European Research Network on Signal Transduction (ERNEST)
COST Action CA18133A

THIRD ERNEST MEETING - Online

Signal transduction:
From the genomic to the systems level

October 12-14, 2020

ABSTRACT BOOKLET

Conference Website: www.ernestcostaction.wixsite.com/website

#3rdERNESTMeeting @ERNESTNetwork

Greetings

We are pleased to announce the 3rd Meeting of the European Research Network on Signal Transduction (ERNEST COST Action CA18133) with the theme of *Signal transduction: From the genomic to the systems level (and everything in between)* on October 12-14, 2020.

We are living in unprecedented times. When ERNEST held its first general meeting a year ago in Belfast, none of us could have anticipated that the most disruptive pandemic in history was just a few months away. As we planned the second ERNEST meeting for late March 2020 in Istanbul, we watched the news worryingly as coronavirus spread quickly throughout Europe. Many ERNEST members had to cancel their meeting registration as they were no longer allowed to travel. Eventually, on the 13th of March, COST cancelled all in-person meetings of its Actions. ERNEST moved quickly to bring its planned meeting online in time, March 28-30, 2020. The virtual meeting was a success with over 200 people tuned in for the keynote address.

The theme of the 3rd ERNEST meeting relates to our main objective: to develop a comprehensive and holistic understanding of signal transduction. By linking the different levels at which GPCR signal transduction is modulated (*i.e.* at the level of genes, proteins, cells/tissues and systems), we can better understand signal transduction as a whole.

Although we will not be meeting in person, we have worked hard to develop an engaging and interactive online meeting program that will foster networking. ERNEST strives to support our community of GPCR researchers in these pandemic times. This meeting will bring together scientists at different career levels from all around Europe and the world and serve as an excellent global platform for researchers from industry and academia to learn, interact, share ideas, network and establish collaborations. Moreover, the meeting will showcase ECI's, their research, and their valuable role in the research community.

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About WuXi DEL

DNA Encoded Library (DEL) technology is a novel affinity selection technology that integrates chemistry, biology, bioinformatics and computational chemistry to facilitate a rapid drug discovery process, allowing access to more chemical entities with lower cost.

In contrast to high throughput screening, DEL requires a very small amount of target protein and minimum assay development. DEL selection can also be performed on the benchtop and does not require complex instrumentation. Most importantly, it disrupts the concept of "cost-per-well" and allows testing billions of compounds in one test tube. The rich data generated by DEL not only generates hits but also provides necessary information into hit-to-lead and lead-optimization processes.

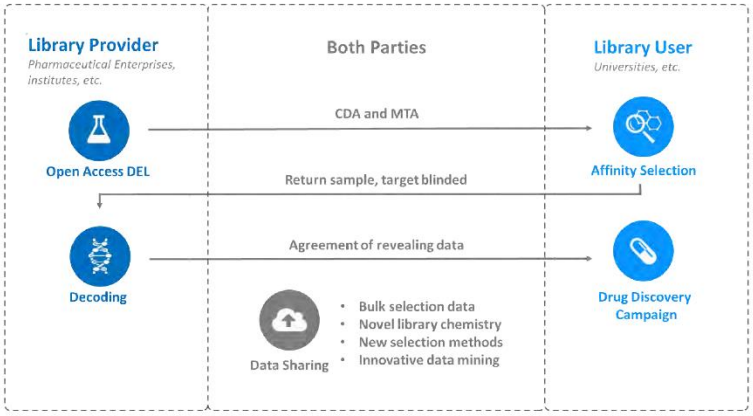
WuXi's DEL mission is to bring high quality DEL service to clients at affordable prices by providing flexible business models, project entry/exit points and customized selection strategies.

- More than 66 billion synthetic compounds and growing
- Proven success with nanomolar hits in various target classes
- Bioinformatics system to collect, process and analyze data, and to facilitate involvement of the client to make collective decisions
- International expert team located in North America, Asia and Europe
- Flexible business model to accommodate different needs

About DELopen

DELopen is a platform enabling industry and academic research institutions to connect and realize effective sharing of scientific research information and protection of intellectual property. Through this platform, researchers and institutions engaged in the development of new drugs with DEL technology can more quickly conduct early new drug development and push the results of early development to commercialization as soon as possible. The blockchain technology in the DELopen platform acts as a tamper-proof digital ledger that records activity in a transparent, secure, and accessible format. As a tool for improving security, transparency, and efficiency, it helps researchers and institutions protect their own target information.

About DELopen kit



- 4.4 billion compound collection
- Multiple solutions to unlock data
- Experiment first, contracts later
- Encouraging data sharing
- Double blinded screening
- IP protection for everyone

Included in the Kit

- Number of Libraries: 30
- Total compounds: 4.4 Billion
- Conditions available: 4
- $0 Initial Commitment

Included with No Additional Cost

- Sample processing at WuXi AppTec DEL platform
- NGS & Sequencing alignment
- Data summary report
- Data package without structure release
- Turn-around Time: 6 weeks

Follow-up Steps

- Pick top five hits
- Resynthesis and activity test by researcher
- Structure release options
- Data for Hits or Fee for Hits

Details / Quotation available via: DEL_service@wuxiapptec.com

COST Action CA18133 ERNEST European Research Network on Signal Transduction

Monday, Oct 12

9:00 - 9:30 (CET)	**Conference opening** Chairs: Antonella Di Pizio, Ali Isbilir & Chloe Peach **Martha Sommer and Jana Selent**
9:30 - 9:45	**BREAK**
9:45 - 11:15	**Session 1: Protein dynamics and the macromolecular interactions in signaling pathways (WG1)** Chair: Natalie Ben Abu & Lior Peri **9:45 - 10:15.** **Alisa Glukhova** (Monash University, Australia) S1.1 *'Structural basis for allostery and bias at the adenosine receptors'* **10:15 - 10:45.** **Alexey Bondar** (University of South Bohemia, Czech Republic) S1.2 *'Preassembly between G protein-coupled receptors and G proteins studied at the single-molecule level'* **10:45 - 11:00.** **Devrim Öz-Arslan** (Acibadem Mehmet Ali Aydinlar University, Turkey) S1.3 *'Investigation of new binding partners for ATG3 protein in a hepatoma cell line'* **11:00 - 11:15.** **Vaithish Velazhahan** (MRC Laboratory of Molecular Biology, Cambridge, UK) S1.4 *'Structure of the Class D GPCR Ste2 dimer coupled to two G proteins'*
11:15 - 11:30	**BREAK**
11:30 - 12:15	**Session 2: Collaboration with Frontiers Journals** Chairs: Milka Vrecl Fazarinc, Marialessandra Contino & Marcello Leopoldo **George Thomas** (Frontiers Media SA, Switzerland) **Session 3: Challenges and Chances of Multi-disciplinarity in GPCR research** Chair: Alexander Hauser **Marcel Bermúdez** (Free University, Berlin Germany)
12:15 - 12:30	**BREAK**
12:30 - 14:00	**Session 4: Biological roles and physiological contexts of signal transduction (WG2)** Chair: Itziar Muneta-Arrate **12:30 - 12:50. Meritxell Canals** (University of Nottingham, UK) S4.1 *'Efficacy and Bias at the mu-opioid receptor'* **12:50 - 13:10. Claudia Stäubert** (University of Leipzig, Germany) S4.2 *'Fermented food derived metabolites mediate innate immune cell function through highly potent activation of HCA3'* **13:10 - 13:30. Julien Hanson** (University of Liège, Belgium) S4.3 *'Functional characterization of the orphan GPCR GPR101'* **13:30 - 13:45. Anemari Horvat** (University of Ljubljana, Slovenia) S4.4 *'Noradrenergic signalling and metabolism are dysregulated in astrocytes with cytoplasmic ALS/FTD-linked TDP-43 inclusions'* **13:45 - 14:00. Andreas Bock** (Max-Delbrück-Center for Molecular Medicine, Berlin, Germany) S4.5 *'Optical mapping of cAMP signaling at the nanometer scale'*
14:00 - 15:00	**BREAK**
15:00 - 16:00	**Meet Today's Speakers & Networking** **Room 1:** *Alisa Glukhova, Alexey Bondar, Devrim Öz-Arslan, Vaithish Velazhahan, Marcel Bermudez* **Room 2:** *Meritxell Canals, Claudia Stäubert, Julien Hanson, Anemari Horvat, Andreas Bock, George Thomas* **Session 5: Dr.GPCR online platform for GPCR researchers** Chair: Alexander Hauser **Yamina Berchiche** (Dr.GPCR Founder, USA) *Beyond the Lab*
16:00 - 16:15	**BREAK**
16:15 - 17:45	**Session 6: Keynote Address, Q&A and Meet the Keynote** Chairs: Ali Isbilir & Chloe Peach **Roshanak Irannejad** (UCSF, USA) - *Regulation of GPCR Signaling by Transporters*
17:45 - 18:00	**BREAK**
18:00 - 19:00	**Poster session 1**

Tuesday, Oct 13

9:00 - 10:30 (CET)	**Session 7: GPCRdb and GPCRmd Introduction, Updates & Workshop** Chair: Jana Selent **Albert Kooistra & David Gloriam** S7.1 GPCRdb **Mariona Torrens & Jana Selent** S7.2 GPCRmd	**Session 8: Workshop HitS DEL platform by WuXi AppTec** Chair: Antonella Di Pizio **9:00 - 9:40. Dennis Gillingham** (University of Basel, Switzerland), S8.1 '*DNA multitasking in DNA encoded libraries*' **9:40 - 10:00. Nuska Tschammer** (WuXi AppTec HitS platform/Crelux, Germany), S8.2 '*DELopen and DELight Promote Early Drug Discovery by Implementing Self-service DNA-Encoded Libraries Platforms*' **10:00 - 10:30. Marco Potowski** (TU Dortmund University, Germany), S8.3 '*Initiating DNA encoded library synthesis on solid phase – chemistry development, library synthesis, and TEAD-YAP inhibitor identification*'
10:30 - 10:45	**BREAK**	
10:45 - 12:15	**Session 9: Design and optimization of molecular modulators of signal transduction (WG3)** Chair: Miriam Scarpa **10:45 - 11:15. György Keserü** (Research Center for Natural Sciences, Hungary) S9.1 *'Conservation of allosteric sites in G-protein coupled receptors'* **11:15 - 11:45. Christofer Tautermann** (Boehringer Ingelheim, Germany) S9.2 *'Drug residence time – and the role of water'* **11:45 - 12:00. Enza Lacivita** (University of Bari Aldo Moro, Italy) S9.3 *'Structure-kinetic relationship studies on serotonin 5-HT7 receptor ligands: role of the lipophilicity on residence time'* **12:00 - 12:15. Margarida Fardilha** (University of Aveiro, Portugal) S9.4 *'Protein phosphatase 1 disrupting peptides as a strategy to modulate sperm motility'*	
12:15 - 12:30	**BREAK**	
12:30 - 14:00	**Session 10: Workshop** Chair: Yaron Ben-Shoshan Galeckzi **Mickey Kosloff** (University of Haifa, Israel) *3D Structures in GPCR Signalling - a primer for non-experts*	**Session 11: Industry Innovators** Chair: Desislava Nesheva **12:30 - 13:00. Eric Trinquet** (CisBio, France) S11.1 '*No-wash technologies to investigate cell signalling pathways in disease relevant cellular models*' **13:00 - 13:30. Frank Birke** (PicoQuant, Germany) S11.2 '*Lifetime for Life Science*' **13:30 - 14:00. Yaroslav Nikolaev** (InterAx Biotech, Switzerland) S11.3 '*AI-meets-RI (Artificial meets REAL intelligence) approach to GPCR drug discovery*'
14:00 - 15:00	**BREAK**	
15:00 - 16:00	**Meet Today's Speakers & Networking** **Room 1:** *György Keserü, Christofer Tautermann, Enza Lacivita, Margarida Fardilh* **Room 2:** *Dennis Gillingham, Nuska Tschammer, Marco Potowski; Eric Trinquet, Frank Birke, Yaroslav Nikolaev*	**Session 12: Academia or Industry?** Chair: Maria Majellaro **Christofer Tautermann** (Boehringer Ingelheim, Germany) **Meritxell Canals** (University of Nottingham, UK) **Willem Jespers** (Uppsala University, Sweden)
16:00 - 16:15	**BREAK**	
16:15 - 17:45	**Session 13: Mapping Group: towards a multidimensional understanding of GPCR signal transduction** Chair: Jana Selent **Opening:** Jana Selent **Numbers & Facts:** Mariona Torrens **Pilot project**: Maria Martí & Ulrike Wittig **Round table:** Maria Martí, Jana Selent, Maria Dost, Ulrike Wittig	
17:45 - 18:00	**BREAK**	
18:00 - 19:00	**Poster session 2**	

