "Controling brain function with metabotropic glutamate receptors"
Jean Philippe Pin (Institut de Génomique Fonctionnelle, Montpellier, France)

11h20 "An orphan GPCR as a novel regulator of adipose tissue function by a non-canonical mechanism - Implication in obesity"
Julie Dam (France)

11h30 "How to turn an African toxin into a drug candidate!"
Nicolas Gilles (France)

11h40 *Q&A*

11h50 ***Round Table I - "Challenges and future directions in the GPCR field"***

12h30 **Poster Session & Lunch**

**Session II (*cont.*) – GPCR function dynamics and modulation**

14h30 "OZITX, a pertussis toxin-like protein for occluding inhibitory G protein signalling including Gαz"
Robert Lane (UK)

14h40 "How the GPCR core and C-terminus differentially influence receptor regulation – GRK-specific β-arrestin interaction with the β2-adrenoceptor, the vasopressin 2 receptor, and their chimeras"
Edda Matthees (Germany)

14h50 "Functional modulation of PTH1R activation and signaling by RAMP2"
Katarina Nemec (Germany)

15h00 *Q&A*

**Session III – GPCR complexes and new technologies to investigate GPCRs**
(Chairs: Robert Quast (FR) & Pierre André Lafon (FR))

15h10 "Advanced imaging to study GCPR pharmacology and organisation at a sub-cellular level"
Steve Briddon (University of Nottingham, UK)

15h35 "Nile-Red-Based GPCR Ligands as Ultrasensitive Probes of the Local Lipid Microenvironment of the Receptor"
Julie Karpenko (France)

15h45 "Single cell studies reveal GPCRs pharmacological profiles depending on their oligomerization states"
Karen Martinez (Denmark)

15h55 *Q&A*

16h00 *Coffee Break*

16h20 ***Round-table 2 - "Career challenges and opportunities in the GPCR field"***

**Session III (*cont.*) – GPCR complexes and new technologies to investigate GPCRs**

17h00 "Regulation of βeta-adrenoceptors activity using synthetic light-regulated molecules"
Xavier Rovira (Spain)

17h10 "Novel single color fluorescent GPCR biosensors to study allosteric coupling in GPCR activation"
Romy Thomas (Germany)

17h20 *Q&A*

17h30 ***Keynote Lecture* – Jean-Pierre Vilardaga** (University of Pittsburgh, PA, USA)
"Druggability of class B GPCRs"

18h30 **Awards & Closing section**

19h00 **Dinner**

# Abstracts

## Selected Talks

## Session I

### The viral-encoded US28 chemokine receptor contributing to glioblastoma constitutively activates Gq from endosomes: does signalling location matter?

Carole Daly(1), Raimond Heukers(2), Emma Evergren(3), Martine J. Smit(2), Bianca Plouffe(1)

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Glioblastoma is the most common and aggressive type of brain cancer. The human cytomegalovirus (HCMV) encodes US28, a chemokine GPCR. The constitutive activation of Gq by US28 promotes and accelerates glioblastoma tumour growth by activating cellular pathways promoting inflammation, proliferation, metabolic reprogramming and angiogenesis. Due to the recent development of molecular technologies allowing to monitor GPCR signalling with a subcellular resolution, accumulating evidence show that several GPCRs are able to activate G proteins from intracellular compartments. Unlike most GPCRs, US28 is mainly located at endosomes due to chronic internalisation. It was previously assumed that US28 signalling was mediated by the minor pool of receptors at the plasma membrane. Using mini G protein technology combined with enhanced bystander BRET and nanobodies in U251 cells, a well-established glioblastoma cell line, we have demonstrated that US28 robustly and constitutively activates Gq from endosomes instead. The contribution of endosomal signalling by US28 on glioblastoma development was also evaluated using a mutant US28 exhibiting a reduced constitutive internalisation. Our findings have important therapeutical relevance as targeting the right subcellular pool of US28 is crucial to optimise the efficacy of therapeutics targeting this receptor to inhibit glioblastoma growth in HCMV-positive patients.

**PDEs define a cAMP signaling nanoarchitecture in intact cells**

Charlotte Kayser, Lisa Maria Martin, Romy Thomas, Martin J. Lohse, and Andreas Bock

A single cell responds to a myriad of extracellular stimuli by relaying extracellular receptor signals to various locations inside the cell, ultimately triggering strictly receptor-specific cell functions. G protein-coupled receptors (GPCRs) form the largest family of such cell membrane receptors in humans. They orchestrate overwhelmingly complex intracellular signaling networks by activating G proteins and subsequently modulating intracellular second messengers and downstream effectors, e.g. cAMP and PKA, respectively.
The architecture of GPCR signaling networks appears to follow an 'hourglass' model where, at the membrane of a single cell, hundreds of different receptors sense specific extracellular cues that translate into hundreds of specific cell functions. Remarkably, a cell uses only a handful of common modules (e.g. cAMP/PKA) to orchestrate these signaling networks which raises the question of how cells achieve signaling specificity. It has been suggested that GPCR/cAMP signaling is compartmentalized. However, the molecular principles of compartmentation have remained enigmatic for decades.
Here, we demonstrate that phosphodiesterases (PDEs), a protein superfamily that hydrolyzes cAMP, are the key molecules responsible for ensuring compartmentation, and thus signaling specificity, of the GPCR/cAMP/PKA signaling network. In my talk, the pivotal role of PDEs in cAMP compartmentation will be illustrated by several examples of our recent work. Collectively, we suggest that PDEs define a cAMP signaling nanoarchitecture that is essential for cell signaling specificity in space and time. Given the central role of PDEs in cell homeostasis, it is not surprising that disruption of cAMP compartmentation can lead to noncommunicable diseases such as heart failure and cancer. We will show and discuss recent data that hint to potential roles of cAMP nanodomain signaling in disease.

**Gi/o and Gq/11 proteins cooperate in asymmetric GPCR dimers to activate ERK signaling**

Erika Cecon 1, Min Chen 1, Irina Tikhonova 2, Angeliki Karamitri 1, Wenwen Gao 1 , Bernard Masri, and Ralf Jockers 1

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G protein-coupled receptor (GPCRs) typically couple to more than one G protein and the balance between the different G protein coupling events determines functional selectivity. However, the molecular basis of this cross-talk remains poorly understood. Here we studied the signal transduction of melatonin MT1 and MT2 receptors, two closely related prototypical Gi-coupled class A GPCRs that recognize the neuro-hormone melatonin. Both MT1 and MT2 receptors signal mainly through inhibition of the cAMP pathway and activation of ERK1/2 pathway. By using selective inhibitors, we dissected the signaling cascade triggered upon melatonin activation of MT1 and MT2, and we observed that ERK1/2 activation by MT2, but not by MT1, depends on both, Gi and Gq proteins. We provide experimental evidence assisted by molecular modeling in support of an asymmetric receptor-G protein complex. This model accommodates binding of both G proteins to the same receptor complex that communicates by trans-conformational changes. The original model might apply to other class A GPCRs.

**Molecular origins and principles governing adrenaline efficacy and potency in the human β2-adrenergic receptor**

Franziska M. Heydenreich, MRC Laboratory of Molecular Biology, United Kindom
Maria Marti Solano, University of Cambridge, United Kingdom
Manbir Sandhu, St. Jude Children's Research Hospital, United States
Brian Kobilka, Stanford University, United States
Michel Bouvier, University of Montreal, Canada
M. Madan Babu, St. Jude Children's Research Hospital, United States

GPCRs translate binding of extracellular ligands into intracellular responses through conformational changes. The resulting intracellular signalling is characterised by its signal strength and the concentration of ligand needed to induce the respective response. Two measures, efficacy and potency, characterise the intracellular signalling for any combination of ligand, receptor, and signalling output.
We use human β2-adrenergic receptor with its endogenous ligand adrenaline as a model to integrate single residue-level pharmacology with intramolecular residue contact data for the inactive and active states. We found that only 20% of the receptor's 412 residues have a strong impact on efficacy, potency, or both. A distinct but overlapping set of residues participates in structural changes of the receptor. Not all residues that undergo structural change are pharmacologically important, and several pharmacologically important residues do not undergo a structural change upon activation. At the intersection, we discovered an allosteric network that translates ligand binding into an intracellular response. This process can be modified by neighbouring residues that are pharmacologically important. Our model allows the classification of structural changes into residue contacts that drive the signal transduction process from secondary structural changes.
We anticipate that our model will help us better understand the complexities of GPCR function beyond macroscopic conformational change and the impact of selected mutations. We envision that the concept can be used for improving the design and understanding of allosteric modulators. If applied to additional ligand-receptor-effector combinations, the model will give us further insights into the molecular basis of biased signalling.

# Session II

**An orphan GPCR as a novel regulator of adipose tissue function by a non-canonical mechanism - Implication in obesity**

Qiang Zhang[1], Ralf Jockers[1], Julie Dam[1]

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Obesity is related to an imbalance between energy intake and expenditure, resulting in the storage of excess energy as fat in white adipose tissue. Hypertrophy of adipose tissue is associated with increased body weight, while brown adipose tissue has the ability to dissipate excess energy in the form of heat production and protects against metabolic disorders. The conversion of specific cells (called "beige" or "brown" adipocytes) residing in white adipose tissue to a brown-like state ("browning") is associated with weight loss and an improved metabolic profile. Our unpublished results reveal that an orphan G protein-coupled receptor regulates the 'browning' of white adipose tissue and could be a new therapeutic target to combat obesity and related metabolic disorders. Here we discover a novel process of controlling adipose thermogenic activity through a non-canonical GPCR signalling mechanism.

**OZITX, a pertussis toxin-like protein for occluding inhibitory G protein signalling including Gαz**

Alastair C. Keen*1,2,3, Maria Hauge Pedersen*4,5,6, Laura Lemel2,3, Daniel J. Scott7,8, Meritxell Canals2,3, Dene R. Littler9, Travis Beddoe10, Yuki Ono11, Lei Shi12, Asuka Inoue11, Jonathan A. Javitch^4,5, J Robert Lane^2,3

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Heterotrimeric G proteins are the main signalling effectors for G protein-coupled receptors. Understanding the distinct functions of different G proteins is key to understanding how their signalling modulates physiological responses. Pertussis toxin, a bacterial AB5 toxin, inhibits Gαi/o G proteins and has proven useful for interrogating inhibitory G protein signalling. Pertussis toxin, however, does not inhibit one member of the inhibitory G protein family, Gαz. The role of Gαz signalling has been neglected largely due to a lack of inhibitors. Recently, the identification of another Pertussis-like AB5 toxin was described. Here we show that this toxin, which we call OZITX, specifically inhibits Gαi/o and Gαz G proteins and that expression of the catalytic S1 subunit is sufficient for this inhibition. We identify mutations that render Gα subunits insensitive to the toxin that, in combination with the toxin, can be used to interrogate the signalling of each inhibitory Gα G protein.

**How the GPCR core and C-terminus differentially influence receptor regulation – GRK-specific β-arrestin interaction with the β2-adrenoceptor, the vasopressin 2 receptor, and their chimeras**

Edda S. F. Matthees1, Raphael S. Haider1, Laura Klement1, Verena Weitzel1, Julia Drube1, Carsten Hoffmann1

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GPCRs form the largest group of human transmembrane receptors and are pharmacologically targeted to treat pathophysiological conditions. β-arrestins are prominent GPCR binding proteins and have been shown to modify receptor signaling, internalization, and trafficking. GPCR—β-arrestin complexes assemble by β-arrestins associating with the phosphorylated receptor C-terminus and by forming a tight interaction with the receptor core. The resulting complex configurations might lead to different signaling outcomes. However, the importance of specific receptor domains and the roles of individual GPCR kinases (GRKs) in this process remain elusive.