Wednesday, Oct 14

9:00 - 10:30 (CET)	**Session 14: Advanced methods and technologies in signal transduction research (WG4)** Chair: Maris-Johanna Tahk **9:00 - 9:30. Rima Budvytyte** (Life Sciences Center, University of Vilnius, Lithuania) S14.1 *'Tethered Bilayer Lipid Membranes - a Platform for Studies of Protein-Lipid Interaction and Biosensing* **9:30 - 10:00. Mat Leveridge** (Sosei Heptares, Cambridge, UK) S14.2 *'A look inside the GPCR Structural Pharmacology toolbox: From hit ID, H2L, LO to Cell Biology for GPCR SBDD'* **10:00 - 10:15. Angela Stefanachi** (University of Bari Aldo Moro, Italy) S14.3 *'CB2 Receptor agonists as important tools for the design of new fluorescent probes and new multitarget directed ligands (MTDL) against multifactorial disease'* **10:15 - 10:30. Yann Lanoiselée** (University of Birmingham, UK) S14.4 *'Detecting transient trapping events from a single trajectory'*
10:30 - 10:45	**BREAK**
10:45 - 12:05	**Session 15: GDR3545 GPCR signaling – from cellular to systemic level (WG-GDR)** Chair: Erika Cecon **10:45 - 10:50. Erika Cecon** (Université de Paris, France) S15.1 *French GPCR network GDR-3545 on G protein-coupled receptors – from physiology to drugs'* **10:50 - 11:10. Frederic Jean-Alphonse** (INRAE, Tours, France) S15.2 *'Mechanisms in GPCR endosomal cAMP signaling'* **11:10 - 11:30. Raphaelle Quillet** (Biotechnology and Cellular Signalling, Illkirch, France) S15.3 *'RFRP-3/NPFF1R is a pronociceptive system involved in long-term effects associated with chronic opioid administration and inflammatory pain'* **11:30 - 11:50. Jordi Haubrich** (Neuroreceptors, dynamics and function, IGF, France) S15.4 *'Nanobody activating mGlu4 receptor discriminates between homo- and heterodimers'* **11:50 - 12:05. Clémentine Philibert** (Institut de Génomique Fonctionnelle, Montpellier, France) S15.5 *'Characterization of endogenous mGlu2 receptor interactome in mouse brain'*
12:05 - 12:30	**BREAK**
12:30 - 14:00	**Session 16: Web-based resources and computational modelling of signal transduction (WG5)** Chair: Brad Hoare **12:30 - 12:55. Roser Vento** (Sanger Institute, Hinxton, UK) S16.1 *'Human Cell Atlas: Mapping the human body one cell at a time'* **12:55 - 13:20. David Gloriam** (University of Copenhagen, Denmark) S16.2 *'GPCRdb G proteins, arrestins and biased signalling'* **13:20 - 13:45. Dóra Kiss** (Research Centre for Natural Sciences, Budapest, Hungary) S16.3 *'Controlling receptor function from the extracellular vestibule of G-protein coupled receptors'* **13:45 - 14:00. Eitan Margulis** (Hebrew University of Jerusalem, Israel) S16.4 *'BitterMatch: Netflix-inspired ligand-receptor matching tool'*
14:00 - 15:00	**BREAK**
15:00 - 16:00	**Meet Today's Speakers & Networking** **Room 1:** *Rima Budvytyte, Mat Leveridge, Angela Stefanachi, Yann Lanoiselée* **Room 2:** *Frederic Jean-Alphonse, Raphaelle Quillet, Jordi Haubrich, Clementine Philibert* **Room 3:** *Roser Vento, David Gloriam, Dóra Kiss, Eitan Margulis* **Session 17: Round Table: AI in GPCR research** Chairs: Yamina Berchiche and Alexander Hauser **Tudor Oprea** (University of New Mexico, USA) **Maria Waldhoer** (InterAx Biotech, Switzerland) **Thomas Sakmar** (Rockefeller University, NY, USA)
16:00 - 16:15	**BREAK**
16:15 - 16:45	**Conference Closing & Poster Prizes** Chairs: Antonella Di Pizio, Ali Isbilir & Chloe Peach **Martha Sommer**
16:45 - Open	**Informal Networking & Socializing** ...a place for people to hang out and chat right after the meeting closes *Room 1: "Beer" Room 2: "Wine" Room 3: "Tea & Coffee"*
17:00 - 18:30	**MC Meeting Management Committee Members only** **Martha Sommer and Jana Selent**

ABSTRACT BOOKLET: TABLE OF CONTENTS

Keynote Speaker

Regulation of GPCR Signaling by Transporters

Author: Roshanak Irannejad

Institution Address: Cardiovascular Research Institute, University of California, San Francisco, 555 Mission Bay Blvd South, Room 352H, San Francisco, CA 94158

Email address of the presenter:
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A common feature of one-third of current drugs on market is their shared family of targets, the G protein-coupled receptors (GPCRs). They are encoded by 700 genes in the human genome and are responsible for sensing a wide range of outside signals such as hormones, neurotransmitters, light, and sense of smell and taste. A long-held tenet of molecular pharmacology is that GPCRs sole site of action is at the cell surface. Therefore, understanding of the immediate consequences of signaling has largely been confined to events that happen at the plasma membrane. Importantly, small molecule drugs have aimed to specifically target the plasma membrane pool of receptors. Our work suggests that these are narrow views of how signaling receptors function. We have pioneered the use of novel single chain cameloid antibody biosensors specific for active GPCR conformations. With this toolkit in-hand, we were able to follow GPCR activity and subsequent G protein signaling in living cells, revealing that 1) critical signaling events extends beyond the plasma membrane to previously unappreciated subcellular compartments, such as the endosomes and the Golgi, 2) efficacies of certain clinically used drugs may be driven from their capacity to cross the plasma membrane and act on internal, spatially segregated receptor pools and 3) impermeable hormones and chemical mediators can reach the subcellular organelles and activate the receptor there, through a mechanism that is facilitated by their specific transporter. These findings provide a potential solution to how small molecules that act on the very same receptor can elicit profoundly different physiological outcomes by showing that these differences correlate with differential abilities of small molecules to access internal sites of signaling.

Session 1: Protein dynamics and the macromolecular interactions in signaling pathways (WG1)

S1.1 Structural basis for allostery and bias at the adenosine receptors

Author: Alisa Glukhova

Institution Address: The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, VIC 3052 Australia

Email address of the presenter: glukhova.a@wehi.edu.au

G protein-coupled receptors (GPCRs) are membrane proteins and are an important drug target class responsible for over one third of current therapeutics on the market. The adenosine A1 receptor (A1AR) is a particularly promising GPCR target for treatment of neuropathic pain and cardiac disease. However, progression of subtype-selective A1AR agonists in the clinic is hindered by both on- and off- target side effects. New promising avenues for targeting this receptor are the use of allosteric modulators and/or signal-pathway "biased agonists". We have used cryo-electron microscopy to identify the first allosteric binding site for synthetic small molecules at the adenosine A1 receptor, as well as the atypical binding pose for a biased A1AR agonist. Insights from these structures provide the first insights into the mechanisms underlying allostery and biased agonism at this important drug target.

S1.2 Preassembly between G protein-coupled receptors and G proteins studied at the single-molecule level

Author: Alexey Bondar

Institution Address: Center for Nanobiology and Structural Biology, Institute of Microbiology, Czech Academy of Sciences, Zamek 136, 37333 Nove Hrady, Czech Republic

Email address of the presenter: bondar@nh.cas.cz

G proteins and G protein-coupled receptors (GPCRs) are the key players of cellular signal transduction present in all eukaryotes from yeast to humans. G protein signaling cascade is responsible for transduction of signals from numerous physical and chemical stimuli, including light, hormones and neurotransmitters. The majority of G protein-coupled receptors (GPCRs) interact with G proteins only upon GPCR activation. However, several GPCRs have been shown to form stable plasma membrane-localized complexes with G proteins in the inactive state. This phenomenon is known as preassembly. The mechanism of preassembly and its role in signaling remain unclear. Using single-molecule imaging of the 5-hydroxytryptamine type 7 receptor (5HT7R) and the G_s protein we show a potential mechanism of interaction between GPCRs and G proteins, which form stable preassembled complexes in the inactive state. These findings will help understanding physiological mechanisms of GPCR signaling and regulation of interactions between receptors and their cognate G proteins.

S1.3 Investigation of new binding partners for ATG3 protein in a hepatoma cell line

Authors: Devrim Öz-Arslan[1] (presenter), Demet ACIKGOZ[2], Beki KAN[1] Merve OZTUG[3], Emel TIMUCIN[4] and Zeynep Aslihan DURER [1,5]

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[2]Acibadem Mehmet Ali Aydinlar University, Institute of Health Sciences, Department of Medical Biotechnology, Istanbul, Turkey
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[4]Acibadem Mehmet Ali Aydinlar University, School of Medicine, Department of Biostatistics and Medical Informatics, Istanbul, Turkey
[5]Acibadem Mehmet Ali Aydinlar University, Faculty of Pharmacy, Biochemistry, Istanbul, Turkey

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Aim: Autophagy is a cellular process for the degradation and recycling of damaged proteins, protein complexes and organelles. Autophagosome formation is one of the major steps in autophagy and ATG3 is one of the critical proteins playing a role in the initiation of the autophagosome. Biophysical characterization of ATG3 indicated that it is an intrinsically disordered protein (IDP). Also, there is growing evidence on the roles of ATG proteins in cellular processes other than autophagosome formation. Therefore, we set out to map the binding interactions of ATG3 in ATG3-overexpressing cells treated with and without inducers of autophagy and mitophagy.

Methods: The human hepatoma cell line Hep3B grown in culture were treated with Torin 1 and CCCP for induction of autophagy and mitophagy, respectively. Cells were transfected with mEmerald-ATG3-N-18 expression plasmid using Lipofectamine or Pei-Max and transfections were confirmed with flow cytometry. ATG3-GFP and its complexes were pulled down using ChromoTek GFP-Trap® agarose beads and analyzed with LC-MS/MS.

Results: Autophagy and mitophagy inductions resulted in dose and time dependent changes in LC3B II/I, p62, and PINK1 proteins. ATG3-GFP fusion proteins and their partners were immunoprecipitated from transfected cells and analyzed with LC-MS/MS to make quantitative comparisons between control, autophagy and mitophagy induced groups.

Conclusion: Proteomic and bioinformatic analyses revealed differences between known and novel binding partners of ATG3 protein in mitophagy and autophagy-induced cells.

This work is supported by Scientific and Technological Research Council of Turkey (TUBITAK) (Grant number:217Z213) and Acibadem University (ABAPKO Grant number:2018/03/08)

S1.4 Structure of the Class D GPCR Ste2 dimer coupled to two G proteins

Authors: Vaithish Velazhahan (presenter), Ning Ma, Gáspár Pándy-Szekeres, Albert J. Kooistra, Yang Lee, David E. Gloriam, Nagarajan Vaidehi, and Christopher G. Tate

Institution Address: MRC Laboratory of Molecular Biology, Cambridge, UK

Email address of the presenter: vaithish@mrc-lmb.cam.ac.uk

G protein-coupled receptors (GPCRs) are divided phylogenetically into six classes, A-F. Over 370 structures of vertebrate GPCRs (classes A, B, C and F) have been determined, leading to a substantial understanding of their function. In contrast, there are no structures of Class D GPCRs, which are found exclusively in fungi where they regulate survival and reproduction. We have determined the first structure of a class D GPCR, the *Saccharomyces cerevisiae* pheromone receptor Ste2, in an active state coupled to the heterotrimeric G protein Gpa1-Ste4-Ste18 using electron cryo-microscopy (cryo-EM). Ste2 was purified as a homodimer that coupled to two G proteins. The dimer interface of Ste2 is formed by a domain-swapped N-terminus, transmembrane helices H1, H2, H7 and the first extracellular loop ECL1. The tridecapeptide pheromone a-factor binds Ste2 in an extended conformation which is different from the peptide binding modes observed in other GPCR structures. The structure of Ste2 bears similarities in overall topology to Class A GPCRs, but H4 is shifted by over 20 Å and the G protein binding site is a shallow groove rather than a cleft. We have established a Class D1 generic residue numbering system (CD1) to enable comparisons with orthologues and other GPCR classes. Overall, the high-resolution Ste2 structure sheds light on how canonical GPCR dimers could form and provides a template for the design of novel drugs targeting fungal GPCRs that could be utilised to treat a number of intractable fungal diseases.

References

Velazhahan, V., Ma, N., Pándy-Szekeres, G., Kooistra, A.J., Lee, Y., Gloriam, D.E., Vaidehi, N., & Tate, C.G. (2020) Structure of the class D GPCR Ste2 dimer coupled to two G proteins. *Nature* In press.

Session 2: Collaboration with Frontiers Journals

Authors: Milka Vrecl[1], Marialessandra Contino[2], Marcello Leopoldo[2], George Thomas[3]

Institution Address:[1]Institute of Preclinical Sciences, Veterinary Faculty, University of Ljubljana, Slovenia; [2]Department of Pharmacy-Drug Sciences, University of Bari Aldo Moro, Bari, Italy**;**
[3] Frontiers Media SA, Lausanne, Switzerland

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The COST action European Research Network on Signal Transduction – ERNEST – has established a 3-year collaboration with Frontiers journals to promote research topics, conferences, or dissemination activities. Frontiers will publish an Article Collection gathering original research articles, reviews, mini-reviews, systematic reviews, methods, hypothesis & theory dealing with the research objectives of the ERNEST COST action (i.e. develop a multidimensional signaling map with molecular, spatial, and temporal information detailing how different signal transduction pathways give rise to distinct cellular responses, and how aberration in signal transduction gives rise to disease and utilize the signaling map to create new pathway-specific chemical modulators of signal transduction). George Thomas (Frontiers Media SA, Switzerland) will illustrate Frontiers' mission and the advantages of publishing in Open Access journals. The collaboration between ERNEST and Frontiers will be focused on the following research areas:

1. Physiology & pathophysiology of GPCR signal transduction
2. Impact of Artificial Intelligence on Computational Biology
3. What the cryo-EM resolution revolution means for GPCR research
4. Advances in high-resolution imaging: understanding spatio-temporal control of signal transduction
5. Molecular Modulators of Signal Transduction
6. Macromolecular interactions in signaling pathways: from classical approaches to virtual reality.

The attendees will be invited to participate in a poll to select research areas from the list above to which they are likely to submit a contribution in the coming ten months.

Session 3: Challenges and Chances of Multidisciplinarity in GPCR Research

Authors: Marcel Bermudez

Institution Address: Freie Universität Berlin, Institute of Pharmacy, Berlin, Germany

Email address of the presenter: m.bermudez@fu-berlin.de

Scientists, policymakers and funding agencies consider multidisciplinary research approaches to be essential to manage the big challenges of our time. While it is generally undoubted that multiple perspectives on a research topic are key to solve the big questions, it also holds some challenges, in particular for Early Career Researchers (ECR).

In the first half of this session, we will give an overview on challenges that ECRs have to face when working in an interdisciplinary field including project timelines, funding, publication of results, getting scientific recognition and being open-minded. Despite the challenges, it is worth to tackle research questions with multidisciplinary approaches and there are many possibilities and chances for ECRs to benefit from collaborations with other fields. In many cases mutual curiosity and good communication is key for successful interdisciplinary projects.

In the second half of the session we will have a discussion with ECRs and PIs about their own experiences with multidisciplinarity in GPCR research and strategies for turning challenges into chances.

Session 4: Biological roles and physiological contexts of signal transduction (WG2)

S4.1 Efficacy and bias at the mu-opioid receptor

Authors: Meritxell Canals

Institution Address: Centre of Membrane Proteins and Receptors, School of Life Sciences, University of Nottingham, Nottingham, NG7 2UH, United Kingdom

Email address of the presenter: m.canals@nottingham.ac.uk

The μ-opioid receptor (MOR) is the target of most commonly used analgesics and a prototypical GPCR in which many pharmacological paradigms have been described. I will present our most recent data investigating agonist-dependent regulation and biased agonism at this receptor.

G protein-biased ligands at the MOR have been proposed to induce less side effects than commonly used opioids. Using cell-based assays not confounded by limited signal window, we have recently shown a strong correlation between measures of efficacy for receptor activation, G protein coupling and arrestin recruitment for a series of opioid ligands. By measuring the anti-nociceptive and respiratory depressant effects of these ligands, we have shown that low intrinsic efficacy of opioid ligands can explain an improved side effect profile in terms of a continuum of existing analgesics, suggesting an alternative mechanism underlying the improved therapeutic windows described for novel opioids.

Previous work from our lab demonstrated how the localization within the plasma membrane of the MOR controlled the spatiotemporal signalling profiles of this receptor. Using fluorescence correlation spectroscopy (FCS) and complementary imaging approaches we have recently highlighted the role of GPCR kinases (GRKs) in mediating such reorganization. Site-directed mutagenesis together with real-time BRET and FRET assays have also allowed us to understand the role that the different phosphorylation motifs play in the dynamics of receptor desensitisation and arrestin and GRK recruitment.

These studies have revealed a complex mechanistic interplay between MOR signalling and regulation, yielded new insights into the biological effects of existing opioid drugs and motivated new mechanistic hypotheses for guiding future development of improved opioid-directed therapeutics.

References

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S4.2 Fermented food derived metabolites mediate innate immune cell functions through highly potent activation of HCA_3

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The interplay of microbiota and its human host is physiologically crucial in health and diseases. The beneficial effects of lactic acid bacteria (LAB), permanently colonizing the human intestine or transiently obtained from food have been extensively reported. The molecular understanding of how LAB modulate host physiology is still limited. G protein-coupled receptors for hydroxycarboxylic acids (HCAR) are regulators of immune functions and energy homeostasis under changing metabolic and dietary conditions. Most mammals have two HCAR but humans and apes contain a third member (HCA_3) in their genomes. A plausible hypothesis why HCA_3 function was advantageous in hominid evolution was lacking.

We used a combination of evolutionary, analytical and functional methods, including pharmacological assays, analyses of human monocytes and pharmacokinetic measurements in human, to unravel the role of HCA_3 *in vitro* and *in vivo*.

We report the discovery of D-phenyllactic acid (D-PLA), a LAB-derived metabolite, interacting with the human host through highly potent activation of HCA_3. We demonstrate that D PLA is well absorbed from the human gut, as reflected in high plasma and urine levels following ingestion and triggers pertussis toxin-sensitive, HCA_3-dependent migration of primary human monocytes.

We provide evidence supporting the hypothesis that HCA_3 was consolidated in hominids as a signaling system that modulates the human host immune system in response to LAB-derived metabolites.

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S4.3 Functional characterization of the orphan GPCR GPR101

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G protein coupled receptors (GPCR) are involved in nearly every physiological process and are one of the main sources of drug target. Despite their important functions, a large number of them remain uncharacterized or orphan for known ligands. The functional characterization of these elusive receptors is of prime importance for the discovery of new physiological regulation and the development of innovative medicines. GPR101 is a class A orphan receptor and the microduplication of the locus containing its gene on the X chromosome has been recently identified as the cause of the X-linked acrogigantism, or X-LAG, syndrome[1]. Carriers of the microduplication develop pituitary adenomas at birth and elevated Growth Hormone (GH) levels that eventually lead to gigantism if left untreated. These findings suggest that GPR101 plays a relevant role in the regulation of GH secretion. We discovered[2] that GPR101 is a constitutively active receptor promiscuously coupled to the Gs, Gq/11 and G12/13 families of G proteins. Furthermore, we uncovered that GPR101 promotes growth hormone secretion through the activation of both Gs and Gq/11, in a PKA and PKC-dependent manner. Interestingly, in stark contrast with other Gs-coupled receptors, GPR101 activation did not lead to the proliferation of somatotrophs. We validated these data with a transgenic mouse model overexpressing GPR101 in the pituitary. We confirmed in pituitary adenomas from X-LAG patients that the activation of PKC is a GPR101 signature. Collectively, our results demonstrate that GPR101 promotes growth hormone secretion in the pituitary somatotrophs through an unconventional mechanism.

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S4.4 Noradrenergic signalling and metabolism are dysregulated in astrocytes with cytoplasmic ALS/FTD-linked TDP-43 inclusions

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Cytoplasmic TDP-43 (TAR DNA-binding protein 43) inclusions are a common pathological hallmark of neurological disorders amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). They are found not only in neurons, but also in glial cells, including astrocytes. Neuronal metabolism largely depends on the activation of astroglial adrenergic receptors (ARs), the target of noradrenaline released from the noradrenergic neurons, which in astrocytes triggers Ca^{2+}/cAMP signalling and augments aerobic glycolysis resulting in production and release of lactate, a neuronal fuel. Cytoplasmic TDP-43 inclusions in astrocytes alone can cause motor neuron death, however, it is unclear whether this affects astroglial metabolism and capacity of astrocytes to metabolically support neurons. By using fluorescent dyes and genetically encoded nanosensors we measured changes in Ca^{2+}/cAMP signalling and glucose and lipid droplet (LD) metabolism in astrocytes expressing inclusion-forming C-terminal fragment of TDP-43 or wild-type TDP-43. LD accumulation was increased by 3-fold in astrocytes with TDP-43 inclusions vs. astrocytes expressing wild-type TDP-43, indicating cellular stress. These cells also exhibited a ~30% reduction in noradrenaline-mediated Ca^{2+} and cAMP signalling, likely due to the downregulation of β_2-ARs. Although noradrenaline-triggered increase in intracellular lactate was similar in astrocytes with and without TDP-43 inclusions, the probability of activating aerobic glycolysis was increased by 1.6-fold in astrocytes with TDP-43 inclusions and lactate MCT1 transporters were downregulated. These data suggest dysregulated metabolism and lactate export in astrocytes with TDP-43 inclusions that may affect astroglial metabolic support of neurons in ALS and FTD.

S4.5 Optical mapping of cAMP signaling at the nanometer scale

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Cells relay signals from hundreds of different G protein-coupled receptors into specific cellular functions by using just a handful of second messengers, most importantly Ca^{2+} and cAMP. Many lines of evidence suggest that cell signaling, including cAMP, should be spatially organized into *signaling compartments* or *domains*.

Whereas this is well documented experimentally for Ca^{2+}, the existence and molecular architecture of cAMP signaling compartments, however, has been repeatedly suggested, but neither been directly demonstrated nor mechanistically explained. This is mainly due to two lines of conflicting evidence: First, cAMP has been shown to *diffuse freely* through the cell. Second, phosphodiesterases (PDEs), the only enzymes degrading cAMP, have very *low turnover rates*. Combined, these properties of cAMP signaling would make the existence of cAMP compartments through targeted cAMP breakdown by PDEs theoretically impossible.

Here, using fluorescence spectroscopy of a designed fluorescent cAMP analog in intact cells, we show that – contrary to earlier data – cAMP at physiological concentrations is largely immobile, because it is quantitatively bound to intracellular cAMP binding sites. This results in so-called *buffered diffusion* which is an essential requirement to allow PDEs to create very small domains of low cAMP concentrations. Using novel FRET-based *nanorulers*, we optically map the size of such cAMP gradients around single molecules of PDE and G protein-coupled receptors. We demonstrate that they have diameters of tens of nanometers. We show that these *cAMP-nanometer domains* control PKA-activation in their immediate vicinity, thereby defining the molecular principles how cells control cAMP access to PKA and downstream targets.

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Session 5: Dr.GPCR online platform for GPCR researchers

S5 Beyond the lab

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GPCRs have played a central role in my scientific career ever since I took Dr. Michel Bouvier's class as an undergraduate student at the University of Montreal in the early 2000. During the past 2 decades, my research mainly focused on chemokine receptor structure/function relationships. For the purposes of this presentation, I will walk you through my various career experiences and include the skills I learned during each experience, which ultimately led me to founding Dr.GPCR. Last, I will give an overview about the various programs we established at DR.GPCR, present our team as well as provide you with a sneak peak of our future podcast guests and more.

Session 7: GPCRdb and GPCRmd Introduction, Updates & Workshop

S7.1 GPCRdb: Integrating GPCR sequence, structure and function

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In this segment, we will introduce the latest and greatest of the GPCR database, GPCRdb, in three ways: 1) a presentation with an overview of the latest updates, 2) a live demonstration, and 3) a Q&A session with the GPCRdb team in which you can ask any GPCRdb question.

The GPCRdb provides reference data, analysis tools, interactive visualization and experiment design tools to ~4,000 users/month (https://gpcrdb.org).[1]

With the latest release, the GPCRdb resources have been improved in several ways that will be further detailed in this session. Here are a few of the highlights: the GPCRdb is now providing an up-to-date overview of all available GPCR structures with monthly updates. A new powerful receptor selection method makes it easier to select the receptors of interest. In addition, the GPCR ligand data has been updated and now contains almost 200K small molecules with related bioactivity data.

Join this session if you want to hear more!

More GPCRdb: On Wed 12.55-13.20, David Gloriam will present GPCRdb G proteins, arrestins and biased signalling

References

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S7.2 GPCRmd is growing: new tools and data coverage

Authors: Mariona Torrens-Fontanals[1], Johanna K.S. Tiemann[2], GPCRmd community[3], Ramon Guixà-González[4,5] and Jana Selent[1]

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GPCRmd[1] (http://gpcrmd.org/) is an online resource that aims to approach the valuable information of molecular dynamics (MD) simulations to all researchers interested in G protein-coupled receptors (GPCRs). As such, GPCRmd provides free access to a vast number of GPCR MD simulations, being the first open-access research resource hosting MD simulations of most GPCR crystal structures solved to date. Moreover, GPCRmd simplifies the analysis of the simulation data. It is equipped with a comprehensive set of intuitive online tools for the interactive visualization and analysis of the MD simulations. Such tools allow, for example, the study of protein motions involving conserved, pharmacologically relevant, or diseased-related residues.

Here, we present the main features of GPCRmd, focusing on recent updates, and discuss the next steps of this project. Importantly, we will provide an update about the progress on our second community-driven simulation round. This round will increase the data coverage of GPCRmd to > 80% of currently solved structures. Our final aim is to provide a resource containing MD data of all GPCRs with known structure, together with a complete set of visualization and analysis tools that covers the needs of the community of GPCR scientists.

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Session 8: Workshop on HitS DEL platform by WuXi AppTec

S8.1 DNA multitasking in DNA encoded libraries

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DNA can be read, written and copied quickly and with near perfect fidelity. These features make DNA a unique polymer in the chemical world since it enables the encoding, storage, and reading of information on a molecular scale. One of the most useful technologies to emerge from this confluence of properties is DNA encoded libraries (DEL). DEL technology helps us keep track of small molecule structure by encoding it in a covalently attached DNA strand. Since each molecule is covalently connected to a unique DNA tag defining its structure, large libraries of molecules can be pooled without losing track of structural information. In this talk I will outline some of the ways we use DNA to store chemical information and to report on the robustness of chemical reactions.

S8.2 DELopen and DELight Promote Early Drug Discovery by Implementing Self-service DNA-Encoded Libraries Platforms

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WuXi AppTec's DNA Encoded Library (DEL) service is a cutting-edge hit identification and optimization platform using an affinity-based selection method against DNA-encoded small molecule libraries. For industry and biotech companies we offer a WuXi DEL Selection (> 90 billion compounds) Service Package or a DELight (8.4 billion compounds) self-service kit. With the self-service DELight kit, the researcher conducts the screen themselves, and does not share any target information with WuXi. The goal of DELopen is to provide free access to the DEL library of more than 2.8 billion compounds to academic users for hit-to-lead research in drug discovery. Through the DELopen or DELight service package, WuXi provides a DEL kit and a user manual with all the necessary protocols to the research organization. Upon receipt of the kit, the researcher performs affinity selection according to the protocol and returns the sample to WuXi. WuXi then completes analysis such as PCR amplification, qPCR determination, purification and NGS (Next Generation Sequencing). A summary report (excluding structural and DNA sequence information) will be provided back to the researcher, who will make an informed decision on follow up actions based on the selection results. We work closely with researchers to enable and optimize early stage Hit ID, drug discovery and development.

S8.3 Initiating DNA encoded library synthesis on solid phase – chemistry development, library synthesis, and TEAD-YAP inhibitor identification

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Selection of DNA-encoded small molecule libraries (DELs) is a fast and economic alternative to brute force screening of discrete compound collections. Nevertheless, the preparation of structurally diverse DELs is still a formidable challenge due to the low stability of DNA under many standard reaction conditions in organic synthesis and the requirement to work with aqueous co-solvents in solution phase DEL synthesis.[1] Alternatively, DEL synthesis can be initiated by micellar catalysis[2] or on solid phase-coupled DNA which allows the usage of dry organic solvents. The later approach was used to adopt a set of four different isocyanide multicomponent reactions (MCRs) to a DNA-encoded format.[3] Further, a screening campaign assessing the stability of fully protected, solid phase-bound DNA in the presence of metal salts and organic reagents that are commonly used as catalysts in organic synthesis, revealed that such DNA tolerated several catalysts under mild conditions.[4] Based on these findings, we have selected catalysts and reaction conditions that can be used in reactions for initiating DEL synthesis. This enabled us to expand the chemical space for DELs by diverse heterocyclic forming reactions like the ®-(-)-BNDHP-mediated Povarov reaction[4] or the Yb(Otf)3-mediated Cushman reaction[5] as well as the CuCl/bpy-mediated Petasis reaction[6] leading to α-(het)aryl glycine conjugates.

Recently, we demonstrated that an indole-focused peptidomimetic DEL produced by the solid phase strategy could lead to the identification of a novel class of protein-protein interaction inhibitors. The DEL synthesis was initiated by the Ugi-multicomponent reaction on a solid phase-coupled chemically stable thymidine adapter oligonucleotide (“tiDEL”).[7] tiDEL synthesis was continued with DNA barcode ligation, and azide-alkyne cycloaddition in the combinatorial step. Selection of the tiDEL on human TEAD4, a tumor-associated transcription factor and effector of Hippo signaling, next generation sequencing and analysis of the sequencing data with a newly developed algorithm indicated a class of compounds binding to the target protein. An exemplary compound disrupted the hTEAD4-YAP interaction in vitro and reduced the expression of a gene under the control of the TEAD-YAP transcription factor complex.