Here, we investigate the role of the GPCR transmembrane core and C-terminus in GRK-specific β-arrestin interaction and signaling outcomes by comparing the β2-adrenoceptor (β2AR), Vasopressin2-receptor (V2R), and their chimeras β2V2 and V2β2, consisting of the β2AR core and the V2R C-terminus and vice versa.

Utilizing our quadruple knockout HEK293 cells lacking GRK2,-3,-5, and -6, we designed NanoBRET-based in cellulo β-arrestin recruitment and conformational change assays to assess GRK-specific functionality of GPCRs. We investigated GRK-specific internalization of the mentioned receptors in living cells by using confocal microscopy.

Our data show that the β2AR is targeted by all GRKs, leading to comparable recruitment of β-arrestin2. In contrast, we observed a pre-coupled complex with arrestin in the absence of agonist for the V2R, mediated by the overexpression of each GRK. Interestingly, no pre-coupling was observed for either of the receptor chimeras. We additionally correlate these findings with GRK-specific conformational change fingerprints of β-arrestins coupling to these receptors and measurements of receptor internalization.

The GPCR core and C-terminus differentially contribute to the interaction with and specificity for distinct GRK isoforms. This becomes evident, as the comparison of β-arrestin2 recruitment, conformational change, and receptor internalization revealed clear differences between parental receptors and their chimeras. Hence, the C-terminus is not sufficient to determine specificity in β-arrestin interaction and downstream receptor signaling.

**How to turn an African toxin into a drug candidate!**

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Venomous animals produce venoms rich in hundreds of naturally occurring peptide toxins that provide a reservoir of potent receptor binding agents. Twelve years ago, the most selective vasopressin type 2 receptor (V2R) antagonist was discovered in the venom of the green mamba. The 57-residue "mambaquaretin", cross-linked by 3 disulfide bridges, has an affinity of 5 nM for the V2R and blocks the 3 signaling pathways related to cAMP production, beta-arrestin mobilization and MAP Kinase activity. V2R controls water homeostasis and its blockade is a validated therapeutic strategy for hyponatremia (HN) and the dominant autonomic polycystic kidney disease (ADPKD). We validated mambaquaretin in a pcy mouse model of ADPKD and a rat model of HN. In a second step, we modified this natural peptide to reduce its risk of immunogenicity. Indeed, any foreign peptide injected in humans can induce the production of anti-drug antibodies. Thanks to an in-house test based on human immune cells, 3 areas of mambaquaretin were identified with a certain risk of immunogenicity. We introduced modifications in these areas and drastically reduced this risk. In addition, a large SAR study identified a lysine which, when changed to alanine, improves the affinity by 9-fold. MQ-LEAD combines these 4 modifications with much less risk of immunogenicity and better affinity than the original peptide. MQ-LEAD is effective and safe with a therapeutic window of over 100 times. The pharmacological qualities of this drug candidate combined with the unmet therapeutic needs of HN and ADPKD prompted us to create a start-up company to take charge of its therapeutic development.

**Functional modulation of PTH1R activation and signaling by RAMP2**

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Receptor-activity-modifying proteins (RAMPs) are ubiquitously expressed membrane proteins that associate with different G protein-coupled receptors (GPCRs), including the parathyroid hormone 1 receptor (PTH1R), a class B GPCR and an important modulator of mineral ion homeostasis and bone metabolism. However, it is unknown whether and how RAMP proteins may affect PTH1R function.

Using different optical biosensors to measure the activation of PTH1R and its downstream signaling, we describe here that RAMP2 acts as a specific allosteric modulator of PTH1R, shifting PTH1R to a unique pre-activated state that permits faster activation in a ligand-specific manner. Moreover, RAMP2 modulates PTH1R downstream signaling in an agonist-dependent manner, most notably increasing the PTH-mediated Gi3 signaling sensitivity. Additionally, RAMP2 increases both PTH- and PTHrP-triggered β-arrestin2 recruitment to PTH1R. Employing homology modeling, we describe the putative structural molecular basis underlying our functional findings.

These data uncover a critical role of RAMPs in the activation and signaling of a GPCR that may provide a new venue for highly specific modulation of GPCR function and advanced drug design.

## Session III

### Nile-Red-Based GPCR Ligands as Ultrasensitive Probes of the Local Lipid Microenvironment of the Receptor

Julie Karpenko (a), Fabien Hanser (a), Claire Marsol (a), Christel Valencia (b), Pascal Villa (b), Andrey S. Klymchenko (c), Dominique Bonnet (a)

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The local lipid microenvironment of transmembrane receptors is an essential factor in G protein-coupled receptor (GPCR) signaling. However, tools are currently missing for studying the lipid microenvironment of endogenously expressed GPCRs in living cells. Environment-sensitive fluorescent membrane probes are powerful tools to study the composition, lipid order and nanodomain organization of biomembranes through shifts in their emission spectra. However, these probes report the average lipid order of the bulk of the cell membrane and are not suitable for probing specifically the receptor microenvironment. Here we introduce receptor-specific fluorescent environment-sensitive probes targeting the oxytocin GPCR. We synthesized them by covalently tethering the ligands targeting the oxytocin receptor to the solvatochromic and fluorogenic dye Nile Red(a,b). We used a combination of steady-state fluorescence spectroscopy and ratiometric confocal fluorescence microscopy to demonstrate the ability of the new probes to embed into the lipid bilayer in the vicinity of the receptor in living cells and to report the properties of the local lipid microenvironment via the color-shifting emission of the Nile Red fluorophore.

References:
(a) J. Karpenko, R. Kreder, C. Valencia, P. Villa, C. Mendre, B. Mouillac, Y. Mély, M. Hibert, D. Bonnet, A. S. Klymchenko, ChemBioChem 2014, 15: 359–363.
(b) F. Hanser, C. Marsol, C. Valencia, P. Villa, A. S. Klymchenko, D. Bonnet, J. Karpenko, ACS Chem. Biol. 2021, 16, 4, 651–660.

**Single cell studies reveal GPCRs pharmacological profiles depending on their oligomerization states**

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Class A GPCRs have been shown to be present as monomers, dimers and even tetramers in cell cultures. Nonetheless, the features of each of these protomers and their physiological relevance remain poorly elucidated.(1,2)
It includes essential questions regarding the functional characterization of each of these protomers such as the ability of a monomer to activate G proteins, the distinct affinities of ligands for monomers and oligomers and ligand cooperativity in oligomers. This fundamental knowledge is limited to a large extend by the technical limitations of state-of-the-art assays based on the averaged responses of large cell populations.
We have developed a method, combining fluorescence based assays with quantitative analysis of images of single cells, which gives access to these open questions. We used this method to determine the pharmacological profile of GPCRs based on the apparent oligomerization state of the receptor, with access to KD, EC50 and stoichiometry.
This generic method, which is suitable for the investigation over time of any GPCR in living cells, is expected to improve our knowledge about the GPCR oligomers and the perspectives they offer in drug discovery.(3)

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2. Ferré, S., Ciruela, F., Casadó, V. & Pardo, L. Oligomerization of G protein-coupled receptors: Still doubted? Prog. Mol. Biol. Transl. Sci. 169, 297–321 (2020).
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**REGULATION OF BETA-ADRENOCEPTORS ACTIVITY USING SYNTHETIC LIGHT-REGULATED MOLECULES**

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Beta-adrenoceptors (β-AR) are prototypical G protein-coupled receptors and important pharmacological targets for many diseases. Indeed, a number of approved drugs target these receptors due to their key role on many physiological functions. Among other examples, we encounter β1-AR antagonists (β-Blockers), which constitute the first-line therapy for the treatment of heart diseases, and β2-AR agonists, which act as bronchodilators for the treatment of breathing pathologies. Considering the relevance of these receptors, achieving a reversible and localised control of their activity would provide a powerful tool, both for its research applications and its clinical potential. In this context, photopharmacology arises as a potent approach.
Photopharmacology is an emerging field based on the use of synthetic light-regulated molecules to allow reversible spatiotemporal control of target receptors in native tissues. These ligands have the potential to provide a precise and controllable therapeutic action with increased efficacy and reduced side effects. Moreover, the fine regulation on demand of the receptor activation state is of great interest for their study in non-modified cells, tissues and organisms. The present project provides the first proof of concept for beta-adrenoceptor photopharmacology.
We first designed and synthesised libraries of light-regulated compounds in order to regulate β-AR activity with spatiotemporal precision. Subsequent testing highlighted the successful development of compounds with promising pharmacological properties which can be reversibly and irreversibly controlled by light. The discovered molecules enable a fine control of β-AR in their native environment that will certainly open the door to innovative research procedures and may inspire future personalized therapies targeting these receptors.

**Novel single color fluorescent GPCR biosensors to study allosteric coupling in GPCR activation**

Romy Thomas (1,3); Chiara De Faveri (4); Pauline Löffler(3); Martin J. Lohse (1,2); Irene Coin (4); Andreas Bock (1,3)

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2: ISAR Bioscience, Munich-Planegg, Germany
3: Leipzig University, Medical Faculty, Rudolf-Boehm-Institute of Pharmacology, Leipzig, Germany
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G protein-coupled receptors (GPCRs) form the largest class of membrane receptors and are among the most important drug targets. GPCRs can couple to various downstream transducers such as different G proteins and arrestins. So called “biased ligands” have been described to preferentially activate one pathway over another. By stimulating therapeutically beneficial signaling pathways, they hold great potential in becoming more specific therapeutics with less side effects. According to the “allosteric coupling” paradigm, binding of an extracellular agonist leads to conformational changes that propagate through the receptor's transmembrane core to the binding sites of intracellular transducers. This process is reciprocal and appears to be transducer-specific and, thus, we hypothesize that it might be linked to biased agonism. A detailed understanding of its molecular basis is of great interest as it might lead to the rational design of biased ligands as next-generation GPCR drugs. To study transducer-specific allosteric coupling, we developed and characterized a novel single color fluorescent GPCR activation sensor. Using genetic code expansion technology and bioorthogonal labeling by click-chemistry, we equipped the entire extracellular surface of the class A muscarinic acetylcholine receptor M2 with small organic fluorophores of different colors. Upon stimulation with various agonists, we monitored reversible variations in sensor fluorescence intensity in intact cells. We found distinct, ligand-dependent fluorescence intensity changes, which, according to our data, indicate distinct, ligand-dependent receptor activation states. Our approach is compatible with usage of fluorophores of different colors, and, thus, can be flexibly combined with other fluorescence-based assays. We anticipate our sensor-design to be transferable to other GPCRs, allowing generalization of our findings. Our results support the hypothesis that binding of different ligands triggers distinct conformational changes which may drive biased signaling. Eventually, utilization of our novel sensor allows to gain deeper insight into the molecular mechanism of ligand-specific GPCR/transducer coupling.

# Poster Abstracts

## 1

### Analgesic effect of Serodolin, a β-arrestin-biased ligand of 5-HT7 receptor, in preclinical models of pain

Chayma El Khamlichi (1), Flora Reverchon (1), Nadège Hervouet-Coste (1), Elodie Robin (1), Nicolas Chopin (2), Emmanuel Deau (2), Fahima Madouri (1), Cyril Guimpied(1), Cyril Colas (1,2), Arnaud Menuet (3), Asuka Inoue (4), Andrzej J. Bojarski (5), Gérald Guillaumet (2), Franck Suzenet (2), Eric Reiter (6) and Séverine Morisset-Lopez( 1)

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Transmembrane signaling through G protein-coupled receptors (GPCR), originally described as requiring coupling to intracellular G-proteins, also uses G-protein-independent pathways through β-arrestins recruitment. Biased ligands, by favoring one of the multiple bioactive conformations of GPCR, allow selective signaling through either of these pathways. This concept of functional selectivity of a ligand has emerged as an interesting property for the development of new therapeutic molecules. Biased ligands are expected to have superior efficacy and/or reduced side-effects by regulating biological functions of GPCRs in a more precise way. In the last decade, 5-HT7 receptor (5-HT7R) has become a promising target for the treatment of neuropsychiatric disorders, sleep and circadian rhythm disorders and pathological pain. We recently identified a small molecular weight compound, named Serodolin, targeting the 5-HT7R with a nanomolar affinity. We showed that Serodolin displays a baised activity : it behaves as an antagonist/inverse agonist on Gs signaling while inducing ERK activation through a β-arrestin-dependent signaling mechanism that requires c-SRC activation. Moreover, we showed that Serodolin clearly decreases hyperalgesia and pain sensation in response to inflammatory, thermal and mechanical stimulation. This anti-nociceptive effect could not be observed in 5-HT7R KO mice and was fully blocked by administration of SB269-970, a specific 5-HT7R antagonist, demonstrating the specificity of action of Serodolin. Physiological effects of 5-HT7R stimulation have been classically shown to result from Gs-dependent adenylyl cyclase activation. In this study, using a novel β-arrestin-biased agonist, we provided new insight into the molecular mechanism triggered by 5-HT7R and revealed its therapeutic potential in the modulation of pain response.

**2**

**Fluorescent protein directionality points to a new direction in cell signaling research**

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Fluorescent proteins (FPs) offer various ways to observe molecular processes of cell signaling. Among the various FP properties exploited in biological research, directionality remains largely overlooked. Here we present a novel, simple, versatile design of FP-based genetically encoded probes that take advantage of FP directionality to allow sensitive, multiplexable, real-time imaging of numerous molecular events of cell signaling with subcellular resolution.

**3**

## A new machine learning based method for ADRB2 agonist detection using single-ligand dynamic interaction data

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The G-protein coupled receptor family is responsible for signaling transduction in many biological processes. The binding of a ligand regulates the signaling by stimulating it (agonist) or inhibiting it (antagonist, inverse agonist). The β2 adrenergic receptor (ADRB2) is one of the most studied GPCR, with many known ligands with an agonistic or antagonistic action. The ligand binding information provided by crystallographic structures of ADRB2 is often used to improve virtual screening performance, by allowing better separation not only of active and inactive ligands, but also of agonists and antagonists [1][2]. Here, we propose a method that takes into account the conformational dynamics of the ADRB2/ligand reference complex with the aim of improving the biased search towards ligands with specific pharmacological properties.
An ensemble of binding poses was obtained from the crystal structure of ADRB2-agonist complex using molecular dynamics (MD) simulations [3][4]. Key interaction-patterns for agonist activity were selected by a machine learning algorithm. As a test, the developed model was used, in combination with protein-ligand docking, to screen a small library containing well-characterized agonists and antagonists targeting ADRB2.
The proposed technique could be used to post-process docking poses to determine if they can be considered as agonist-like. It can be applied as a filter to remove non relevant poses, non-active ligands, and ligands with an undesired pharmacological effect.

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**4**

**Tracking receptor motions at the plasma membrane reveals distinct effects of ligands on CCR5 dynamics depending on its dimerization status**

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G protein-coupled receptors (GPCR) are present at the cell surface in different conformational and oligomeric states. However, how these states impact GPCRs biological function and therapeutic targeting remains incompletely known. Here, we investigated this issue in living cells for the CC chemokine receptor 5 (CCR5), a major receptor in inflammation and the principal entry co-receptor for Human Immunodeficiency Viruses type 1 (HIV-1). We used TIRF microscopy and a statistical method to track and classify the motion of different receptor subpopulations. We showed a diversity of ligand-free forms of CCR5 at the cell surface constituted of various oligomeric states and exhibiting transient Brownian and restricted motions. These forms were stabilized differently by distinct ligands (native agonist, inverse agonist, HIV-1 envelope glycoproteins), revealing a link between receptor activation and immobilization. Using a dimerization-compromized mutant, we showed that dimerization impact the fate of activated receptors. This study demonstrates that tracking the dynamic behavior of a GPCR is an efficient way to link GPCR conformations to their functions, therefore improving the development of drugs targeting specific receptor conformations.

**5**

**A Classification Model for the Second Extracellular Loop of Class A GPCRs**

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The extracellular loop 2 (ECL2) is the longest and the most diverse loop among class A G protein-coupled receptors (GPCRs) [1]. The ECL2 affects the binding of both orthosteric and allosteric modulators and determines ligand selectivity, ligand entrance, and exit [1]. The recent advancements in GPCR structural biology are leading to an increased number of structures, calling for a need for tools for structural analyses.
We propose a classification model of ECL2 loops based on shape (volume overlap) similarity [2]. The reorganization of structural data of this highly diverse loop may be valuable to support structure-based GPCR drug design campaigns. We analyzed experimental ECL2 structures and enriched our analysis with frames from the Molecular Dynamics (MD) trajectories available on the GPCRmd website. To reduce the number of structures, we clustered and extracted 10 frames from each MD trajectory.
Despite the high sequence variability, the analyzed structures could be clustered into seven main groups based on volume overlaps [3]: singletons, the long unstructured ECL2 of P2Y12 and the short ECL2 of MC4R; structures with a β-sheets fold oriented over the binding site (i.e. peptide GPCRs), and those with a β-sheets fold oriented over the binding site; the ECL2 of the adenosine A2a and A1 receptors; the ECL2 of the aminergic receptors and the longest ECL2 of the FFA1; and the ECL2 of lipid GPCRs, which group structures that form an intra-loop disulfide bridge instead of the conserved ECL2-TM3 disulfide bridge. We then explored the contribution of side chains to the ECL2 folding through a contact analysis whose results are available at: http://ecl2.giorginolab.it/.

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**6**

**Epitope Identification, a case study: Nanobodies binding to mGlu5 receptor**

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Schizophrenia is a mental disorder that affects approximately 1% of the worldwide population. Scientists finally developed a hypothesis that may interpret the pathophysiology behind this disorder, which is related to the deficiency of glutamate in the brain. Amongst glutamate targets, in this study we have focused on metabotropic glutamate receptor 5 (mGlu5). Due to the highly conserved orthosteric binding site of mGlu receptor subtypes, scientists developed an alternative approach to target mGluRs using nanobodies as allosteric modulators. A nanobody is defined as the variable fragment of the heavy chain-only antibody. Its small size and stability under extreme physical conditions make it an interesting therapeutic agent. In the present study, based on biological data stating that nanobody Nb5A acts as a positive allosteric modulator of rat mGlu5, we performed an epitope identification of Nb5A at mGlu5 receptor using molecular modeling techniques like docking and molecular dynamics and artificial intelligence algorithms. Two poses resulting from the docking of Nb5A to the active conformation of rat mGlu5 receptor were selected. Then, among the 2 poses, one was confirmed experimentally.

**7**

**G protein selectivity and gene regulation by US28 are shaped by the presence or absence of chemokines.**

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US28, a GPCR encoded by the cytomegalovirus, has an oncomodulatory role in glioblastoma (GBM) by regulating the transcription of genes associated to cancer. US28 binds to a wide range of chemokines, but also has a particularly high constitutive activity. Studies have focused primarily on the Gαq isoform associated to high levels of agonist-independent activation of PLCβ. However, the relative constitutive and agonist-dependent activation of other G protein isoforms activated by US28 have not been explored yet and their oncomodulatory role is largely unknown. Using BRET-based biosensors, we compared the G protein activation profile of US28 and US28-Δ2-16, a mutant lacking the first 16 residues in N-terminal critical for chemokine binding. Our results show that while US28 activates the four G protein families, US28-Δ2-16 does not activate Gαs nor Gα12/13 but conserves the ability to activate Gαq and some Gαi/o isoforms. These results suggest that Gαs and Gα12/13 are not constitutively activated by US28 and require chemokine binding, while Gαq and some Gαi/o isoforms do not. We took advantage of these differences in agonist requirement between Gαs/12/13 and Gαq/i/o isoforms to identify the genes regulated by Gαs or Gα12/13 and by Gαq or Gαi/o. We sequenced the RNA of human U251 cells, a well-established GBM cell line, expressing US28 or US28-Δ2-16 and performed a differential gene expression analysis. We identified several genes regulated by US28 but not US28-Δ2-16, suggesting that these genes requires the presence of chemokines and are regulated by activation of Gαs or Gα12/13 by US28. We also identified many genes not differently regulated by US28 and US28-Δ2-16, which are therefore dependent on Gαq or Gαi/o activation. Overall, our data demonstrate that the presence or absence of chemokines determines the subset of G protein isoforms activated by US28 and therefore dictates the subset genes regulated by this oncomodulatory receptor.

**8**

## β-arrestin1 and 2 exhibit distinct phosphorylation-dependent conformations when coupling to the same GPCR in living cells

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β-arrestin1 and 2 are hypothesized to mediate targeted processes for more than 800 different G protein-coupled receptors (GPCRs). They have been shown to act as important regulators of GPCR signaling, internalization, and trafficking. To facilitate this, the individual β-arrestin isoforms have been shown to adopt different active conformations resulting from the geometry of the specific GPCR–β-arrestin complex. However, whether β-arrestin1 and 2 respond differently upon binding to the same GPCR is still unknown. The aim of the study was to compare the complex configurations of β-arrestin1 and 2 for binding to the parathyroid hormone 1 receptor (PTH1R), including the assessment of β-arrestin conformational changes and their initial downstream functionality.
With advanced NanoLuc/FlAsH-based β-arrestin1 and 2 biosensors which were labelled in eight independent positions, we reveal the comprehensive signature of conformational changes for both β-arrestin isoforms when interacting with the ligand-activated and phosphorylated PTH1R (P-R* receptor state) in living cells. Using our recently published CRISPR/Cas9 mediated GRK2,3,5,6 knockout cell line, we repeated these measurements in the absence of GPCR kinases (GRKs) (R* receptor state). Hence, this approach enables the differentiation between conformational changes that are induced by P-R* or R* receptor states. Additionally we used two distinct phosphorylation-deficient receptor variants (devoid of either proximal or distal C-terminal phosphorylation sites). This approach further discloses the impact of site-specific GPCR phosphorylation on arrestin-coupling and function. Furthermore, we use live-cell confocal microscopy, cellular localized bystander-BRET assays and additional knockout cell lines to characterize formed complexes and their intracellular localization.
With this study, we demonstrate that the GPCR phosphorylation state not only regulates differences in affinity between β-arrestin1 and 2 but also translates into specific conformational rearrangements that determine the functional diversity between the two isoforms.