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Session 9: Design and optimization of molecular modulators of signal transduction (WG3)

S9.1 Conservation of allosteric sites in G-protein coupled receptors

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Allosteric modulation of G protein-coupled receptors represent a promising mechanism of pharmacological intervention. The large number of GPCR structures published during the last decade revealed that the binding sites of allosteric modulators are widely distributed. Here we show that protein mapping tools FTMap and FTSite identify 83% and 88% of such experimentally confirmed allosteric sites located at intrahelical and intracellular sites and at the allosteric conformational locks [1]. The methods were also able to find partially hidden allosteric sites that were not fully formed in X-ray structures crystallized in the absence of allosteric ligands. Comparative analysis of the explored allosteric sites to hotspots predicted for all available GPCR structures revealed overlapping sites that suggests the conservation of allosteric sites in GPCRs. These results confirm that the intrahelical sites capable of binding druglike allosteric modulators are among the strongest and probably conserved ligand recognition sites in a large fraction of GPCRs and suggest that both FTMap and FTSite are useful tools for identifying allosteric sites and to aid in the design of such compounds in a range of GPCR targets.

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S9.2 Drug residence time and the role of water

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Drug residence time on a GPCR has emerged as an optimization parameter in drug design. Long residence times can counterbalance unfavorable pharmacokinetic parameters, contributing to compound safety. Short residence times can be beneficial to avoid target related side effects. In contrast to the prediction and interpretation of structure-activity relationships (SAR), the structure-kinetics relationships (SKR) are much less well understood. This means that the detection and understanding of SKR in a project is a great challenge, but also a great opportunity.

In this talk, I will present a general framework of how to identify and analyze unexpected behavior in SKR. Experimental data on antagonists of CCR2 and M3 receptors hint to residence times of ligands, which are much higher than to be expected from the binding affinity. The reason for these over proportionally long residence times is investigated by MD-simulations and can eventually be found in water networks close to the ligand binding site on the GPCR.

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Molecular basis for the long duration of action and kinetic selectivity of tiotropium for the muscarinic M3 receptor, *J Med Chem*, (2013) 56, 8746-8756.

S9.3 Structure-kinetic relationship studies on serotonin 5-HT$_7$ receptor ligands: role of the lipophilicity on residence time

Authors: Enza Lacivita[1], Edoardo Penna[2], Sabina Podlewska[3], Mauro Niso[1], Carla Perrone-Capano[2], Marianna Crispino[2], Andrzej J. Bojarski[3], Marcello Leopoldo[1]

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The serotonin receptor 7 (5-HT7R) is a G protein-coupled receptor (GPCR) involved in many physiological processes and in neurological and neurodevelopmental disorders characterized by abnormal neuronal connectivity and consequent intellectual disabilities. To unveil the molecular pathway linking 5-HT7R to these diseases, the use of adequate pharmacological tools, such as the brain penetrant and selective 5-HT7R receptor agonist LP-211, has been important [1]. Usually, the properties of pharmacological tools are defined by the affinity for the target receptor (K_i) and by the agonist (EC_{50}) or antagonist (IC_{50}) potency. During the last years, there is a growing interest in the temporal aspects of ligand-receptor interactions in determining drug pharmacodynamics *in vivo* and on the exploitation of binding kinetics in drug development [2].
Previous studies on LP-211 and related analogs have suggested that the residence time of these classes of 5-HT7R ligands might be structure-dependent. In this study, we assessed the binding kinetic parameters of a series of 5-HT7R ligands characterized by different lipophilic properties (ClogP). We have found that it is not the overall lipophilicity of the molecule that determines its dissociation rate but rather the lipophilicity at a specific position of the scaffold. Next, we combined docking and molecular dynamic (MD) simulations to explain the structure-kinetic relationships in a subset of the studied 5-HT7R ligands. As a result of a statistical analysis of the ligand-protein interactions occurring during MD simulations, a series of contacts correlated with the residence time of the compounds was determined.

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S9.4 Protein phosphatase 1 disrupting peptides as a strategy to modulate sperm motility

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The mechanism by which sperm acquire motility is a vital process occurring at a late phase of post-testicular sperm maturation (1). Protein phosphatase 1 (PP1) catalyses a considerable fraction of phosphoserine/threonine dephosphorylation reactions in eukaryotic cells and inhibition of its catalytic activity is essential for sperm motility acquisition and regulation (1, 2). Regulation of PP1 catalytic activity and specificity is mediated by forming holoenzymes with regulatory interactors of protein phosphatase one (RIPPOs) (2-4). The aim of this study was to design PP1-disrupting peptides as a strategy to modulate sperm motility. PP1-disrupting peptides were designed based on the sequences from (i) a sperm-specific PP1 interactor (A kinase anchor protein 4, AKAP4) and (ii) a PP1 inhibitor (protein phosphatase inhibitor 2, PPP1R2). Those sequences were covalently coupled to inert cell penetrating peptides (CPPs) as bioportide constructs, which were successfully delivered to the flagellum of sperm cells to induce a marked impact upon PP1g2 activity and sperm motility. Molecular modelling studies further facilitated the identification of an optimized PP1 binding sequence and enabled the development of a Modified Stop Sperm (MSS1) bioportide with reduced size and increased potency of action. Additionally, a bioportide mimetic of the unique 22-amino acid C-terminus of PP1g2, a testis- and sperm-specific isoform, accumulated within spermatozoa to significantly reduce sperm motility and further define the PP1g2-specific interactome. These investigations demonstrate the utility of CPPs to deliver peptide sequences that target unique protein-protein interactions in spermatozoa to achieve a significant impact upon spermatozoa motility, a key prognostic indicator of male fertility.

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Session 10: Workshop

S10 3D Structures in GPCR Signalling - a primer for non-experts

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The 3D structure of signaling proteins have always been central to understanding how they interact and function in signaling cascades. Indeed, over the recent decades structures of individual proteins or complexes involved in G protein coupled signaling cascades have repeatedly transformed sub-fields of research related to these cascades. In this primer to 3D Structures in GPCR Signaling, we will briefly cover the methodological basis for experimentally determining the 3D structure of proteins. We will then delve into selected structures that provided the field with insights into how these proteins look like, how they function, and how they interact. Lastly, we will briefly examine how computational methods can further inform us of how 3D structure relates to function.

Session 11: Industry Innovators

S11.1 No-wash technologies to investigate cell signalling pathways in disease-relevant cellular models

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Historically, G protein coupled receptors (GPCR) have been one of the most druggable classes of cellular proteins. The primary effectors of GPCR are G proteins, which initiate signal propagation into the cells. In recent years, other GPCR effectors, in particular arrestin proteins have been found to induce cell signaling in the absence of G protein interactions. This key observation led to a new paradigm in GPCR research and drug discovery that relies on multidimensionality of GPCR signaling. In addition, some GPCR ligands were found to selectively activate one pathway over another, and this phenomenon has been referred to as biased agonism or functional selectivity.

All the new paradigms will have a profound influence on the future of the GPCR drug discovery. To address them, Cisbio Bioassays / Perkin Elmer has developed a unique set of cell-based assays to investigate signaling events. These assays are based on no-wash assay platforms called HTRF® and AlphaLISA®.

This talk will highlight how these platforms are superior solutions to accurately determine the pharmacological profile of GPCR ligands. It will also illustrate how the combination of HTRF® and/or AlphaLISA® assays allows the investigation of the multidimensionality of GPCR signaling in disease-relevant cellular models.

S11.2 Lifetime for Life Science

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With more than 20 years, PicoQuant has high experiences in time-resolved emission measurements. Based on the technique of TCSPC (Time Correlated Single Photon Counting), PicoQuant offers a broad range of different solutions starting from stand-alone devices, e.g. different laser, counting electronics and detectors, up to full automated systems for microscopy and spectroscopy. With this setups additional information are accessible for samples in biology and life science applications.

In the talk, PicoQuant will present a brief overview about these techniques and applications for characterization of biological and similar samples like protein interactions in the field of life science research.

S11.3 AI-meets-RI (Artificial meets REAL intelligence) approach to GPCR drug discovery

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GPCRs stand out in their ability to activate a range of intracellular signaling pathways from the same receptor (via G-proteins, beta-arrestins, etc). This poses a challenge in GPCR drug discovery – drug molecule can trigger a range of cellular responses, including both therapeutically beneficial responses and those with undesirable effects. Designing GPCR drugs which generate desired cellular responses requires quantitative mechanistic understanding of the types of responses a given receptor can generate in presence of drugs. InterAx uses *in vitro* experiments and mathematical modeling (systems biology) to re-create and quantify a part of the *in vivo* GPCR activation complexity upon perturbation by endogenous ligands and drug molecules. This allows to cluster and rank drug molecules based on in vivo-like signaling profiles and thereby speed-up the design of drugs with desired signaling properties.

A holy grail on this path would be to further connect the chemistry and structural biology of drug-GPCR complexes to the drug *in vivo* signaling profiles and phenotypes. I.e. being able to computationally predict not just the affinity of a drug candidate, but its efficacy and signaling parameters by analyzing the structure of drug-GPCR complex. Using beta-2 adrenergic (B2A) GPCR as an example, we will show preliminary results on connecting human intelligence with machine-learning-based AI to predict the systems biology / signaling parameters from the structural data of the drug-receptor complex.

Session 13: Mapping Group

S13 Towards a multidimensional understanding of GPCR signal transduction

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An important goal of ERNEST and the Mapping Group is to establish the framework for a holistic understanding of GPCR signal transduction by uniting the diverse data and perspectives of the research community into a single multidimensional map (see Figure 1). Such global insights hold great promise for improved drug design and therapeutic targeting.

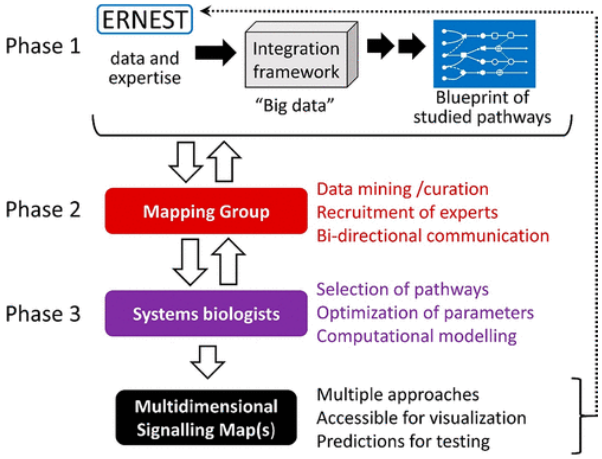

Figure 1. Schematic overview of how ERNEST will develop a holistic multidimensional map of GPCR-mediated signal transduction[1] .

In this session, we will provide an update about the progress and activities of the mapping group. We will present a first pilot study which lays out a framework on how to create a comprehensive model to address complex GPCR signaling linking the genomic to the systems level. Finally, in a roundtable discussion we will open up for questions and discussion about ideas, alternative approaches and future steps.

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Session 14: Advanced methods and technologies in signal transduction research (WG4)

S14.1 Tethered Bilayer Lipid Membranes- a Platform for Studies of Protein-Lipid Interaction and Biosensing

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Tethered bilayer membranes (tBLMs) constitute a class of biomimetic self- assembled structures that provide a generic platform for a broad spectrum of biophysical experiments such as peptide(protein)/membrane interactions, reconstitution of functioning proteins, antibody/antigene interactions and other applications. We have developed tethered bilayer lipid membranes (tBLMs) as a long-term stable and versatile experimental model in which thiolated anchor lipids span a hydrated layer that separates the membrane from its solid support [1,2].
Owing to their completeness, insulating properties and high in-plane fluidity, tBLMs are attractive matrices for the functional incorporation of membrane proteins as we demonstrate in the reconstitution of pore forming toxins and proteins [3, 4].
The loss of the structural integrity and accompanying decrease or total removal of the dielectric barrier of tBLM may be monitored by electrochemical impedance spectroscopy (EIS). Neutron reflectometry provides high-resolution structural information on the interaction between the protein and the disordered membrane. The robust nature of the membrane enabled the precise localization of the protein within 1.1 Å [5]. The results suggest that these techniques could be used to elucidate molecular details of the association of other proteins with membranes and may provide structural information on domain organization and stimuli-responsive reorganization for transmembrane proteins in membrane mimics.

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S14.2 A look inside the GPCR Structural Pharmacology toolbox: From hit ID, H2L, LO to Cell Biology for GPCR SBDD

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G protein-coupled receptors (GPCRs) are an important and long-standing family of drug targets. Despite many historical success stories, today there are still a significant number of GPCRs with compelling pre-clinical validation that remain highly challenging for drug discovery.

Over the last 10 years there has been great progress in the structural biology of GPCRs facilitating Structure-Based Drug Design (SBDD) approaches. Sosei Heptares uses its proprietary StaR® technology to thermostabilise GPCRs by mutagenesis into a chosen conformational state. These purified proteins can then be used for biophysical screening techniques and crystallisation to yield X-ray structures with multiple ligands.

This presentation will illustrate how at Sosei Heptares we use our StaR® approach for GPCR structure and hit identification. Case studies will demonstrate how we have used the structural identification of orthosteric and allosteric binding sites for SBDD and highlight how this approach has provided differentiated series over empirical methods.

S14.3 CB2 Receptor agonists as important tools for the design of new fluorescent probes and new multitarget directed ligands (MTDL) against multifactorial disease.

Authors: Angela Stefanachi,[a]* Carmen Abate,[a] Giuseppe F. Mangiatordi,[b] Chiara Riganti,[c] José Brea,[d] María I. Loza, [d] and Marialessandra Contino.[a]

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The Cannabinoid subtype 2 receptor, CB2R, belongs to the endocannabinoid system, and it is involved in the onset of neurodegeneration and cancer evolution and progression; thus, its modulation may have a great impact both on the therapeutic and diagnostic fields. Several studies, focused on different types of tumours, report a promising anticancer activity induced by CB2R agonists due to their ability to reduce inflammation and cell proliferation. Moreover, in neuroinflammation, the stimulation of CB2R, overexpressed in microglial cells, exerts beneficial effects in neurodegenerative disorders. However, CB2Rs distribution and their signalling pathways in physiological and pathological conditions are still controversial mainly because of the lack of reliable diagnostic tools. Our aims are 1) to develop ligands to reliably image CB2R and 2) identify new strategies to overcome the limitations of the current treatment of multifactorial disease. In accordance with the first aim to allow a green and safe strategy to image CB2R, we designed a series of fluorescent ligands linking to the N-adamantyl-1-pentyl-4-oxo-1,4-dihydroquinoline-3-carboxamide core (responsible for the CB2R affinity), three different green emitting fluorescent tags (4-dimethylaminophthalimide, 4-DMAP, 7-nitro-4-yl-aminobenzoxadiazole, NBD, and Fluorescein-thiourea, FTU) through polymethylene linkers at the N1-position of the core. We identified and studied compound SM15 [1] endowed with good pharmacodynamic and fluorescent properties. Our second aim prompted us to develop new Multitarget Directed Ligands (MTDL) targeting CB2R. We propose new promising targets association involving CB2R with the histone deacetylases (HDACs) and sigma receptors by the aid of modern approaches as molecular hybridization, computational polypharmacology and artificial intelligence.

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S14.4 Detecting transient trapping events from a single trajectory

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Single-molecule microscopy experiments have the potential to provide a detailed description of the dynamics and interaction kinetics of G protein-coupled receptors [1]. These methods give access to large datasets containing the trajectories of thousands of individual receptors diffusing on the cell membrane. Given the complexity of the obtain datasets, powerful automated analysis tools are needed to extract biologically relevant information from these trajectories.

We introduce a new method [2] to detect transient trapping events within single molecule trajectories. This offers a highly temporally resolved description of molecular motion and allows one to explicitly quantify dynamic changes in the diffusion of individual molecules over time. Such method is suitable for investigating both intracellular and membrane compartmentalisation at the single molecule level.

Our method can account for different trapping region sizes and relies on a Monte-Carlo approach to provide a statistical assessment of the trapping regions. To do so, we introduce a novel measure on a recurrence matrix that takes in account both the spatial and temporal dynamics of each trajectory. Thus, our method avoids several common pitfalls in trapping detection. First, the trapping assessment is independent of the diffusion coefficient of the molecule. Second, it is robust to the presence of potential errors introduced by tracking algorithms. Finally, it is able to account for complex binding kinetics, for instance due to a molecule revisiting a previous trapping site.

Finally, we applied the new method to single particle trajectory data of β_2-adrenergic receptors. Our study reveals that under basal condition receptors spend 30% of their time confined on the plasma membrane, increasing up to 45% after agonist stimulation. The increase is explained by 4/3-fold longer confinement duration and increased proportion of receptors undergoing hop diffusion. Detected size of confinement domains is in average 0.1 µm and is found to be independent of stimulation time.

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Session 15: GDR3545 GPCR signaling – from cellular to systemic level (WG-GDR)

S15.1 French GPCR network GDR-3545 on G protein-coupled receptors – from physiology to drugs'

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The GDR3545 on “G Protein-coupled Receptors: from physiology to drugs (RCPG-Physio-Med)” was created in 2012 by the CNRS, a world-leading French research organization. The GDR3545 is composed of approximately 600-700 members working in 65 French and associated research teams from around the world, all working in GPCR-relevant fields as diverse as chemistry, cell biology, physiology, immunology, infectious diseases, structural biology, molecular modeling, systems biology and pharmacology.

The GDR3545 has 4 main objectives:

-to structure and organize the GPCR community in France;

-to promote exchange between academia and industry;

-to coordinate research on GPCRs between different organisms (CNRS, Inserm, CEA, INRA, university);

-to support young scientists in the field.

The GDR3545 is currently directed by Ralf Jockers (Institut Cochin, Paris).

S15.2 Mechanisms in GPCR cAMP endosomal signaling

Authors: Frédéric Jean-Alphonse, Alex White, Fei Fang, Karina Pena, Shi Liu, Gabriel Konig, Auka Inoue, Despoina Aslanoglou, Samuel Gellman, Evi Kostenis, Kunhong Xiao, Jean-Pierre Vilardaga

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For a growing number of GPCRs, second messenger production is regulated in a temporal and spatial manner. Indeed, in the case of the PTH1R, following an initial phase of cAMP signaling from the plasma membrane, internalization allows a second phase of G protein activation and signaling from early endosomes. PTH1R assembles with b-arrestin and Gbg in endosome to form a multimeric functional complex which regulates the Gs-dependent endosomal cAMP production. However, our knowledge of the molecular mechanisms underlying this endosomal activity remains incomplete. Because PTH1R also couples to Gq/11, we investigated the role of Gq/11 activation in mediating endosomal cAMP responses. Interestingly, the pharmacological inhibition of Gq/11 activity or its depletion in cells markedly reduced endosomal cAMP signaling. We determined that modulation of cAMP generation by Gq/11 occurs at the level of the heterotrimeric G protein via cell surface Gβγ subunits released, which, in turn, act in a phosphoinositide-3 kinase-dependent manner to promote the assembly of PTHR-βarrestin-Gβγ functional complexes that mediate endosomal cAMP responses.

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S15.3 RFRP-3/NPFF1R is a pronociceptive system involved in long-term effects associated with chronic opioids administration and inflammatory pain

Authors: Raphaelle Quillet, Severine Schneider, Valerie Utard, Armand Drieu la Rochelle, Jo Beldring Henningsen, Patrick Gizzy, Valerie Kugler, Tania Sorg-Guss, Marie-France Champy, Hamid Meziane, Benoit Petit-Demouliere, Valerie Simonneaux Brigitte Ilien, Frederic Bihel, Frederic Simonin

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RF-amide-related peptide-3 (RFRP-3) is involved in the modulation of several functions including nociception. It targets two different G protein coupled receptor subtypes called Neuropeptide FF1 receptor (NPFF1R) and Neuropeptide FF2 receptor (NPFF2R). However, the study of their functions *in vivo* is severely limited by the lack of highly specific tools. In this work, we describe the identification of a highly selective antagonist for NPFF1R. We demonstrate that RFRP-3/NPFF1R system is involved in the development of pain hypersensitivity and analgesic tolerance induced by chronic administration of morphine. For the first time, we observed the expression of NPFF1R and RFRP-3 transcripts in the dorsal horn of spinal cord. Finally, we demonstrate that NPFF1R was involved in the development of inflammatory hyperalgesia. Altogether, our data show that NPFF1R/RFRP-3 is a pronociceptive and anti-opioid system and further suggest that antagonists of this receptor might represent interesting therapeutic tools.

S15.4 Nanobody activating mGlu4 receptor discriminates between homo- and heterodimers

Authors: Jordi Haubrich[1], Joan Font[1], Anne Goupil[2], Pauline Schoeller[1], Damien Nevoltris[3], Francine Acher[4], Patrick Chames[3], Philippe Rondard[1], Laurent Prézeau[1] & Jean-Philippe Pin[1]

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Metabotropic glutamate receptors (mGluRs) are glutamate-activated G protein-coupled receptors that control the activity of many synapses in the brain. These eight receptor subtypes are promising targets for the treatment of many brain diseases including neurodegenerative diseases such as Parkinson's disease. Although mainly considered as homodimers, heterodimeric mGluRs received much attention in the recent years. Here we describe DN45, a single chain antibody (nanobody) specifically activating human $mGlu_4$ receptor. Mutagenesis and modelling studies revealed that the binding epitope covered residues from both lobes of the venus flytrap domain (VFT), leading to full receptor activation of the mGlu4 homodimer. DN45 did not activate the heterodimeric mGlu2-4 and acted as a pure allosteric modulator, largely potentiating the effect of an mGlu2 agonist or glutamate. As DN45 discriminates between homodimers and heterodimers, it possesses unique pharmacological properties. These unique properties could be used in uncovering the specific functional roles of the recently discovered heterodimer mGluRs.

S15.5 Characterization of endogenous $mGlu_2$ receptor interactome in mouse brain

Authors: Clémentine Philibert, Mathieu Oosterlaken, Julie Kniazeff, Philippe Rondard, Jean-Philippe Pin, Philippe Marin, Franck Vandermoere

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The metabotropic glutamate $mGlu_2$ receptor keeps on attracting particular attention given its implication in psychosis associated with schizophrenia. If this G protein-coupled receptor (GPCR) is the main target of a new generation of antipsychotics currently under clinical trial [1], its signaling in the brain and its pathological disturbances remain poorly characterized. Specific characterization of $mGlu_2$ receptor signaling in the brain was previously challenging due to a high homology with the metabotropic glutamate $mGlu_3$ receptor [2], which makes problematic the production of specific ligands or antibodies. Thanks to a new technology, our collaborators developed lama single chain antibodies (nanobodies) specifically targeting $mGlu_2$ receptor [3]. I have therefore taken advantage of these nanobodies to purify the endogenous receptor and its interacting proteins from mouse prefrontal cortex, a brain region disturbed in schizophrenia. This interactome was characterized by high resolution mass spectrometry. Bioinformatics annotations of candidate protein partners revealed high relevance to $mGlu_2$ receptor functions. Currently, the importance of several candidate partners in the response to $mGlu_2$ receptor stimulation is currently being tested.

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Session 16: Web-based resources and computational modelling of signal transduction (WG5)

S16.1 Human Cell Atlas: Mapping the human body one cell at a time

Mapping the temporal and spatial dynamics of the human endometrium in vivo and in vitro

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The endometrium, the mucosal lining of the uterus, undergoes dynamic changes throughout the menstrual cycle in response to ovarian hormones. We have generated a single-cell and spatial reference map of the human uterus and 3D endometrial organoid cultures. We dissect the signalling pathways that determine cell fate of the epithelial lineages in the lumenal and glandular microenvironments. We show in vitro that downregulation of specific pathways increases the differentiation efficiency along the secretory and ciliated lineages, respectively. In addition, some of the ligands that are expressed by distinct fibroblast populations are revealed to be linked genetically with endometrial disorders. Taken together, these mechanistic insights provide a platform for the future development of treatments for a range of common endometrial disorders including endometriosis and carcinoma.

S16.2 GPCRdb G proteins, arrestins and biased signalling

Albert Kooistra, Alibek Mamyrbekov, Sahar Gardizi, Gaspar P.-Szekeres, Mauricio Esguerra, Kasper Harpsøe, Alexander Hauser, Stefan Mordalski, David Gloriam

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The GPCR database, GPCRdb, provides reference data, analysis tools, interactive visualisation and experiment design tools to ~4,000 users/month. (www.gpcrdb.org)[1].

This talk will cover new pages in the signalling sections:

1) G proteins: G protein sequence alignments, snake-like residue topology diagrams, intersection or dissection of receptors based on their coupling to the four main G protein families and *in vitro* GPCR and G protein mutations/chimeras with effect on signalling. GPCR couplings and structure interactions & models. This will expand the resource from a previous study on GPCR-G protein selectivity[2].

2) Arrestins: Arrestin pages, alignments, structures and resources coming soon.

3) Biased signalling: Biased ligands: ligand counts and properties (analysis of annotated literature ligands), pathway-function atlas and resources coming soon.

Contribute: GPCRdb seeks user feedback and annotation help (for joint publication).

More: On Tues 13 Oct at 9:00, Albert Kooistra will present new GPCRdb resources part of the next database issue of *Nucleic Acids Research*.

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S16.3 Controlling receptor function from the extracellular vestibule of G-protein coupled receptors

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Receptor function is traditionally controlled from the orthosteric binding site of G-protein coupled receptors.

Here, we systematically demonstrate how introducing interactions in the extracellular secondary binding pocket (SBP) located far from the signalling interface can fine-tune the functional activity and signalling of human dopamine hD2 and hD3 receptor. Functional assays following the Go1 and arrestin pathways proved that with only small modifications in the SBP head group we can fine-tune the functional activity. Furthermore, we provide a plausible structural explanation for the observed effects based on molecular dynamics simulations, which revealed that the SBP interactions do not directly modulate the G-protein signalling in these ligands; rather they influence the positioning of the orthosteric binding motif.

These findings suggest optimization of the SBP binding part of bitopic ligands might be a useful strategy to develop GPCR ligands with designed functional and signalling profile.

S16.4 BitterMatch: Netflix-inspired ligand-receptor matching tool

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Work group section: Advanced Methodologies and Technologies

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Bitter taste receptors (T2Rs), are a subfamily of class A GPCRs,[1] capable of recognizing over 1000 chemically diverse ligands.[2] While some T2Rs are broadly tuned with more than 100 ligands, others are very selective with very few known ligands.[3] It is not easy to predict which T2R will recognize a particular ligand, and assignment of orthologs in other species is not straightforward.

Here we present BitterMatch, a novel algorithm that is capable of matching ligands with their putative human and mouse T2Rs. The algorithm uses chemical and structural features of the ligands and the receptors. In addition, it uses the already known ligand receptor ("customer-product") associations, similarly to recommendation systems used in Netflix, YouTube and more.

Despite the challenges in training the model on unbalanced and sparse data, BitterMatch achieves an excellent AUC of 0.984 compared to random assignment, with precision of 80% and recall of 48%. For some of the T2Rs i.e. T2R14 and T2R46 precision and recall are over 90% and 70% accordingly. Furthermore, BitterMatch outperformed the prior benchmark, which emphasises the significance of our algorithm. Analysis of the contributing features revealed that the recommendation part of the algorithm is an important contribution to the prediction, as well as structural features of the receptor and it's binding site (exposed surface area, hydrophobicity and more) and ligand features (heavy atoms, number of rings, etc).

BitterMatch enables the discovery of new ligands for T2Rs and will be used to shed light on possible evolutionary roles of T2Rs, connections between T2Rs and other GPCRs and other aspects of polypharmacology.

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Session 17: Roundtable Discussion

S17 AI in GPCR research – a roundtable discussion

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The explosion of biomedical data such as in genomics, structural biology and pharmacology can provide new opportunities to improve our understanding of human physiology and disease. In recent years, machine learning (ML) and artificial intelligence (AI) methods have received a significant boost of attention. ML/AI can be powerful for identifying abstract patterns within large data where traditional methods would be oblivious to. This comes without the need for manual feature engineering as systems can learn through implicit rules from the data provided [1]. G protein-coupled receptors (GPCRs) mediate a vast variety of critical biological processes and provide an ideal case study for quantitative, and multi-scale integration of these amounts of data to gain novel insights into receptor biology. How can we best leverage these exciting new techniques in areas such as protein structure prediction, bioactive ligand discovery, in vivo translationability or in our understanding of signalling determinants? Here, we would like discuss opportunities, weaknesses and advantages of these new technologies, which may contribute to probe our favourite targets at all scales.