**9**

**FRET-based Kappa Opioid Receptor-Sensor reveals basal activity and kinetics of voltage sensitivity**

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Several GPCRs are already classified as voltage sensitive. A modulation of the membrane potential influences the activity of the receptor. An important type of voltage sensitive GPCRs are the opioid-receptors. We recently investigated the μ-opioid receptor and found a ligand specific voltage sensitivity, which had a considerable effect on the ability of opioid analgesics to activate μ-receptors.

In this study, we investigated the kappa-opioid-receptor (KOR) in terms of voltage dependence with a combination of FRET and patch clamp. For further investigations of the KOR, we established a FRET-based receptor-sensor. With this sensor we, on the one hand, were able to perform FRET measurements of multiple cells at a plate reader and, on the other hand, were able to characterize kinetics of the voltage effect displayed by the KOR.

In a FRET-based G-protein activation assay we found that the high level of constitutional activity of KOR caused reduced G-protein signaling. With preincubation of the opioid antagonist naloxone, we observed a recovery in G-protein signaling, which allowed for measurements of the KORs voltage dependence. Surprisingly we observed a voltage dependence of the KOR in presence and absence of the endogenous agonist dynorphin A. These findings could be confirmed in other assays. To our knowledge, this is the first observation of a ligand free voltage dependence of an unmutated GPCR. Further, by the use of the FRET-based receptor-sensor, we were able to resolve kinetics of the voltage induced activation with and without agonist. In a further step, we investigated the naloxone effect at different assays and observed inverse agonism of naloxone.

In conclusion, KOR showed activation upon depolarization in presence and absence of agonist. Further, KOR displayed basal activity, which could be detected directly at the conformation level. This might be linked to our observation of the voltage dependence in absence of agonist.

**10**

**Ligand-induced changes in the plasma membrane organisation of the chemokine receptor CXCR4**

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Introduction:
CXCR4 is a G protein-coupled receptor that binds to the chemokine CXCL12. Receptor desensitisation and regulation of signal duration can be controlled by receptor trafficking and membrane redistribution. In this study, we use Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Recovery After Photobleaching (FRAP) to assess the organization of CXCR4 within the plasma membrane in basal, agonist- (CXCL12) or antagonist- (IT1t)-treated conditions.
Methods:
HEKG SNAP-CXCR4 cells were labelled with SNAP Surface-AlexaFluor 488. Cells were treated with vehicle, IT1t (1µM, 30 minutes) or CXCL12 (10nM, 10 minutes) and FCS measurements were recorded for 30s on the upper cell membrane. Autocorrelation curves were fitted to a two-component 2D model for autocorrelation analysis to determine particle number, dwell time of the receptor and Photon Counting Histogram (PCH) used to determine molecular brightness. Macro-diffusion and the immobile fraction of CXCR4 were determined using FRAP on the basal membrane. Data were analysed and corrected to the background and reference cells.
Results:
FCS measurements showed CXCR4 density under basal conditions was 230±10 N/mm2 and its diffusion coefficient (D) was 0.287±0.011 µm2/s (n=31-37 cells from ≥6 independent experiments). FRAP measurements gave a lower value of (D)=0.054±0.003 µm2/s (n=32-38 from ≥ 5 independent experiments). Ligand addition did not significantly change the receptor density or diffusion coefficient (one-way ANOVA). However, the percentage of the cells with a second PCH component was increased to 34% in CXCL12-treated cells compared to vehicle (3%). The immobile proportion of receptors was increased significantly in both the CXCL12 (56.8 ± 2.2%) and IT1t conditions (52.3 ± 2.2%), compared to the vehicle (40.0 ± 1.6%, p

**11**

**Structure of the vasopressin hormone-V2 receptor-b-arrestin1 ternary complex**

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Arrestins interact with G protein-coupled receptors (GPCRs) to stop G protein activation and to initiate key signaling pathways. Recent structural studies shed light on the molecular mechanisms involved in GPCR-arrestin coupling, but whether this process is conserved among GPCRs is poorly understood. Here, we report the cryo-electron microscopy active structure of the wild-type arginine-vasopressin V2 receptor (V2R) in complex with b-arrestin1. It reveals an atypical position of b-arrestin1 compared to previously described GPCR-arrestin assemblies, associated with an original V2R/b-arrestin1 interface involving all receptor intracellular loops. Phosphorylated sites of the V2R C-terminus are clearly identified and interact extensively with the b-arrestin1 N-lobe, in agreement with structural data obtained with chimeric or synthetic systems. Overall, these findings highlight a striking structural variability among GPCR-arrestin signaling complexes.

**12**

## Structure-activity Relationship analysis of subtype selective bitopic ligands of the M1 muscarinic receptor

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Muscarinic acetylcholine receptors (mAChR1-5) belong to the family of G protein-coupled receptors. They are considered pharmaceutical targets for therapeutics to treat Alzheimer`s disease and other diseases with impaired cognitive function. In this project we aim to study the M1 receptor, as it is the most abundantly expressed in the central nervous system. The orthosteric acetylcholine-binding site in the mAChRs is highly conserved and the efficacy of drugs used to enhance cognition is limited. Nonetheless, the existence of topographically distinct allosteric binding sites allows the modulation of the receptor affinity, efficacy and the cellular signaling processes by using ligands structurally different from orthosteric ligands. To study the receptor response upon the interaction on the allosteric binding sites, dualsteric ligands are required. These structures merge two biologically active molecules into a single chemical entity, retaining the basic properties of the parent compounds. This project explores the allosteric, M1-selective BQCAd scaffold derived from the benzylquinolone carboxylic acid as a starting point for the design, synthesis, and pharmacological evaluation of a series of novel bitopic ligands in which the orthosteric moieties and linker lengths are systematically varied. To derive structure−activity relationships, the set of compounds was tested for G protein signaling activation, with a luminescence-based complementation assay and β-Arrestin recruitment measured by BRET. As a result, we found that various bitopic ligands are partial to full agonists for G protein activation, some activate β-arrestin recruitment, and the degree of β-arrestin recruitment varies according to the respective modifications. Also was observed that the allosteric BQCAd scaffold controls the positioning of the orthosteric ammonium group of all ligands, suggesting that this interaction is essential for stimulating G protein activation. The novel set of bitopic ligands may constitute a toolbox to study the requirements of β-arrestin recruitment and G protein activation during ligand design for therapeutic usage.

**13**

**Development and characterization of nanobody targeting serotonin 5-HT7 receptor for therapeutic and pharmacological applications.**

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The serotonin 5-HT7 (R5-HT7) receptor belongs to the superfamily of G protein-coupled receptors (GPCRs). In recent years this receptor has become a promising target for the treatment of neuropathic pain or neurodegenerative and inflammatory diseases. Usage of small synthetic molecules in various animal models has increased the interest of the receptor for the treatment of these pathologies. Owned to some limitations of synthetic molecules (lack of bioavailability, selectivity) an alternative for targeting GPCRs has emerged and it is based on the development of nanobodies, antibody fragments with high affinity and selectivity. Compared to conventional antibodies, nanobodies (VHH) are small (~12 kDa) and lack a light chain (Muyldermans, 2013). According to their convex shape they are particularly interesting for targeting buried epitopes such as those found in ligand-binding pockets of receptors (De Genst et al, 2006; Muyldermans et al, 2009). To identify nanobodies to human 5-HT7R, we used different antigen format for in vivo immunization of Llama. Here we report the successful selection of a few potential candidates, after performing phage display screening, that showed specific binding to targeted receptor 5-HT7 (ELISA, interferometry and flow cytometry). This allow us to do characterization of selected candidates /nanobodies targeting 5HT7, as pharmacological tools in different cellular tests (Western blot, BRET, Alpha Lisa, immunocytochemistry). We are now studying their actions on different signaling pathways in living HEK293 cells expressing BRET biosensors. This work provides novel pharmacological tools that should allow deciphering of signaling and functions of 5-HT7R. By achieving these aims, we will gain insight into 5HT7 biology that could be leveraged toward developing novel interventions for diseases in brain.

**14**

**Modeling of CCR2 homodimers and CCR5/CCR2 heterodimers**

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Class A G protein coupled receptors (GPCRs) can form homo- and heterodimers. Among these, the chemokine receptors CCR2 and CCR5 are crucial regulators of leukocyte trafficking, and the improper regulation or exploitation of these receptors contribute to a wide variety of inflammatory and autoimmune diseases, cancer, atherosclerosis, and HIV, making them an attractive target for drug discovery [1,2].
Three symmetrical dimer organizations have been validated with CCR5, and dimerization was shown to be (1) required for cell-surface delivery and (2) influenced by ligand binding [3].
Here we built three CCR2 homodimer models and three CCR5/CCR2 heterodimer models based on the CCR5-validated dimer interfaces, and a fourth CCR2 homodimer model based on an asymmetrical dimer arrangement present in a CCR2 crystal structure [4]. All seven dimer systems were placed into a hydrated lipid bilayer and subjected MD simulations. The trajectories were analyzed to assess the stability of structures in the different arrangements of dimers and to design experiments with testable hypotheses to validate structurally relevant models.

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**15**

**Modulation of Dimerization and Signaling in Melanocortin-4 Receptor (MC4R) by accessory protein MRAP2.**

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Leptin-melanocortinergic pathway is critical in regulation of energy homeostasis. One pivotal player of this regulation system, melanocortin-4 receptor (MC4R), belongs to a subfamily of class A GPCRs. MRAP2 is an accessory protein related to MC4R, that was shown to form antiparallel and parallel dimers. Alterations in expression of MRAP2 have been shown to lead to functional alterations in receptor activity.

We explore here, by a toolbox of fluorescence microscopy and spectroscopy tools, the role of MRAP2 in influencing MC4R signaling, trafficking and oligomerization at the plasma membrane of single, living cells. We observe effects of MRAP2 onto Gs-dependent cAMP production by MC4R, receptor internalization as well as an increased oligomerization of MC4R upon MRAP2 overexpression. Our results thus shed light into underappreciated roles for MRAP2 in modulating MC4R biophysics.

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**16**

**Dissecting the GPCR signaling cascade one step at the time: a fluorescence spectroscopy investigation of adrenergic-mediated signaling upon cell swelling.**

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G-protein coupled receptors are among the most important transmembrane proteins to regulate cellular processes. They have been observed to be functionally modulated, e.g. through their biophysical environment, while adapting to changed extra-/intracellular conditions. Here we employ a comprehensive and novel set of fluorescence-based methods to dissect the signaling cascade originating from stimulation of ß-adrenergic receptors (ß-AR) down to cAMP production.
Our aim is to determine whether receptor signaling can be affected by alterations to the subplasmalemmal biophysical context. We approach this by exposing cells to osmotic swelling, a physiological process which takes place in several pathological conditions like ischemia and acidosis, which is known to cause substantial alteration to intracellular architecture, ranging from membrane curvature to altered actin polymerization.
Notably, we observe that swollen cells yield increased cAMP production in response to stimulation of Gαs-coupled receptors. Further studying the ß2-AR, we don't observe any changes to ligand binding affinity nor to conformational activation of the receptor via a cpGFP sensor, whereas the recruitment of Nb80 to the ß2-AR in active conformation is strongly increased in swollen cells. Moreover, upon direct adenylyl cyclase stimulation with forskolin, cell swelling increases cAMP production, but fails to elicit comparable effects when using a Gαs-knockout cell line. Therefore, we propose signal transduction by Gαs-protein to be increased by the intracellular reorganization arising upon cell swelling.
This opens interesting perspectives on whether this is a general mechanism common to other GPCRs, with Gαs being the molecular switch that the cell uses to tune downstream signaling while readapting to pleiotropic changes of its local environment.

**17**

**Decoding the function of PfSR25 and homodimerization of PfSR12 as GPCR candidates in the human malaria parasite Plasmodium falciparum.**

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Our group identified for the first time in the malaria parasite genome, Plasmodium falciparum, four candidates for potential GPCRs: PfSR1, PfSR10, PfSR12, and PfSR25. We have recently characterized the PfSR25 as a sensor for potassium (K+) shift, increasing the concentration of cytosolic Ca2+ in the WT parasites but not in PfSR25 knockout parasites. Moreover, the PfSR25 knockout parasite also showed greater susceptibility to oxidative stress and compounds with antimalarial activity such as piperaquine, lumefantrine and chloroquine (Santos et al. 2020, 2021). As for PfSR12 we discovered through biosensors based on bioluminescence resonance energy transfer (BRET) in HEK293 cells that it can form homodimers. When HEK293 cells were transfected using fixed concentrations of RLuc tagged PfSR12 and increasing concentrations of GFP10 tagged PfSR12, an increase in BRET was observed until saturating, which indicate homodimerization. To identify which region of PfSR12 was responsible for the dimerization, two truncated versions were produced: one lacking the
C-terminal sequence and one lacking the whole N-terminal sequence. While the absence of C-terminal showed no interference in the dimerization, the N-terminal was found to be essential for dimerization of PfSR12, as revealed by a lower BRET between RLuc and GFP tagged PfSR12 truncated from its 232 N-terminal residues. We modeled PfSR12 homodimer using Alphafold2. The model shows a dimer interface based mainly in transmembrane 1 and in the extracellular N-terminal region supporting the BRET- based observartion. The resulting model resembles the recently described Cryo-EM structure of Ste2 as a class D GPCR dimer (Velazhahan, V. 2022).

**18**

**Nanobody-based sensors reveal a high proportion of mGlu heterodimers in the brain.**

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Membrane proteins, including ion channels, receptors and transporters, are often composed of multiple subunits and can form large complexes. Their specific composition in native tissues is difficult to determine and remains largely unknown. In this study, we developed a method for determining the subunit composition of endogenous cell surface protein complexes from isolated native tissues. Our method relies on nanobody-based sensors, which enable proximity detection between subunits in time-resolved Förster resonance energy transfer (FRET) measurements. Additionally, given conformation-specific nanobodies, the activation of these complexes can be recorded in native brain tissue. Applied to the metabotropic glutamate receptors in different brain regions, this approach revealed the clear existence of functional metabotropic glutamate (mGlu)2-mGlu4 heterodimers in addition to mGlu2 and mGlu4 homodimers. Strikingly, the mGlu4 subunits appear to be mainly heterodimers in the brain. Overall, these versatile biosensors can determine the presence and activity of endogenous membrane proteins in native tissues with high fidelity and convenience.

**19**

**Hypertension causing PDE3A catalytic mutation leads to alteration of cAMP signaling.**

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Phosphodiesterases (PDEs) are the only enzymes that hydrolyze cyclic adenosine monophosphate (cAMP), the ubiquitous second messenger downstream of many G protein-coupled receptors (GPCRs). GPCRs play a fundamental role in vital signaling pathways, for instance heartbeat regulation and metabolism. We could recently show that PDEs are pivotal for generating low-cAMP nanodomains, i.e. small membraneless regions in the cell that have a size of nanometers. This so-called compartmentation of cAMP in low and high cAMP nanodomains allows a precise spatiotemporal activation of downstream targets and thereby GPCR signaling specificity [1,2].

Our previous work suggests that the catalytic activity of PDEs plays an important role in the size of low-cAMP nanodomains. To explore the pathophysiological relevance of low-cAMP nanodomains, we here analyze a novel point mutation in the catalytic pocket of the cGMP-inhibited PDE, PDE3A. This mutation causes a severe disease phenotype called hypertension with brachydactyly (HTNB), a rare form of a monogenetic, autosomal-dominant hypertension. Untreated, the increase in blood pressure leads to death by stroke in affected individuals at the age of 50 [3]. To characterize the influence of this mutation on PDE3As' catalytic properties we developed a FRET-based in vitro assay to estimate the hydrolytic activity of PDEs. Here we find a massive increase in enzyme velocity directly caused by the catalytic pocket mutation. Furthermore, we looked into PDE3A's ability to generate low-cAMP nanodomains. We hypothesize that the increase in catalytic activity might influence either the radius or the depth of cAMP gradients in living cells, thereby dysregulating the crucial nanoarchitecture of cAMP in cells.

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## 20

**The extracellular N-terminus of natural receptor isoforms allosterically modulates GPR35-transducer coupling and mediates intracellular pathway bias.**

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The human G protein-coupled receptor (GPCR) GPR35 is expressed as two distinct isoforms that differ only in the length of their extracellular N-termini by 31 amino acids. Through gene expression analysis in immune and gastrointestinal cells, we show that these isoforms emerge from distinct promoter usage and alternative splicing but detailed insights into their functional differences are lacking. Therefore, we additionally employed optical assays in living cells to thoroughly profile both GPR35 isoforms for constitutive and ligand-induced activation and signaling of ten different heterotrimeric G proteins, ligand-induced arrestin recruitment and receptor internalization. Our results reveal that the extended N-terminus of the long isoform limits G protein activation, yet elevates receptor-beta-arrestin interaction. To better understand the structural basis for this bias, we examined structural models of GPR35 and conducted experiments with mutants of both isoforms. We found that a proposed disulfide bridge between the N-terminus and extracellular loop 3, present in both isoforms, is crucial for constitutive G13 activation, while an additional cysteine contributed by the extended N-terminus of GPR35 long limits the extent of agonist-induced receptor-beta-arrestin2 interaction. The pharmacological profiles and mechanistic insights of our study provide clues for the future design of isoform-specific GPR35 ligands that selectively modulate GPR35-transducer interactions and allow for mechanism-based therapies against, for example, inflammatory bowel disease or bacterial infections of the gastrointestinal system.

**21**

**Distinct roles of β-arrestins in GPCR endocytosis.**

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Internalization plays a pivotal role in controlling the density of cell surface receptors for modulating cell signaling and physiological responses. Beta-arrestins (βarrs) are believed to be major regulators for most G protein-coupled receptors (GPCRs) internalization, but not for some which can also recruit βarrs, such as the glucagon-like peptide-1 receptor (GLP-1R). Here, by developing innovative time-resolved FRET assays and using βarrs knock-out HEK293 cells, we analyzed internalization profiles of 60 GPCRs dissecting the contribution of βarrs to this process. Based on our agonist-induced internalization results, GPCRs can be classified into four groups: the receptors that cannot internalize, those that fully or partially require βarrs for internalization, and finally the GPCRs that are βarrs independent for endocytosis. By exchanging the C-terminal domain (CTD) of receptors from different groups, their internalization profiles were changed, indicating that the CTD acts as a major molecular determinant for controlling agonist-induced internalization by βarrs. Finally, we identified the molecular determinants in the CTD of GLP-1R that control βarrs dependent- and independent internalization. Overall, our study provides unique data resources and uncovers an unexpected role of βarrs in GPCR internalization.

## 22

**Biased signaling due to oligomerization of a G protein-coupled receptor.**

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G protein-coupled receptors (GPCRs) are important drug targets that mediate various signaling pathways by activating G proteins and engaging -arrestin proteins. Despite its importance for the development of therapeutics with fewer side effects, the underlying mechanism that controls the balance between these signaling modes of GPCRs remains largely unclear. Here, we show that assembly into dimers and oligomers can largely influence the signaling mode of the platelet-activating factor receptor (PAFR). Single-particle analysis results show that PAFR can form oligomers at low densities through two possible dimer interfaces. Stabilization of PAFR oligomers through cross-linking increases G protein activity, and decreases -arrestin recruitment and agonist-induced internalization significantly. Reciprocally, -arrestin prevents PAFR oligomerization. Our results highlight a novel mechanism involved in the control of receptor signaling, and thereby provide important insights into the relationship between GPCR oligomerization and downstream signaling.

**23**

**Luminogenic HiBiT peptide-based NanoBRET ligand binding assays for melatonin receptors.**

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The two human melatonin receptors, MT1 and MT2, which belong to the G protein-coupled receptor (GPCR) family are important drug targets with approved indications for circadian rhythm- and sleep-related disorders and major depression. Currently, most of the pharmacological studies were performed using [3H]melatonin and 2-[125I]iodomelatonin (2-[125I]-MLT) radioligands. Recently, NanoLuc-based bioluminescence resonance energy transfer (NanoBRET) monitoring competitive binding between fluorescent tracers and unmodified test compounds has emerged as a sensitive, non-radioactive alternative for quantifying GPCR ligand engagement on the surface of living cells in equilibrium and real time. However, developing such assays for the two melatonin receptors depends on availability of fluorescent tracers, which has been challenging predominantly due to their narrow ligand entry channel and small ligand binding pocket. Here, we generated a set of melatonergic fluorescent tracers and used NanoBRET to evaluate their engagement with MT1 and MT2 receptors that are genetically fused to an N-terminal luminogenic HiBiT-peptide. We identified several non-selective and sub-type selective tracers. Among the selective tracers, PBI-8238 exhibited high nanomolar affinity to MT1 and PBI-8192 exhibited low nanomolar affinity to MT2. The pharmacological profiles of both tracers were in good agreement with those obtained with the current standard 2-[125I]-MLT radioligand. Molecular docking and mutagenesis studies suggested the binding mode of PBI-8192 in the MT2 and its selectivity over MT1.
In conclusion, we describe the development of the first non-radioactive, real-time binding assays for melatonin receptors expressed at the cell surface of living cells that are likely to accelerate drug discovery for melatonin receptors.

**24**

**Mambaquaretin, a novel toxin from snake venom for the potential diagnosis and treatment of renal cancer by targeting the vasopressin V2 receptor.**

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The vasopressin V2 receptor (V2R) is expressed in the kidneys and its activation by vasopressin induces production of cAMP responsible to the antidiuretic effect. V2R is also abnormally expressed in renal cancer and could be an interesting therapeutic target. Many studies shown that the use of V2R antagonists such as Tolvaptan can inhibit cell proliferation as well as tumor formation in mice models. But due to a lack of selectivity these antagonists have undesirable effects, and it is therefore necessary to develop new ligands.
Mambaquaretin (MQ) is a toxin from green mamba characterized as a selective V2R antagonist. This molecule is able to inhibit the formation of cysts in mice with polycystic kidney disease.
The aim is to evaluate the effect of MQ on different renal cancer models to characterize this molecule as a new therapeutic tool for renal cancer.
Our RT-PCR experiments shown the expression of RNA of the V2R in human renal cancer cells. Then, labelling experiments with fluorescent MQ highlighted the presence of the protein of V2R on cell surface. In addition, MQ has a significant inhibitory effect on cell viability by MTT assay and on cell migration. We validated the effect of MQ on xenografts nude mouse with a very significant decrease of tumor growth. All results suggest that V2R is a very relevant target and MQ could be a promising molecule for the diagnosis and treatment of renal cancer.