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POSTER PROGRAMME

(Central European Time)

Poster Session 1 – Monday, Oct 12

Room 1.1

Protein dynamics and the macromolecular interactions in signaling pathways (WG1)

Poster ID	Submitter	Email	Time
1.1.1	Iu Raïch	iuraichipanisello@gmail.com	18:00
1.1.2	Mohammed Safar	mohammed.safar@strath.ac.uk	18:10
1.1.3	Alejandro Lillo Marquez	alilloma55@gmail.com	18:20
1.1.4	Rafael Rivas-Santisteban	rrivasbioq@gmail.com	18:30
1.1.5	Pedro Renault	pedro.renault@uab.cat	18:40
1.1.6	Romain Gerbier	romain.gerbier@college-de-france.fr	18:50

Room 1.2

Biological roles and physiological contexts of signal transduction (WG2)

Poster ID	Submitter	Email	Time
1.2.1	Miriam Scarpa	m.scarpa.1@research.gla.ac.uk	18:00
1.2.2	Gemma Navarro Brugal	g.navarro@ub.edu	18:10
1.2.3	Itziar Muneta-Arrate	itziar.muneta@ehu.eus	18:20
1.2.4	Margarida Fardilha	mfardilha@ua.pt	18:30
1.2.5	Philipp Rabe	Philipp.Rabe@medizin.uni-leipzig.de	18:40

Room 1.3

Design and optimization of molecular modulators of signal transduction (WG3)

Poster ID	Submitter	Email	Time
1.3.1	Suli-Anne Laurin	suli-anne.laurin@umontreal.ca	18:00
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1.3.3	Katarzyna Kucwaj-Brysz	katarzyna.kucwaj@uj.edu.pl	18:20
1.3.4	Kristoffer Sahlholm	kristoffer.sahlholm@umu.se	18:30
1.3.5	Margaret Cunningham	margaret.cunningham@strath.ac.uk	18:40

Room 1.4

Design and optimization of molecular modulators of signal transduction (WG3)

Poster ID	Submitter	Email	Time
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1.4.3	Marta López-Balastegu	marta.lopez01@estudiant.upf.edu	18:20
1.4.4	Tianyi Ding	tding02@qub.ac.uk	18:30
1.4.5	Romy Thomas	romy.thomas@mdc-berlin.de	18:40
1.4.6	Isabel Alves	i.alves@cbmn.u-bordeaux.fr	18:50

Room 1.5

Advanced methods and technologies in signal transduction research (WG4)/
Web-based resources and computational modelling of signal transduction (WG5)

Poster ID	Submitter	Email	Time
1.5.1	Brian Medel	brianmedelmo@gmail.com	18:00
1.5.2	Marek Grosicki	marek.grosicki@uj.edu.pl	18:10
1.5.3	Atsuro Oishi	aoishi@ks.kyorin-u.ac.jp	18:20
1.5.4	Maris-Johanna Tahk	maris-johanna.tahk@ut.ee	18:30

Poster Session 2 – Tuesday, Oct 13

Room 2.1

Protein dynamics and the macromolecular interactions in signaling pathways (WG1)

Poster ID	Submitter	Email	Time
2.1.1	Clare Harwood	clare.harwood@nottingham.ac.uk	18:00
2.1.2	Tomasz Maciej Stepniewski	tm.stepniewski@gmail.com	18:10
2.1.3	Jaume Lillo Jové	lillojaume@gmail.com	18:20
2.1.4	Agnieszka Sztyler	agnieszka.sztyler@charite.de	18:30
2.1.5	Alexander S. Hauser	alexander.hauser@sund.ku.dk	18:40

Room 2.2

Biological roles and physiological contexts of signal transduction (WG2)

Poster ID	Submitter	Email	Time
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2.2.3	Veronika Oralová	211812@mail.muni.cz	18:20
2.2.4	Samuel Mailhot-Larouche	samuel.mailhot.larouche@umontreal.ca	18:30
2.2.5	Victoria Elisabeth Groß	victoria.gross@medizin.uni-leipzig.de	18:40

Room 2.3

Design and optimization of molecular modulators of signal transduction (WG3)

Poster ID	Submitter	Email	Time
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2.3.2	Natalie Ben Abu	natalielysn@gmail.com	18:10
2.3.3	Ewelina Honkisz-Orzechowska	ewelina.honkisz@uj.edu.pl	18:20
2.3.4	Noey Boldizsar	Nmb41@duke.edu	18:30
2.3.5	Julie Dam	julie.dam@inserm.fr	18:40

Room 2.4

Design and optimization of molecular modulators of signal transduction (WG3)

Poster ID	Submitter	Email	Time
2.4.1	Nipuna Weerasinghe	Nipunawe@email.arizona.edu	18:00
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2.4.4	Marcel Bermudez	m.bermudez@fu-berlin.de	18:30
2.4.5	Natasa Tomasevic	natasa.tomasevic@meduniwien.ac.at	18:40
2.4.6	Ben Veerman	ben.veerman@strath.ac.uk	18:50

Room 2.5

Advanced methods and technologies in signal transduction research (WG4)/
Web-based resources and computational modelling of signal transduction (WG5)

Poster ID	Submitter	Email	Time
2.5.1	Célien Jacquemard	jacquemard@unistra.fr	18:00
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2.5.3	Tõnis Laasfeld	laasfeld@ut.ee	18:20

P1.1.1 Different cannabidiol- and cannabigerol-type phytocannabinoids acting on cannabinoid CB1, CB2 and CB1/CB2 heteromer receptors

Authors: Iu Raïch[b,d], Alejandro Lillo[a,d] Gemma Navarro[a,b], Katia Varani[c] , Fabrizio Vincenzi[c] , Rafael Rivas-Santisteban[b,d] , Irene Reyes-Resin[ab,d], Carlos Ferreiro-Vera[e] , Pier Andrea Borea[f] , Veronica Sanchez de Medina[e] , Xavier Nadal[g], Rafael Franco[b,d],*

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Work group section: Macromolecular Interactions in Signalling Pathways

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Recent approved medicines whose active principles are Δ9 Tetrahidrocannabinol (Δ9 - THC) and/or cannabidiol (CBD) open novel perspectives for other phytocannabinoids also present in Cannabis sativa L. varieties. Furthermore, solid data on the potential benefits of acidic and varinic phytocannabinoids in a variety of diseases are already available. Mode of action of cannabigerol (CBG), cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), cannabidivarin (CBDV) and cannabigerivarin (CBGV) is, to the very least, partial. Cannabinoid CB1 or CB2 receptors, which belong to the G protein-coupled receptor (GPCR) family, are important mediators of the action of those cannabinoids. Pure CBG, CBDA, CBGA, CBDV and CBGV from Cannabis sativa L. are differentially acting on CB1 or CB2 cannabinoid receptors. To determine the affinity of phytocannabinoids for cannabinoid receptors and functional assessment of effects promoted by these compounds when interacting with cannabinoid receptors, we performed four different functional outputs were assayed: determination of cAMP levels and of extracellular-signal-related-kinase phosphorylation, label-free dynamic mass redistribution (DMR) and ß-arrestin recruitment. Results: Affinity of cannabinoids depend on the ligand of reference and may be different in membranes and in living cells. All tested phytocannabinoids have agonist-like behavior but behaved as inverse agonists in the presence of selective receptor agonists. CBGV displayed enhanced potency in many of the functional outputs. However, the most interesting result was a biased signaling that correlated with differential affinity, i.e. the overall results suggest that the binding mode of each ligand leads to specific receptor conformations underlying biased signaling outputs.

P1.1.2 Interrogating purinergic $P2Y_1$ and $P2Y_{12}$ heterodimerisation

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Work group section: Macromolecular Interactions in Signalling Pathways

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ADP is an endogenous agonist for some G protein-coupled receptors (GPCRs) such as $P2Y_1$ and $P2Y_{12}$ purinoceptors. Previous studies demonstrated that AR-C69931MX, which is a $P2Y_{12}$ antagonist, inhibits ADP-induced intracellular Ca^{2+} in recombinant $P2Y_1$ and $P2Y_{12}$ receptors. In this study, we examined the effect of AR-C69931MX on native $P2Y_1$ receptor signalling. Also, test the physical interaction between both receptors in the recombinant and native system. In the recombinant system (tSA201 cells), the physical interaction between the receptors was determined using co-immunoprecipitation (co-IP), proximity ligation assay (PLA) and the combination of lifetime and FRET (FLIM-FRET) technique that was developed and optimised with the cooperation with the physics department at the University of Strathclyde[1]. While in the native system (BV-2 microglial cells), PLA was used to determine the physical interaction. To investigate the influence of this dimer on receptors function, we measured the intracellular Ca^{2+} influx using Cal-520 dye in FlexStation Microplate Reader. We found that $P2Y_1$ and $P2Y_{12}$ can form a heterodimer that locates on the cell membrane in the recombinant system while it locates intracellularly in the native system. Native $P2Y_1$ receptor signalling was inhibited using $P2Y_{12}$ antagonist, which is a similar finding from the Kennedy lab that used a recombinant system. Further work is underway to investigate the interaction between both receptors in disease models, and investigate the signalling relevance for the formed dimer.

References

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P1.1.3 Adenosine A2A and A3 Receptors Are Able to Interact with Each Other. A Further Piece in the Puzzle of Adenosine Receptor-Mediated Signaling.

Authors: Alejandro Lillo[1], Eva Martínez-Pinilla[2, 3,4], Irene Reyes-Resina[1], Gemma Navarro[1,5]*, Rafael Franco[5,6]*.

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Work group section: Macromolecular Interactions in Signaling Pathways

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The aim of this study was to check the possible interaction of two of the four purinergic P1 receptors, the A2A and the A3. Discovery of the A2A-A3 receptor complex was achieved by means of immunocytochemistry and of bioluminescence resonance energy transfer (BRET). The functional properties and heteromer print identification were addressed by combining binding and signaling assays in an heterologous expression system (HEK-293T transfected cells). The physiological role of the novel heteromer, which is also expressed in primary cultures of neurons, is to provide a differential signaling depending on the pre-coupling to signal transduction components and/or on the concentration of the endogenous agonist. The main feature was that the heteromeric context led to a marked decrease of the signaling originating at A3 receptors. Interestingly from a therapeutic point of view, A2A receptor antagonists overrode the blockade, thus allowing robust A3 receptor-mediated signaling. These and previous results suggest that all four adenosine receptors may interact with each other leading to heteromers with unique properties. Heteromer functionality expands the signaling outputs derived from the binding of adenosine to its cognate receptors.

Keywords: BRET, adenosine, heteromer print, purinergic P1 receptors, G-protein coupled receptors.

P1.1.4 Alterations in angiotensin AT_1 and AT_2 receptors heteromer levels in hemilesioned rat model of Parkinson's disease

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Work group section: Macromolecular Interactions in Signalling Pathways

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Angiotensin II and angiotensin AT_1 and AT_2 receptors are mainly known for their role in regulating water homeostasis and blood pressure. However, receptors are present in the central nervous system, where the renin-angiotensin system (RAS) plays a relevant role. RAS is dysbalanced in Parkinson's disease (PD), a disease due to *substantia nigra* neurodegeneration and whose dopamine-replacement therapy, using the precursor levodopa, leads to dyskinesias as the main side effect. We here confirm that AT_1 and AT_2 receptors form heterodimers ($AT_{1/2}$Hets) that are expressed in cells of the central nervous system. $AT_{1/2}$Hets are novel functional units with particular signaling properties. Importantly the coactivation of the two receptors in the heteromer reduces the signaling output of angiotensin. Remarkably, $AT_{1/2}$Hets, which are expressed in both striatal neurons and microglia, show a tight cross-modulation. In fact, candesartan, the antagonist of AT_1, increases the effect of AT_2 receptor agonists. In addition, using the unilateral 6-OH-dopamine lesion rat PD model we observed increases in $AT_{1/2}$Het expression upon disease progression and is maximal in dyskinetic animals. The results indicate that, boosting the action of neuroprotective AT_2 receptors using an AT_1 receptor antagonist, constitutes a promising therapeutic strategy in PD.

P1.1.5 Dynamical Correlations Reveal Allosteric Sites in the β_2 Adrenergic Receptor

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Work group section: Macromolecular Interactions in Signalling Pathways

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G Protein-Coupled Receptors (GPCRs) play a central role in many physiological processes and, consequently, constitute very important drug targets. Conformational changes underlie the biological function of GPCRs and drug design efforts should take into account the dynamical character of these proteins. In particular, the search for allosteric drugs has recently drawn attention, since they could be more selective, provide a finer control over the dynamical equilibrium of the receptor and lead to fewer side effects [1]. Accordingly, computational tools have been used to estimate the druggability of allosteric sites in GPCRs [2]. Although successful in many cases, these estimations are less effective in the case of hydrophobic sites in the interface between the protein and the membrane, suggesting the usefulness of complementary approaches to search for allosteric sites in these receptors. In this work, we investigated the coupling of binding sites in a GPCR to the collective motion of activation of the receptor, and verified if these couplings could reveal allosteric sites. In an ensemble of experimental structures of the β_2 adrenergic receptor (β_2AR), a prototypical class A GPCR, known allosteric sites were correctly identified on the basis of dynamical correlations. Our results indicate that this dynamics-based approach can be a complementary tool to characterize allosteric sites in GPCRs.

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2. Wakefield AE, Mason JS, Vajda S, Keserü GM (2019) Analysis of tractable allosteric sites in G protein-coupled receptors. Sci Rep 9:1-14

P.1.1.6 In vivo characterization of MTR/5-HT2c heteromers in mouse

Authors: Romain Gerbier[1], Florence Gbahou[1], Delphine Ndiaye-Lobry[1],, Philippe Delagrange[2], Ralf Jockers[1]

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Work group section: Macromolecular Interactions in Signalling Pathways

Position of the presenter: Postdoc

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Association of G protein-coupled receptors into heterodimeric complexes has been reported for over 50 receptor pairs in vitro but functional in vivo validation remains a challenge. Our recent in vitro studies (1) defined the functional fingerprint of heteromers composed of Gi-coupled melatonin MT2 receptors and Gq-coupled serotonin 5-HT2C receptors, in which melatonin transactivates phospholipase C (PLC) through 5-HT2C. Here, we identified this functional fingerprint in the mouse brain. For that purpose, we determined the activation of Gi and Gq proteins in mouse brain by measuring [35S]GTPγS incorporation into immunoprecipitated Gα subunits. Melatonin induced the activation of both proteins in cerebellum and hypothalamus. Whereas S20928, a MTRs antagonist, inhibited both, Gi and Gq activation ; SB243213, a specific 5-HT2C inverse agonist, only inhibited Gq activation, suggesting that melatonin receptor signaling through Gq is dependent on 5-HT2C transactivation. When using MT1 and MT2 knock-out mice melatonin-induced Gq activation was only seen in MT1 KO mice.These data indicate that MT2, and not MT1, functionally interacts with the Gq-

coupled 5-HT2C in mouse cerebellum and hypothalamus. Activation of the Gq/PLC pathway was further studied by measuring inositol-1 phosphate (IP1) levels by HTRF® in brain structures of melatonin-treated mice. Melatonin, increased IP1 levels in mouse cerebellum and hypothalamus unlike in mouse hippocampus and cortex. These effects were inhibited by the SB243,213, and were absent in MT2 and 5-HT2C knockout mice,.Taken together, our study provides for the first time in vivo evidence for the existence of functional MT2/5-HT2C heteromers in different mouse brain structures.