## 25

**POPDC proteins – potential regulators of compartmentalized cAMP signaling.**

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The popeye domain-containig protein (POPDC) family consists of three subtypes: POPDC1, POPDC2 and POPDC3. These transmembrane proteins are mainly expressed in heart- and skeletal muscle cells and contain a highly conserved C-terminal “Popeye Domain”. It appears to play a crucial role in cAMP signaling and cAMP-dependent protein-protein interactions (1,2). Intriguingly, it shares almost no sequence homologies with cyclic nucleotide binding domains (CNBDs) of all other mammalian cAMP effector proteins. Therefore, the mode of direct cAMP binding remains elusive, as well as the function in GPCR signaling (2,3). Studies have shown that point mutations in the Popeye Domain can lead to cardiac arrhythmia and heart failure in animal models (4), and to cardiac arrhythmia and muscular dystrophy in patients (2,5). In hippocampal neurons, POPDC1 was shown to be involved in phosphodiesterase (PDE)4-dependent memory consolidation processes (6). PDEs shape the specificity of GPCR signaling by creating domains of low cAMP around effector proteins, and PDE4 is essential for modulating downstream beta-adrenergic receptor signaling (7).
In this regard, our goal was to monitor cAMP signaling dynamics in the vicinity of POPDC1 following beta-adrenergic receptor stimulation, and to investigate the functional interaction between POPDC1 and PDE4. Therefore, we transiently expressed a novel FRET-based biosensor fused to POPDC1 in HEK293 cells. The results show a strongly reduced FRET-response to beta-adrenergic stimulation in the vicinity of POPDC1, as well as an amplified response to PDE4 inhibition. These results were further substantiated in PDE4D knock-out cells, where the aforementioned effect was diminished. The same effect could not be observed following inhibition of PDE8, another PDE long isoform, suggesting a possible PDE4 specific interaction and, with that, a containment of POPDC function to cAMP signaling pathways modulated by PDE4. This makes POPDC a candidate for regulating the precision of the beta-adrenergic signaling cascade.

## 26

**Fluorescence spectroscopy of low-level endogenous β-adrenergic receptor expression at the plasma membrane of differentiating human iPSC-derived cardiomyocytes.**

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The potential of human induced pluripotent stem cells (hiPSCs) to be differentiated into cardio-myocytes (CMs) mimicking the adult CMs functional morphology, marker genes and signaling characteristics has been investigated since over a decade. The evolution of the membrane localiza-tion of CM-specific G protein-coupled receptors throughout differentiation has received, however, only limited attention to date. We employ here advanced fluorescent spectroscopy, namely lines-can Fluorescence Correlation Spectroscopy (FCS), to observe how the plasma membrane abun-dance of the β1- and β2-adrenergic receptors (β1/2-ARs), labelled using a bright and photostable fluorescent antagonist, evolves during long-term monolayer culture of hiPSC-derived CMs. We compare it to the kinetic of observed mRNA levels in wildtype (WT) hiPSCs and in two CRISPR/Cas9 knock-in clones. We conduct these observations against the backdrop of our recent report that β2-ARs, as opposed to β1-ARs, specifically segregate to the T-Tubular system of adult CMs.

**27**

**Structural dynamic of a class C G-protein coupled receptor: single molecule FRET study.**

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Abstract:
Metabotropic glutamate receptors (mGluRs) are class C G-protein coupled receptors (RPCG) essential for the fine tuning of the glutamate response in synapses of the central nervous system. Upon activation by glutamate binding on the extracellular domain, these dimeric receptors undergo a multidomain conformational reorganization that allows for G-protein interaction. Understanding these conformational changes is key for the design and discovery of innovative drugs.
Here we use genetic code expansion to introduce two distinct non-canonical amino acids in response to the introduction of two different stop codons in mammalian cells. This allows us to control the positioning of donor and acceptor fluorophores to produce orthogonally labeled receptors for single molecule FRET measurements. Single molecule FRET changes in presence of different ligands give information about the conformational changes that the receptors undergo while lifetime measurements uncover the dynamic of these changes(1). Our double labeling strategy has the potential to accurately account for photophysical changes of the fluorophores upon attachment to the receptor to perform accurate FRET and distance measurements(2).
Measuring accurate distances by single molecule FRET will be central for the characterization and modeling of the conformational transformations as well as intermediate states explored by mGluRs and induced by both orthosteric and allosteric ligands(1).

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**28**

**Mitochondrial-targeted caged melatonin supports the presence of melatonin MT1 receptors in mitochondria inhibiting mitochondrial respiration.**

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Melatonin is a hormone that modulates physiological functions such as circadian rhythms, sleep and seasonal reproduction. These effects are mainly exerted through the activation of the G protein-coupled MT1 and MT2 receptors. Recent evidence revealed the presence of melatonin MT1 receptors in mitochondria, in addition to the plasma membrane. However, the different functions of the mitochondrial vs. plasma membrane MT1 receptors are poorly understood. Melatonin is a highly lipophilic molecule that binds both plasma membrane and mitochondrial receptors without distinction. Therefore, developing innovative tools that selectively target mitochondrial MT1 receptors could be helpful to elucidate the role of these intracellular receptors. One interesting approach is to design ligands with photo-activable properties, using light to modulate biological systems with spatiotemporal precision.

In this context, we describe the synthesis and validation of MCS-1145; a mitochondria-targeted caged melatonin. MCS-1145 selectively accumulates in mitochondria and releases melatonin upon light exposure. Exogenous melatonin and the uncaged light-activated form of MCS-1145 inhibit the respiration of mitochondria isolated from HEK293 cells expressing MT1, but not in mock cells or MT2-expressing cells. Similar effects were observed in mitochondria isolated from mouse cerebellum of WT mice but not in MT1 knockout mice. Overall, these results show that melatonin inhibits mitochondrial respiration through the activation of mitochondrial MT1 receptors.

**29**

**Pleiotropic prodrugs with potential therapeutic interest in Down Syndrome and Alzheimer's disease.**

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With age, people with Down syndrome (DS) develop dementia due to Alzheimer's disease (AD). Both pathologies share a high production of beta-amyloid peptides from the APP gene located on human chromosome 21 and several of studies suggest that a common pathogenic mechanism exists between AD and DS, involving alterations in neurotransmitter systems (the cholinergic, GABA-ergic, serotonergic, glutamatergic and adrenergic). Therefore, some common therapeutic targets have been identified. Faced with the multifactorial origin of these diseases, a pleiotropic intervention is now widely recommended.
This project aims at designing new pleiotropic prodrugs for intranasal administration, with potential therapeutic interest in DS and AD. The prodrugs will be activated by the covalent inhibition of butyrylcholinesterase (BuChE), capable of counteracting cholinergic neurodegeneration in AD. The released drugs will then target either the beta2-Adrenergic or 5-HT4 serotonin receptors or the pro-neuroinflammatory HMGB1 protein, in order to display a potential additional neuroprotective effect. Liposomal formulation and intranasal administration will enhance CNS distribution of prodrugs for selective activation by brain BuChE, to enhance central effects of released drugs and potentially avoid their peripheral side effects. The pharmacological evaluation of first compounds will be presented.

## 30

**TRPV1 modulation of MOR signalling.**

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Opioids are still the mainstay treatments for pain relief. However, their use is associated with severe adverse effects. Despite significant drug discovery efforts, the search for safer opioids has not been successful yet. This highlights the need for novel strategies that promote antinociceptive signalling while sparing the regulation processes associated with tolerance. Transient receptor potential vanilloid 1 (TRPV1) channel, the cation channel activated by capsaicin, is co-expressed with the mu-opioid receptor (MOR) in dorsal root ganglia neurons (DRGs) and has been proposed to modulate MOR signalling, however, the cellular mechanisms involved in MOR/TRPV1 regulation are not yet understood.

We used transfected HEK 293 cells to study how activation of TRPV1 affects MOR signalling using a range of BRET sensors. To study co-expression patterns, we used a ligand-directed fluorescent probe NAI-A594 and anti-TRPV1 and anti-MOR antibodies. Mouse DRGs were dissected and cultured overnight before being labelled.

BRET assays showed that activation of TRPV1 fully prevents agonist-induced arrestin-3 and GRK2 recruitment to the MOR and partially reduces agonist-mediated G protein activation. TRPV1 activation does not influence the recruitment of a conformationally selective nanobody (Nb33) or mini Gi protein recruitment. TRPV1 activation also affected the regulation of other GPCRs like the delta opioid (DOR) and neurokinin NK1 receptors. Similarly, activation of transient receptor potential ankyrin 1 (TRPA1) channel produced the same effects on MOR signalling as TRPV1 activation.

Our results demonstrate that TRPV1 activation modulates signalling pathways downstream of G protein activation without directly affecting MOR activation. This indirect interaction between MOR and TRPV1 is supported by the fact that TRPV1 modulates other GPCRs comparably, and TRPA1 activation modulates MOR signalling in a similar manner, suggesting that the specificity of TRPV1 modulation may arise from co-expression patterns of receptors and signalling proteins in specific cell types including DRGs rather than a direct interaction.

**31**

**Spatiotemporal signalling and location of fractalkine receptor CX3CR1 and its natural genetic variants associated to disease CX3CR1-V249I/T280M and CX3CR1-A55T.**

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Fractalkine receptor CX3CR1 plays important roles in inflammation and immunity, as well as in neuron-microglia communication. Natural genetic variants of CX3CR1 have been found associated to disease, among them the double polymorphic variant CX3CR1-V249I/T280M of not fully described pharmacology, and the rare genetic variant CX3CR1-A55T, proposed to be deficient in G protein coupling.
Activation of ERK1/2 signalling by CX3CR1 promotes relevant cellular responses. We aimed at studying the spatio-temporal compartmentalization of ERK1/2 signalling by CX3CR1 in response to fractalkine as well as at deciphering its signalling components. The signalling and trafficking of CX3CR1 natural genetic variants is also investigated.
Fractalkine promoted recruitment of β-arrestin 1, β-arrestin 2, and GRK2 by CX3CR1 in transfected HEK293 cells as by assessed by BRET-based assays, with potency similar for the three partners (EC50 values ≈ 1 nM). While CX3CR1-V249I/T280M variant displayed increased efficacy at engaging β-arrestins and GRK2 (146% to 220% of wild-type receptor), CX3CR1-A55T displayed a diminished capacity to promote β-arrestin 2 recruitment (46% of wild-type). The three receptor variants trafficked from the plasma membrane to intracellular compartments in response to fractalkine, with slight differences in trafficking dynamics among the receptor variants.
Fractalkine elicited extracellular-signal regulated kinase (ERK) 1/2 signalling in the cytosolic and in the nuclear compartments as assessed by FRET-based biosensors of ERK1/2 activity. Both the cytosolic and nuclear ERK activation were abolished by disruption of Gi/o signalling. While cytosolic ERK signalling was completely abolished by downregulation of β-arrestins, the nuclear signalling resulted only partially dependent on β-arrestins. More importantly, nuclear ERK signalling was fully dependent on dynamin function, opposite to cytosolic ERK activity that was fully independent.
Our results show compartmentalized ERK signalling by CX3CR1 with involvement of different signalling components. The altered engagement of signalling partners/scaffolds and trafficking by CX3CR1 genetic variants associated to disease might have pathophysiological implications.

## 32

**Development of tool compounds for the orphan MAS-related G protein-coupled receptor MRGPRX2.**

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The orphan MAS-related G protein-coupled receptor (GPCR) X2 (MRGPRX2) is a member of the primate-specific MRGPRX family, which belongs to the δ-branch of rhodopsin-like, class A GPCRs.1 MRGPRX2 is mainly expressed on sensory neurons2,3 and immune cells (e.g. mast cells, eosinophils, and basophils).2,3 It was recently identified as an important mast cell receptor responsible for anaphylactoid drug reactions and is involved in skin and mucosal diseases including urticaria, atopic dermatitis, rosacea, and allergic rhinitis.4,5 Thus, MRGPRX2 antagonists likely have great potential as therapeutic drugs. So far, only weakly potent MRGPRX2 antagonists have been described in the literature. Our research project has been aimed at developing drug-like molecules as pharmacological tool compounds to study MRGPRX2 signaling and to validate MRGPRX2 as a novel drug target.