References

1. Kamal et al. (2015) Convergence of melatonin and serotonin (5-HT) signaling at MT2/5-HT2C receptor heteromers. J Biol Chem, 290(18):11537-46

P1.2.1 Phosphorylation of the M1 muscarinic acetylcholine receptor protects from neurodegeneration

Authors: Miriam Scarpa (presenter), Colin Molloy, Lisa Finlayson, Andrew B. Tobin, Sophie J. Bradley

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Work group section: Biological Roles of Signal Transduction

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Aims: Pharmacological activation of the M1 muscarinic acetylcholine receptor (mAChR) can improve cognitive deficits and exert disease-modifying effects in models of neurodegeneration and Alzheimer's disease [1]. This study provides evidence that the M1 mAChR-mediated phosphorylation-dependent pathway protects from prion neurodegeneration.
Methods: Knock-in mouse strains were generated whereby the WT M1 mAChR gene (Chrm1) is replaced by HA-tagged WT or phosphorylation-deficient (PD) mutant of the M1 mAChR [2]. Mice were inoculated with 1% control or prion (RML)-infected brain homogenates. Hippocampal function was assessed using burrowing test. Western blots and RT-qPCR were performed on dissected, frozen hippocampus and cortex. Immunohistochemistry was performed on slices of paraffin waxed, perfused brains.
Results: M1-PD mice with prion show earlier symptom onset (WT:22 w.p.i.; M1-PD:20 w.p.i.), faster decline in hippocampal function ($p<0.05$), and shorter lifespan compared with WT with prion (WT:25 w.p.i.; M1-PD:21 w.p.i.). Prion-infected M1-PD mice show higher levels of misfolded PrP compared to WT with prion, and higher expression of disease markers compared to WT (APO-E, Clusterin, SerpinA3N, Galectin-1), and significantly increased neuroinflammation.
Conclusion(s): Phosphorylation-dependent signalling of the M1 mAChR is protective in prion-induced neurodegeneration. This suggests that M1-activating ligands with a signalling bias towards the phosphorylation-dependent signalling pathway of the M1 mAChR are more likely to exert disease-modification of neurodegenerative diseases

References

1. Scarpa, M., et al.: Adv Pharmacol, 88, 277-310 (2020).
2. Bradley, S. J., et al.: Nat Chem Biol, 16(3), 240-249 (2020).

P1.2.2 Modulators of NMDA Glutamate Receptor Function

Authors: Navarro, G.[1,2]; Rivas-Santisteban, R.[2,3]; Casanovas, M. [2,3] Lillo, A. Saura, C.[2,4] and Franco, R. [2,3]

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Work group section: Molecular Modulators of Signal Transduction

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N-methyl d-aspartate (NMDA) ionotropic glutamate receptor (NMDAR), which is one of the main targets to combat Alzheimer's disease (AD), is expressed in both neurons and glial cells. The aim of this paper was to assess whether the adenosine A2A receptor (A2AR), which is a target in neurodegeneration, may affect NMDAR functionality.

On the one hand, NMDA and A2A receptors were able to physically interact forming complexes, mainly in microglia. Furthermore, the amount of complexes was markedly enhanced in activated microglia. On the other hand, the interaction resulted in a novel functional entity that displayed a cross-antagonism, that could be useful to prevent the exacerbation of NMDAR function by using A2AR antagonists. Interestingly, the amount of complexes was markedly higher in the hippocampal cells from the APPSw,Ind than from the control mice. In neurons, the number of complexes was lesser, probably due to NMDAR not interacting with the A2AR. However, the activation of the A2AR receptors resulted in higher NMDAR functionality in neurons, probably by indirect mechanisms. To sum up, it can be concluded that A2AR antagonists such as istradefylline, which is already approved for Parkinson's disease (Nouriast® in Japan and Nourianz® in the US), have potential to afford neuroprotection in AD in a synergistic-like fashion, via both neurons and microglia.

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P1.2.3 Enhanced coupling of the 5-HT_{2A}R on $G_{\alpha i1}$-protein in post-mortem frontal cortex of subjects with schizophrenia

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In animal and cellular models, activation of $G\alpha_{i1}$-proteins by serotonin 5-HT_{2A} receptor (5-HT_{2A}R) agonist drugs represents a molecular fingerprint of hallucinogenic properties. On the other hand, supersensitive 5-HT_{2A}Rs signalling through $G\alpha_{i1}$-, but not $G\alpha_{q/11}$–proteins, has been observed in schizophrenia.

The aim of the present study was to test whether such 5-HT_{2A}R supersensitivity represents the existence of enhanced constitutive activity of 5-HT_{2A}R on $G\alpha_{i1}$-proteins in post-mortem human frontal cortex of schizophrenic subjects. Pimavanserin (10^{-10}-10^{-6} M) was used as a 5-HT_{2A}R inverse agonist to promote uncoupling between receptor and G-protein. Finally, the selectivity of pimavanserin effect on the 5-HT_{2A}R was measured by using the selective 5-HT_{2A}R antagonist MDL-11,939 (10 µM). Scintillation Proximity Assay (SPA) was used as methodological approach.

Pimavanserin induced a higher inhibitory effect on 5-HT_{2A}R coupling to $G\alpha_{i1}$-protein in schizophrenic subjects (I_{max}=17±2%, n=23) than in controls (I_{max}=11±1%, n=23). In contrast, pimavanserin did not induce effects on 5-HT_{2A}R coupling to $G\alpha_{q/11}$-protein (I_{max}=0±1%, n=23 and I_{max}=-1±1%, n=23, respectively). In all the cases, co-incubation of pimavanserin with MDL-11,939 abolished the inhibitory effect on the $G\alpha_{i1}$-protein and no changes were found for $G\alpha_{q/11}$-protein.

These findings demonstrate an enhanced constitutive activity of 5-HT_{2A}R through the pro-hallucinogenic pathway in prefrontal cortex of schizophrenic subjects. The constitutive 5-HT_{2A}R hyperactivity could contribute to predispose to psychotic symptoms in schizophrenia.

P1.2.4 PP1 regenerative potential in tooth decay

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Tooth decay, or dental caries, is the most common infectious disease worldwide. It results from a complex interplay between host-related factors, acid-producing bacteria and fermentable sugars, resulting in the destruction of susceptible dental hard tissues, further affecting pulp and periapical tissues if untreated(1). Following damage, the dental pulp initiates a regenerative/reparative response, through odontoblastic activation, triggered to combat the injuries present in the tooth and dental pulp itself(2). Serine/threonine phosphatase 1 (PP1)(3,4) seems to play a role in odontoblastic differentiation and angiogenesis(3). Furthermore, the regenerative properties of PP1 have been shown in the cardiac and nervous systems, with PP1 found to be expressed in oral tissues (tongue and dental pulp)(3). It is well known that PP1 specific functions rely on PP1 complexes with different interactors known as RIPPOs(5). Taking this into consideration we investigated the involvement of PP1 in regenerative pathways of dental pulp-derived cells by characterizing PP1 isoforms' expression and interactome. We were able to show that PP1α, PP1β and PP1γ isoforms are expressed in pulp cells, but no PP1γ2 expression was observed, as expected. Also, the expression of RIPPOs involved in the signalling pathways that lead to the activation of regenerative and repair processes like AKT, p38 MAPK and MAPK1, was confirmed in dental pulp cells. Our preliminary results suggest that PP1γ is important in regulating the signalling pathways involved in tooth regeneration, which will be essential to ensure survivance, proliferation and differentiation of dental pulp cells, with potential modulatory capabilities in regenerative therapeutic strategies for tooth decay.

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P.1.2.5 The Relevance of SUCNR1 for Cancer Cell Survival and Metabolism

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Cancer cells often display metabolic alterations because of an increased energy demand due to high proliferation rates [1]. That is why main pathways of cellular metabolism like glycolysis and tricarboxylic acid (TCA) cycle are perturbed. Some metabolites of these pathways, like e.g. succinate, a central TCA cycle metabolite, also act as signaling molecules specifically activating G protein-coupled receptors (GPCRs). Since the altered metabolism is crucial for cancer cells, these GPCRs might also play an important role for cancer cell survival and proliferation.

Here, we analyzed the role of the Gi/Gq-coupled succinate receptor (SUCNR1) for cancer cell metabolism and survival as well as its signaling using a broad spectrum of signal transduction, imaging and biochemical methods. We found SUCNR1 mRNA expression in different cancer cell lines originating from various tissues. SUCNR1 signaling in these cell lines was confirmed using classical second messenger assays and the dynamic mass distribution technology. Using adherent 2D and spheroid 3D cell culture models, we found that the siRNA-induced knockdown of SUCNR1 differentially affected the viability of the analyzed cancer cell lines. Metabolic analyses of oxygen consumption rate and extracellular acidification rate revealed a higher basal and maximal respiration in cells with SUCNR1 knockdown suggesting a role in TCA cycle regulation.

In summary, our findings provide evidence that SUCNR1 is involved in the regulation of the metabolic activity in cancer cells, thus being crucial for their survival and proliferation.

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P1.3.1 Effects of the pharmacological chaperone UM0130866 on the cell-surface expression and signalling profile of MC4R variants associated with severe early-onset obesity

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Work group section: Molecular Modulators of Signal Transduction

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Melanocortin type 4 receptor (MC4R) is a G-protein coupled receptor which central activation increases energy expenditure and decreases appetite. Individuals bearing a MC4R loss-of-function mutation can develop severe early-onset obesity. Many of those mutated receptors are misfolded and retained in the cell by its quality control system, unable to reach their site of action. Pharmacological chaperones (PC) are membrane-permeable selective chemicals that can promote proper folding and subsequent targeting of a protein of interest, thus having the potential to rescue the function of mutants. Our goal is to develop MC4R PC that would promote agonist-induced activation toward signaling pathways important for weight loss in order to improve treatment efficiency and to reduce adverse effects. Here, we report that stimulation of the wild-type MC4R can strongly activate Gαs, Gα15 and Gαz, and weakly activate Gα14, GαoB and both β-arrestins signaling pathways, whereas no activation of Gαq/11 or Gα12/13 was detectable. Dose-response curves with endogenous agonists αMSH and βMSH, and synthetic super agonists NDP-αMSH and melanotan-II showed (1) that both endogenous agonists induce similar signaling profiles, (2) that Gαs and Gα15 pathways have similar potencies of activation and (3) that Gαz and βarr2 pathways have lower potencies of activation. The MC4R PC UM0130866 can strongly restore defective expression of the intracellularly retained R165W MC4R variant, as well as Gαs and Gα15 signaling pathways, whereas rescue of β-arr2 and Gαz signaling pathways were respectively weaker or not observed. Interestingly, UM0130866 also seemed to have a partial agonist effect on the rescued pathways.

P1.3.2 Novel Rimonabant-like peripheral selective CB1 ligands

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Rimonabant is a CB_1 inverse agonist approved for the treatment of obesity that was removed from the market in 2008 due to serious psychiatric side effects.[1] It was reported that Rimonabant´s neuropsychiatric liabilities are consequence of its binding to central CB_1 receptors. Blocking exclusively the peripheral CB_1 receptor it is possible to achieve a similar appetite suppression and weight loss effect,[2] therefore the search for novel peripheral selective CB_1 ligands have emerged as a promising approach for obesity control.[3] In the frame of a project aimed to identify new lead compounds by using MCR-based approaches, we herein document the discovery and optimization of novel series of brain non-penetrant Rimonabant analogues, which were assembled by Ugi or Ugi-split reactions. Some of the new ligands exhibit high CB1 affinity and selectivity profiles which is accompanied by high polar surface area, low Log P and micromolar activity toward PGP (MDR1). The most promising ammonium derivative and its non ionic precursor were tested in obese rats. One of these compounds showed a body weight loss effect as well as a reduction in circulating glucose, triglycerides, cholesterol, HDL and LDL levels comparable to rimonabant at the same doses.

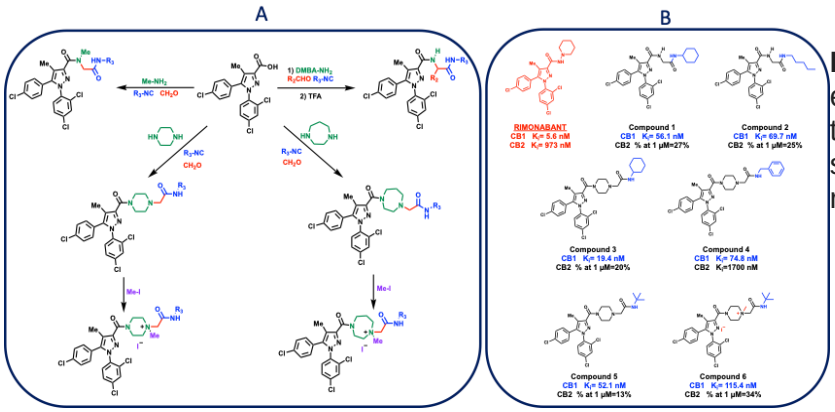

Figure 1. A) Synthetic scheme employed; B) Molecular structure of the most promising compounds of the series and their affinity for CB receptors.

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P1.3.3 Dual 5-HT$_6$R/5-HT$_{2A}$R action as a strategy towards effective therapy of cognitive impairment in Alzheimers's disease.

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder with complex etiology still being under investigation by many research groups in the world. Despite the huge number of molecules with promising preclinical activity only four compounds are currently authorized for treating cognitive impairment of AD with the last compound approved by FDA in 2003[1]. As the available therapies are not sufficiently effective, there is an urgent need to search for novel strategy. During last years, serotonin 5-HT$_6$ receptor was a promising target in this subject, due to many preclinical studies which indicated that both 5-HT$_6$R agonists and antagonists are able to improve cognitive impairment in animal models. On the other hand, none of the selective 5-HT$_6$R agents which reached clinical trials, showed enough efficacy to be approved by FDA, thus suggesting that such complex disorder requires not selective but multitargeted therapy which influence on more than one signaling pathway[2].

Our previous studies led to characterization of the original triazine-based selective 5-HT$_6$R antagonists[3]. Herein, we present our success in enhancement the pharmacological profile into dual action. As a second protein target, we selected serotonin 5-HT$_{2A}$ receptor, of which ligands are also known to play important role in cognition processes[4].

The interdisciplinary approach enabled us to define the structural requirements which are responsible for balancing between selective 5-HT$_6$R and dual 5-HT$_6$R/5-HT$_{2A}$R action. The most potent dual antagonist (5-HT$_6$ K_i = 11 nM; 5-HT$_{2A}$ K_i = 39 nM) was selected for *in vivo* pharmacological evaluation which confirmed its procognitive potency in Novel Objection Recognition test.