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**33**

**Bioluminescence Resonance Energy Transfer and NanoLuciferase-based complementation assays for the real-time monitoring of mitochondrial protein interactions.**

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Mitochondria are central organelles in the mammalian cell that contains proteins which interact together to produce varied biological activities including ATP synthesis, fatty-acid oxidation, reactive oxygen species generation, calcium homeostasis among others. However, dysfunction in any of these biological processes due to poor protein-protein interactions have been implicated in many metabolic diseases. In this study, we applied the Bioluminescence Resonance Energy Transfer (BRET) and the split NanoLuciferase assays to study the interactions between mitochondrial proteins which are located at the outer-membrane, intermembrane space and in the matrix respectively in live HEK 293 cells and in real time. Our results show that the BRET and NanoLuciferase-based assays are powerful tools that can be successfully used to query the interactions between different mitochondrial proteins including GPCRs and their functional localization in their specific sub-compartments.

**34**

**Kinetic analysis of endogenous β2-adrenoceptor-mediated cAMP GloSensorTM responses in HEK293 cells.**

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Standard pharmacological analysis of ligand activity utilises measurements of receptor-mediated responses at set time-points, or peak response levels, to determine empirical parameters such as potency (EC50) and efficacy (EMAX). However, the occurrence of non-equilibrium conditions and differential effects of regulatory mechanisms on the signal may account for large discrepancies between ligand parameters in the literature. Derivation of new kinetic equations for curve fitting time-course data has enabled quantification of new ligand parameters for kinetic potency (L50) and maximal initial rate (IRMAX), related to efficacy[1]. In this study, the kinetics of ligand-mediated cAMP responses elicited by endogenous β2-adrenoceptor (β2AR) in HEK293 cells, which express β2ARs at extremely low levels[2], were examined by curve fitting of cAMP GloSensorTM luminescence time-course data. Kinetic parameters were compared with equivalent standard pharmacological parameters. The rank order of efficacy of the tested β2AR agonists remained the same by both methods of analysis (isoprenaline > formoterol > salmeterol), but the partial agonist salmeterol displayed both a reduced IRMAX value compared with its EMAX value, and a reduced L50 value compared with its EC50 value. This is likely to be a consequence of salmeterol's slow onset of action and slow dissociation rate at the β2AR. Additionally, the effect of preincubation of β2AR antagonists with distinct receptor dissociation rates on isoprenaline-mediated responses was examined. Except the fast-dissociating bisoprolol, the antagonists concentration-dependently reduced the isoprenaline peak response, due to a state of hemi-equilibrium. This effect was exacerbated in terms of isoprenaline initial rate, whereby even bisoprolol reduced the maxima. The slowly dissociating antagonist carvedilol abolished the isoprenaline initial rate due to a lack of receptor reserve. Kinetic analysis of signalling data can improve our understanding of pharmacological properties and mechanisms of action of ligands at their target receptors. This should enable more accurate ligand characterisation and ultimately improve therapeutic development.

**35**

**FRET-based Kappa Opioid Receptor-Sensor reveals basal activity and kinetics of voltage sensitivity.**

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Several GPCRs are already classified as voltage sensitive. A modulation of the membrane potential influences the activity of the receptor. An important type of voltage sensitive GPCRs are the opioid-receptors. We recently investigated the μ-opioid receptor and found a ligand specific voltage sensitivity, which had a considerable effect on the ability of opioid analgesics to activate μ-receptors.

In this study, we investigated the kappa-opioid-receptor (KOR) in terms of voltage dependence with a combination of FRET and patch clamp. For further investigations of the KOR, we established a FRET-based receptor-sensor. With this sensor we, on the one hand, were able to perform FRET measurements of multiple cells at a plate reader and, on the other hand, were able to characterize kinetics of the voltage effect displayed by the KOR.

In a FRET-based G-protein activation assay we found that the high level of constitutional activity of KOR caused reduced G-protein signaling. With preincubation of the opioid antagonist naloxone, we observed a recovery in G-protein signaling, which allowed for measurements of the KORs voltage dependence. Surprisingly we observed a voltage dependence of the KOR in presence and absence of the endogenous agonist dynorphin A. These findings could be confirmed in other assays. To our knowledge, this is the first observation of a ligand free voltage dependence of an unmutated GPCR. Further, by the use of the FRET-based receptor-sensor, we were able to resolve kinetics of the voltage induced activation with and without agonist. In a further step, we investigated the naloxone effect at different assays and observed inverse agonism of naloxone.

In conclusion, KOR showed activation upon depolarization in presence and absence of agonist. Further, KOR displayed basal activity, which could be detected directly at the conformation level. This might be linked to our observation of the voltage dependence in absence of agonist.

**36**

**Application of BRET Method to Investigate the Functional Properties of GPCR-like Proteins of the Plasmodium falciparum.**

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Malaria is one of the deadliest diseases in the world and accountable for the death of more than 400,000 people in tropical and sub-tropical regions. The responsible pathogens belong to the Plasmodium spp. of apicomplexan protozoa and among them; Plasmodium falciparum is the most virulent with highest fatalities. The clinical symptoms of malaria are associated with the periodic rupture and exponential increase of parasites in the host circulation system. The host hormone melatonin has been reported to synchronize the asexual cell cycle of P. falciparum. Melatonin also modulates the intracellular calcium, and interplay between melatonin and calcium is crucial for parasite maturation. However, the mechanism underlying the melatonin-induced signaling is still lacking including its receptor and signaling pathways. In mammals, melatonin binds to G-protein coupled, 7-transmembrane (7TM) receptors MT1 and MT2. No such homologs have been identified in Plasmodium. Genome-wide analysis leads us to identify four GPCR candidates (SR1, SR10, SR12 and SR25) in P. falciparum. Little is known about the functionality of these GPCR candidates. Direct immunofluorescent assays indicates that both SR1 and SR10 express on the surface of parasites. Nanoluciferase complementation experiments of SR1 and SR10 tagged with HiBiT at their N-termini and addition of extracellular LgBiT indicates that the N-termini of both SR1 and SR10 face the extracellular milieu and that SR10 facilitates surface expression of SR1. Further signaling and bioluminescence resonance energy transfer (BRET)) experiments are ongoing in HEK293 cells to decipher the function of SR1 and SR10. Our investigation will help to unravel the signaling mechanism of Plasmodium SR1 and SR10. More importantly this will allow us to understand the modulatory effect of melatonin in parasites. Disrupting melatonin-induced synchronization could be a potential therapeutic avenue for malaria intervention since current antimalarials are exhausting rapidly due to emerging drug resistant Plasmodium strains.

**37**

**Ligand screening for underexplored epitopes of G protein coupled receptors (GPCRs) with focus on free fatty acid receptor 3 (FFAR3) and endothelin receptor type B (ETBR).**

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G protein coupled receptors play an important role regarding the pharmacotherapy of many diseases. Nearly all the drugs that are in use bind to the orthosteric pocket. Hence, finding ligands bind to allosteric pockets to modulate the receptors signaling opens up new potential. As part of the GPCR ligands for underexplored epitopes (GLUE) interdisciplinary project, we are screening for novel ligands with a combined in silico and in vitro approach.

Small molecules identified as top candidates by molecular docking analysis were synthesized and evaluated in different Förster resonance energy transfer (FRET)-based single cell assays. Those assays were performed either in intact or permeabilized cells and differ in their part of the signal cascade.

We started to differentiate between ligands that could influence the signaling of the GPCRs of those that showed no effect. Therefore, all the ligands were tested in two main assays, in a receptor conformation sensor and the G protein binding assay in two model receptors, the FFAR3 and ETBR. Since we are also addressing epitopes like the G protein binding pocket, we need to make sure, the ligand can reach this region. So, we permeabilize the cells in the first testing part. The conformation sensor can show, whether the ligand can influence the receptors conformation by itself. The G protein binding assay under nucleotide-free conditions can elucidate its impact on the direct interaction between receptor and the heterotrimeric G proteins. Moreover, ligands that show an interaction are further characterized in downstream assays.

Our assays can identify ligands that have an impact on the functionality of GPCRs and their interaction proteins. Addressing alternative epitopes with impact on receptor function is a challenging and important new route to develop better drugs.

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**β-arrestin1 and 2 exhibit distinct phosphorylation-dependent conformations when coupling to the same GPCR in living cells.**

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β-arrestin1 and 2 are hypothesized to mediate targeted processes for more than 800 different G protein-coupled receptors (GPCRs). They have been shown to act as important regulators of GPCR signaling, internalization, and trafficking. To facilitate this, the individual β-arrestin isoforms have been shown to adopt different active conformations resulting from the geometry of the specific GPCR–β-arrestin complex. However, whether β-arrestin1 and 2 respond differently upon binding to the same GPCR is still unknown. The aim of the study was to compare the complex configurations of β-arrestin1 and 2 for binding to the parathyroid hormone 1 receptor (PTH1R), including the assessment of β-arrestin conformational changes and their initial downstream functionality.
With advanced NanoLuc/FlAsH-based β-arrestin1 and 2 biosensors which were labelled in eight independent positions, we reveal the comprehensive signature of conformational changes for both β-arrestin isoforms when interacting with the ligand-activated and phosphorylated PTH1R (P-R* receptor state) in living cells. Using our recently published CRISPR/Cas9 mediated GRK2,3,5,6 knockout cell line, we repeated these measurements in the absence of GPCR kinases (GRKs) (R* receptor state). Hence, this approach enables the differentiation between conformational changes that are induced by P-R* or R* receptor states. Additionally we used two distinct phosphorylation-deficient receptor variants (devoid of either proximal or distal C-terminal phosphorylation sites). This approach further discloses the impact of site-specific GPCR phosphorylation on arrestin-coupling and function. Furthermore, we use live-cell confocal microscopy, cellular localized bystander-BRET assays and additional knockout cell lines to characterize formed complexes and their intracellular localization.
With this study, we demonstrate that the GPCR phosphorylation state not only regulates differences in affinity between β-arrestin1 and 2 but also translates into specific conformational rearrangements that determine the functional diversity between the two isoforms.

**39**

**Identification of an N-acylated-D-Arg-L-Leu-NH2 Dipeptide as a Highly Selective Neuropeptide FF1 Receptor Antagonist That Potently Prevents Opioid-Induced Hyperalgesia.**

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RFamide-related peptide-3 (RFRP-3) and neuropeptide FF (NPFF) target two different receptor subtypes called neuropeptide FF1 (NPFF1R) and neuropeptide FF2 (NPFF2R) that modulate several functions. However, the study of their respective role is severely limited by the absence of selective blockers. We describe here the design of a highly selective NPFF1R antagonist called RF3286, which potently blocks RFRP-3-induced hyperalgesia in mice and luteinizing hormone release in hamsters. We then showed that the pharmacological blockade of NPFF1R in mice prevents the development of fentanyl-induced hyperalgesia while preserving its analgesic effect. Altogether, our data indicate that RF3286 represents a useful pharmacological tool to study the involvement of the NPFF1R/RFRP-3 system in different functions and different species. Thanks to this compound, we showed that this system is critically involved in the development of opioid-induced hyperalgesia, suggesting that NPFF1R antagonists might represent promising therapeutic tools to improve the use of opioids in the treatment of chronic pain.

**40**

**Characterization of the melatonin type 1 receptor / cannabinoid type 1 receptor (MT1/CB1) heteromer.**

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Membrane receptors belonging to the G proteins coupled receptors (GPCRs) form the largest family of proteins in the human genome with more than 800 members. GPCR functions were initially thought to take place at the plasma membrane upon ligand binding to single monomeric receptor units. This scheme was complexified after identification of cross-talks between GPCRs upon heteromerization and identification of functional GPCRs within intracellular organelles as nucleus or endosomes. Among a few others receptors, functional melatonin receptor type 1 (MT1) and cannabinoid receptor type 1 (CB1) were found in neuronal mitochondria, suggesting a cross-talk between them. Here, we are identifying and characterizing the MT1/CB1 complex as a new GPCR heteromer.
Confocal analysis of immunofluorescence experiments of cells coexpressing both receptors pinpointed a high degree of colocalization in transfected cells. Coimmunoprecipitation experiments performed on transfected cells and on mice cerebellum from wild-type, MT1 or CB1 knock-out mice identified a constitutive heteromerization between MT1 and CB1 receptors. The existence of the MT1/CB1 complex was further confirmed by Proximity Ligation Assay (PLA) and Nanoluciferase complementation experiments performed on transfected cells. Use of interfering peptides targeting the suspected CB1 dimerization interface provoked MT1/CB1 dissociation. Subcellular distribution studies by immunofluorescence and electronic microscopy revealed the presence of the MT1/CB1 complex within the mitochondrial network and the plasma membrane. Co-stimulation by melatonin and Win 55,212-2, a synthetic CB1 agonist, of HEK293 cells coexpressing MT1 and CB1 resulted in a Win 55,212-2 dependent abrogation of MT1-dependent cAMP inhibition. Measure of mitochondrial respiration performed on semi-purified mitochondria obtained from cerebellum of wild-type, MT1 or CB1 knock-out mice or transfected cells show a synergistic effect of both ligands. Characterization of actions caused by a direct cross-talk on the level of MT1 or CB1 receptors or by downstream cross-talk is ongoing.

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**Chemogenetic G protein-ligand pairs for causal investigation of cellular biology in vitro and in vivo.**

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Chemogenetics collectively refers to approaches employing pairs of engineered proteins and their specific ligands, which together enable non-invasive chemical control over cell populations in vitro and in vivo. A fundamental requirement for the functionality of chemogenetic toolboxes is the amenability of the engineered proteins to activation or inhibition by ligands otherwise inert in the absence of said protein.
In this project, we aim to elucidate whether heterotrimeric G protein subunit Gαq and its highly selective inhibitor FR900359 (FR) qualify for the development of a chemogenetic toolbox for the family of heterotrimeric G proteins, so far unexplored by chemogenetic approaches.
Previously, we reported on engineered variants of Gq that retain wildtype-like biological activity, yet are refractory to FR inhibition, and we have employed these to verify that FR acts in cells by specific inhibition of Gαq, Gα11 and Gα14 proteins without any overt off-target effects or toxicities (Patt et al., JBC 2021). We also successfully transplanted FR inhibition to G proteins that are naturally not FR regulated. (Malfacini et al., JBC 2019; Boesgaard et al., JBC 2020). Thus, our protein-ligand pair is compatible with in vitro chemogenetic applications.
Based on these findings, we put forward the hypothesis that FR qualifies to enable chemical control over G protein pathways in vivo. We tested our assumption utilizing Gαq proteins in the fruit fly, Drosophila melanogaster, a genetically tractable model organism which enables detailed dissection of signaling routes in vivo. Here, I will show that engineering of Drosophila Gαq variants with the desired phenotypes is more challenging than initially anticipated. Despite all challenges, we expect our protein-ligand pairs to be no less compatible with chemogenetic applications in vivo.

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**42**

**The off-rate of agonists does not influence the stability of G-protein-coupled receptor—arrestin complexes**.

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Many G-protein-coupled receptors (GPCRs) interact with arrestins upon agonist stimulation and are subsequently internalized. Some GPCRs internalize on their own, leaving arrestin at the plasma membrane (class A) whereas others co-internalize with arrestins into endosomes (class B). It has been hypothesized that class B GPCRs have a higher affinity to arrestins than class A GPCRs. The dissociation rate of the receptor-arrestin complex depends on both the affinity of arrestin to the GPCR and the dissociation rate of the agonist from the GPCR. Hence the question appears whether agonists with a very slow dissociation rate from the receptor are able to form particularly stable GPCR-arrestin complexes and thus might convert a class A to a class B GPCR.

We followed the dissociation rate of GPCR-arrestin3 complexes upon agonist washout using fluorescence resonance energy transfer (FRET) between YFP-labelled GPCRs and CFP-labelled arrestin3 and used fluorescence recovery after photobleaching (FRAP) to investigate the stability of GPCR-arrestin3 receptors in the continuous presence of agonists. We employed two class A GPCRs, the β2-adrenergic (β2AR) and the μ-opioid receptor (μOR). At the β2AR we employed the full agonists isoprenaline, adrenaline and BI-167,107. At the μOR we used the full agonists DAMGO and etorphine and the partial agonist morphine.

Upon agonist washout in FRET experiments we observed washout kinetics for all ligands employed except BI-167,107. However, in FRAP experiments the stability of β2AR-arrestin complexes was independent of the employed agonist. BI-167,107 could not convert the β2AR into a class B receptor, and etorphine could not convert the μOR into a class B receptor. In FRAP experiments, the μOR-arrestin3 complex in the presence of morphine was substantially less stable than the μOR-arrestin3 complex in the presence of DAMGO.

We conclude that agonists with slow off-rate cannot convert a class A into a class B receptor.

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**Development of subtype-selective photoswitchable positive allosteric modulators for mGlu receptors.**

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Positive allosteric modulators (PAMs) for metabotropic glutamate receptors have been postulated to treat neuropsychiatric diseases. Besides, obtaining a reversible and efficient spatiotemporal control of mGlu activity would be therapeutically advantageous. Photopharmacology may provide a solution on this topic, since it is based on the use of light and photoswitchable ligands to modulate a protein activity. This approach offers new perspectives for drug discovery and promises a better drug action control reducing side effects to unattained levels.

Optogluram is active as a PAM of mGlu4 and mGlu6 in its trans configuration and loses activity with 380 nm illumination in its trans configuration and loses activity with 380 nm illumination. The active isomer was recovered upon illumination with 500 nm light. Here, we present a new series of 6 different analogues of Optogluram, from which Optogluram-2 emerges as a new more selective photoswitchable PAM of mGlu4 receptor. The potency of Optogluram-2 in mGlu4 is slightly lower than that for Optogluram. However, the selectivity versus mGlu6 is higher.

We have also developed photoswitchable PAMs for mGlu1 and mGlu4 in order to precisely switch on/off the activity of the receptor with light. Replacing the phtalimide moiety of a known mGlu1 PAM with a N=N bond led to azobenzene candidates. Subsequent in vitro assays revealed that Photoglurax-2 is a mGlu1 PAM ligand in the dark, whereas it loses activity under 380 nm light. It derived from the equipotent mGlu1/4 PAM Photoglurax-1 converted into a highly selective mGlu1 PAM.

Overall, we are offering to the scientific community tool compounds to fine control the activity of a mGlu subtype with light and with a high selectivity. This will allow to study pharmacological and physiological implications those mGlu subtypes with an unprecedented precision, which may lead to unexpected findings in neuroscience.

**44**

**Upregulated apelin signaling in pancreatic cancer activates oncogenic signaling pathways and glycolytic metabolism to promote tumor development.**

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Despite decades of effort and medical advances in understanding pancreatic ductal adenocarcinoma (PDAC), there is still a lack of innovative targeted therapies for this aggressive and devastating disease. Herein we report the expression of apelin and its receptor, the G protein-coupled receptor APJ, in human pancreatic adenocarcinoma and its protumoral function. Characterization of the spatio-temporal pattern of expression of apelin signaling in an engineered mouse model of PDAC revealed different localization of apelin signaling. In premalignant lesions, apelin was expressed in epithelial lesions whereas APJ was found in isolated extrainsular endocrine cells tightly attached to premalignant lesions. However, in invasive stage, apelin and APJ were co-expressed by tumor cells as observed in human PDAC. In human tumor cells, apelin induced a long-lasting activation of PI3K/Akt/GSK-3, which was Gi protein-independent but relied instead on apelin receptor internalization. Through this signaling cascade, apelin upregulated -catenin as well as the oncogenes c-myc and cyclin D1, thereby fostering pancreatic tumor growth through its capacity to promote proliferation and migration of cancer cells. Moreover, apelin positively regulated expression of Glut1 and hexokinase II, two key components of aerobic glycolysis, thereby increasing glucose uptake by tumor cells. Apelin receptor blockade by shRNA-mediated silencing reduced cancer cells proliferation induced by endogenous secreted apelin along with a subsequent reduction in pancreatic tumor burden. These findings identify apelin signaling pathway as a new actor of pancreatic cancer development and a novel therapeutic target for this incurable disease.

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## Characterisation of the transducer coupling profiles of PAC1 receptor splice isoforms.

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The PAC1R is a promising drug target for the treatment of numerous CNS disorders where there is an unmet clinical need. This may be due to the complexity of signalling downstream of PAC1R activation and is further complicated by the existence of PAC1R alternative splicing in the N-terminal extracellular domain (ECD) and intracellular loop 3 (ICL3). N-terminal ECD is important for peptide recognition while ICL3 is critical for transducer activation. Hence, alternative splicing in these regions may alter ligand selectivity and intracellular transducer engagement.

Here, G-protein activation and β-arrestin recruitment profiles of the PAC1R splice isoforms were characterised, including the most common isoform (PAC1nR), variants with a truncated ECD (PAC1sR), and/or variants containing ICL3 insertions (hip, hop or hiphop). PAC1R isoforms and TRUPATH G-protein or β-arrestin BRET biosensors were transiently transfected into COS-7 cells and treated with increasing concentrations of PAC1R agonists: PACAP38, PACAP27, VIP and maxadilan. G-protein dissociation profiles of Gs, Gi, Gq/11 and G12 and β-arrestin recruitment profiles of β-arrestin 1 and 2 were measured at 37°C.

Truncation of the N-terminal ECD (PAC1sR) enhanced G-protein activation and β-arrestin recruitment mediated by the low-affinity agonist, VIP. Alternative splicing in ICL3 resulted in distinct G-protein activation and beta-arrestin recruitment profiles induced by all four agonists investigated. PAC1nR-hop displayed a three-fold increase in PACAP38 potency for Gq coupling compared to PAC1nR. While PAC1nR-hip displayed weaker Gi1 coupling and β-arrestin1/2 responses compared to PAC1nR.

Altered G-protein activation and β-arrestin profiles of the PAC1R ICL3 variants contribute to their overall signalling profile, while splice isoforms in the N-terminal ECD enhanced transducer coupling of the low-affinity ligand, VIP, which may be indicative of enhanced ligand affinity. The results from this study provide a comprehensive analysis describing how alternative splicing affects PAC1R transducer engagement and provide insight into the signalling mechanisms of PAC1R.