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P1.3.4 Voltage-dependent dopamine potency at D_1-like dopamine receptors

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In recent years, transmembrane voltage has been found to modify agonist potencies at several G protein-coupled receptors (GPCRs)[1,2,3,4]. Whereas the voltage sensitivities of the $G\alpha_{i/o}$-coupled dopamine D_2-like receptors (D_2R, D_3R, D_4R) have previously been investigated, the putative impact of transmembrane voltage on agonist potency at the mainly $G\alpha_{s/olf}$-coupled dopamine D_1-like receptors (D_1R, D_5R) has hitherto not been reported.

Here, we assayed the potency of dopamine in activating G protein-coupled inward rectifier potassium (GIRK) channels co-expressed with D_1R and D_5R in *Xenopus* oocytes, at -80 mV and at 0 mV. Furthermore, GIRK response deactivation rates upon dopamine washout were measured to estimate dopamine dissociation rate (k_{off}) constants.

Depolarization from -80 to 0 mV was found to reduce dopamine potency by about 7-fold at both D_1R and D_5R. This potency reduction was accompanied by an increase in estimated dopamine k_{off}s at both receptors. While the GIRK response elicited via D_1R was insensitive to pertussis toxin (PTX), the response evoked via D_5R was reduced by 64% (-80 mV) and 71% (0 mV) in the presence of PTX. Injection of oocytes with $G\alpha_s$ antisense oligonucleotide inhibited the D_1R-mediated response by 62% (-80 mV) and 76% (0 mV) and abolished the D_5R response when combined with PTX.

Our results suggest that depolarization decreases dopamine affinity at D_1R and D_5R. The voltage-dependent affinities of dopamine at D_1R and D_5R may be relevant to the functions of these receptors in learning and memory[5].

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P1.3.5 Extracellular release of microglial high molecular weight (HMW) high mobility group box 1 (HMGB1) by the serine protease thrombin

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High mobility group box 1 (HMGB1) is a key clinical biomarker in acute and chronic inflammatory conditions, cancer and autoimmune diseases [1] and is considered an attractive therapeutic target. HMGB1 is a nuclear protein that can translocate to the cytoplasm and be passively or actively released from the cell. Interestingly, despite lacking a nucleus, platelets are a rich source of HMGB1, where upon its release contributes to the development of thrombotic diseases [2-4]. While the mechanism of platelet HMGB1 activity has been primarily focused upon the role of Toll-like receptors (TLRs), evidence has emerged in other cellular contexts to suggest that HMGB1 release can also be induced by thrombin through proteinase-activated receptor (PAR) [5]. PARs are key platelet G-protein-coupled receptors well known for their role in thrombosis through activation via thrombin. Interestingly, thrombin has also been implicated in the proteolytic cleavage of HMGB1 [6], with new data demonstrating the extracellular release of a novel form of high molecular weight HMGB1 (HMW-HMGB1) (Cunningham, in preparation). This work has identified a distinct mechanism of extracellular release of HMGB1, which may be integral to the inflammatory role this protein plays in the coagulation context.

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P1.4.1 Discovery of new 5-HT$_{2A}$ receptor antagonists with a strategy of combining ligand and target-based drug design methodologies

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Serotonin 5-HT$_{2A}$ receptors (5-HT$_{2A}$R) are highly expressed in human prefrontal cortex, essential for learning and cognition [1]. Consequently, antagonists of these receptors are effective in treatment of various neuropsychiatric disorders, such as depression, insomnia, schizophrenia, anxiety, and Parkinson's disease [2]. Recent progress in molecular modelling studies has led to significant success in drug discovery using ligand and target-based methods. To design novel potent 5-HT$_{2A}$R antagonists, we report a strategy of combining three-dimensional quantitative structure-activity relationship (3D-QSAR) modelling with molecular docking and molecular dynamic (MD) simulation. Based on the common structural features, data set of 75 compounds was divided into three clusters. Firstly, MD simulations were carried out for each cluster representative in complex with 5-HT$_{2A}$R, providing important molecular level insight into their structure and dynamics. Afterward, to provide more accurate information about binding modes in the active site of the receptor, obtained conformations were used for docking studies and generation of the virtually bioactive conformations of all studied ligands. In addition, 3D-QSAR study, utilizing selected conformers, was carried out to gain further insights into the structural requirements that affect their antagonistic activity. Besides, some commercially available 5-HT$_{2A}$R antagonists were examined through *in vitro* PAMPA essay, as well as *in silico* computational methods not only to improve BBB permeability of new designed compounds, but also to establish promising tool to study their membrane permeability in detail. Overall, these and future results will provide new methodologies that could be used as guidelines for rational drug design of novel 5-HT$_{2A}$R antagonists.

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P1.4.2 Low basicity as a characteristic for atypical serotonin receptor ligands

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Serotonin receptors are extensively examined by academic and industrial researchers, due to their vital roles, which they play in the organism and constituting therefore important drug targets. Up to very recent times, it was assumed that the basic nitrogen in compound structure is a necessary component to make it active within this receptor system. Such nitrogen was supposed to interact in its protonated form with the aspartic acid from the third transmembrane helix (D 3x32) forming a hydrogen bond tightly fitting the ligand in the protein binding site. However, there are several recent studies that report strong serotonin receptor affinity also for compounds without a basic moiety in their structures. In the study, we carried out a comprehensive *in silico* analysis of the low-basicity phenomenon of serotonin receptor ligands. We focused on the crystallized representatives of the proteins of 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, and examined the problem both from the ligand- and structure-based perspectives. The study was performed for the native proteins, and for D3x32A mutants. The investigation resulted in the determination of non-standard structural requirements for activity towards serotonin receptors, which can be used in the design of new non-basic ligands.

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P1.4.3 Uncovering the high impact variants affecting GPCRs

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Although some G protein-coupled receptors (GPCRs) are known to be very polymorphic due to natural genetic variation, the development of drugs is typically based on the most frequent form of these receptors. Hence, variants affecting GPCRs may impact on the response to certain drugs, leading to important consequences such as adverse effects or overdose. In fact, ineffective prescriptions due to GPCR variants generate an economic burden of ~30 million British Pounds per year only in the UK [1]. In this study, we try to uncover some of the variants that are responsible for ineffective prescriptions which decrease the quality of life of the patients and generate unnecessary economic costs.

With this objective, we first selected a set of potentially 'high-impact' variants by studying the variants located in 'sensitive' regions, such as the binding sites, conserved motifs, or the intracellular binding sites. Secondly, this original set is reduced filtering only the variants present more frequently in the population and predicted to have functional impact, this is achieved using *impact scores*. Finally, the reduced set of selected variants, which are more likely to generate differences in drug response compared to the wild-type, are analyzed with molecular simulations. This last step will be the one used to confirm the different behaviour produced by the high-impact variant in the GPCR receptor when bound to a certain drug.

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P1.4.4 Exploring Allosteric Binding Cavities in the Chemokine Receptors from Computer Simulations

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Chemokine receptors are drug targets against immune and autoimmune diseases, as well as cancer (1). In recent years, several X-ray structures of chemokine receptors (CCR2, CCR5, CCR7, CCR9) in complex with allosteric ligands have been solved revealing the different location of allosteric sites (2-5). These structures provide new opportunities to explore the properties and behaviour of allosteric binding cavities in the chemokine receptor family and predict allosteric sites for the receptors for which structural knowledge is unknown (CCR4 and CXCR4). To do this, we conduct molecular dynamics simulations of the CCR7, CCR5, CCR4 and CXCR4 receptors in a water-lipid bilayer. The receptors are simulated in an empty form and the presence of allosteric ligands and probe molecules. The results of simulations highlight key conserved and non-conserved amino acid residues holding a ligand in allosteric sites at extracellular and intracellular sides of the receptors. Analysis of druggability and physicochemical properties of allosteric cavities; and topology and functional groups of structures of allosteric ligands indicate unique features of allosteric binding. The location and amino acid residues of allosteric binding sites at the CCR4 and CXCR4 receptors are predicted for experimental tests. Subtype selectivity of allosteric binding sites is discussed. The characterization of allosteric binding sites may provide new structure-based strategies in identifying a novel type of chemokine receptor modulators.

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P1.4.5 Genetically-encoded FRET-based biosensors to monitor cAMP dynamics in nanodomains

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The ubiquitous second messenger cAMP regulates a vast range of physiological processes such as cardiac function by relaying extracellular stimuli (by many different G protein-coupled receptors) to specific cellular functions. To achieve such specificity, we have recently shown that cAMP signaling is confined into nanometer-sized compartments in intact cells. The very small size of these compartments suggests that nanodomain signaling is controlled by only a few molecules of cAMP. In order to precisely monitor these single-molecule events in real time and in intact cells, we set out to develop genetically-encoded cAMP-biosensors with high dynamic ranges and fast kinetics. Based on a universally-taggable, positive cAMP switch, comprising a split cAMP-binding domain (CNBD), i.e. CUTie [1], we here report cloning and characterization of a family of FRET-based cAMP-biosensors. We found that using different combinations of several cyan and yellow fluorescent proteins within the same scaffold results in sensors with varying dynamic ranges, kinetics and affinities for cAMP. From our small library, we selected two sensors with dynamic ranges that exceed existing sensors by 2-3 fold, allowing to detect even subtle changes in cellular cAMP levels. We demonstrate that targeting these sensors to several nanodomains facilitates monitoring spatiotemporal cAMP signaling of the entire GPCR/cAMP/PKA signaling axis. Moreover, we show that the sensor design can be extended to green/red fluorescent protein pairs and that the split- CNBD design is applicable to other cAMP switches. In conclusion, we present a state-of-the-art toolbox to monitor cellular cAMP signaling in nanodomains.

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P1.4.6 Impact of lipid polyunsaturation on dopamine D2 receptor activation and signaling

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The dopamine D2 receptor (D2R) has been implicated in the etiology of several psychiatric disorders and is a main target of most antipsychotics. Interestingly, a “whole-body” decrease in long-chain polyunsaturated fatty acids (PUFA) levels – n-3 PUFAs such as docosahexaenoic acid (DHA) in particular - has been consistently described in these psychiatric disorders [1]. However, the mechanisms by which alteration in PUFA levels may contribute to pathogeneses and could alter the functionality and pharmacology of the D2R are unknown. Our project aims at unraveling the impact of membrane PUFAs on D2R pharmacological properties and conformation through biochemical, biophysical and modeling studies in both PUFA enriched cells and membrane model systems of controlled lipid composition. To this aim, we have investigated the impact of membrane PUFAs in the first stages of receptor activation, that is in the receptor/ligand interaction using fluorescence anisotropy and plasmon waveguide resonance (PWR) [2]. Moreover, PUFAs impact on the recruitment and activity of D2R signaling effectors was investigated by BRET approaches. Overall the data indicate that membrane PUFA composition impacts both agonist and antagonist affinity for D2R. This effect could be related to a preferential interaction of PUFA-containing phospholipids with the D2R leading to the formation of microdomains around the receptor as revealed by molecular modeling. Regarding the signaling cascades, preliminary data indicates an absence of influence of PUFAs on cAMP production but an impact on β-arrestin recruitment. The results could have a significant impact in the development of adjuvant therapeutic strategies for psychiatric disorders implicating the D2R.

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P1.5.1 Exploring the energetic landscape of GPCR activation using enhanced sampling methods

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Although G protein-coupled receptors (GPCRs) are involved in many physiological processes and diseases, the underlying mechanism responsible for their functionality and activation still remains poorly understood due to its complexity. Molecular dynamics (MD) is a promising approach for understanding atomistically the functionality of GPCRs. However, events such as GPCR activation are beyond the time scale of classical unbiased MD.
In this scenario, we take advantage of metadynamics, an enhanced sampling technique which allows us to overcome the time scale barrier. Here, we present an efficient metadynamics approach based on Path Collective Variables, which is able to define the β_2 adrenergic receptor activation mechanism in its apo form. We provide insights about the behaviour of the highly conserved motifs of the receptor along the transition between active and inactive states.

P1.5.2 Cellular phenotypic drug screening using eosinophils adhesion assay – the JN-35 histamine receptor ligand case study.

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Human eosinophils adhesion assay was developed as a new method used to evaluate the activity of histamine receptor ligands, that could be used as potential drugs in the treatment of many inflammatory and immunomodulatory diseases, including allergic rhinitis, asthma, atopic dermatitis, colitis, or pruritus [1]. This assay, in line with reemerging phenotypic-based drug discovery, allows studying the effects of GPCR ligands on native, non-modified human cells.

In this study, the triazine derivative JN-35 affinity and functional activity were studied at classical recombinant cell-based tests, including radioligand displacement, and cellular aequorin-based functional assays. Obtained data were confronted with the JN-35 activity in human eosinophils adhesion assay. Additionally, this compound was tested against its permeability, cytotoxicity, and metabolic stability. Finally, the JN-35 activity was confirmed during the *in vivo* studies.

Eosinophils adhesion assay detected robust anti-inflammatory activity of the JN-35 compound, although this ligand showed low affinity at histamine receptor subtypes and exhibited only mediocre receptor-mediated potency when examining during classical recombinant cell-based assays. Interestingly, the JN-35 mediated reduction of eosinophils adhesion was independent of the presence of histamine. JN-35 was also active during the *in vivo* examination, during which it inhibited both the croton oil-induced ear edema and pruritus in CD-1 mice.

In conclusion, eosinophils adhesion assay uncovered the pharmacological activity of JN-35 ligand, which was later confirmed *in vivo*, underscoring the value of a well-suited cell-based phenotypic screening approach in drug discovery.

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P1.5.3 β-Arrestin-2 BRET Biosensors Detect Different β-Arrestin-2 Conformations in Interaction with GPCRs

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β-Arrestins are critical regulators of G protein-coupled receptors (GPCRs) that desensitize G protein signaling, promote receptor internalization, and initiate signaling on their own. Recent structural findings indicate that β-arrestins adopt different conformations upon interaction with agonist-activated GPCRs. Here, we established a β-arrestin-2 conformational bioluminescence resonance energy transfer (BRET) sensor composed of the bright Nanoluc BRET donor and the red-shifted CyOFP1 BRET acceptor. The sensor monitors early intramolecular conformational changes of β-arrestin-2 in complex with a wide panel of different class A and class B GPCRs upon agonist activation and with orphan GPCRs known to spontaneously recruit β-arrestin-2. The introduction of the R170E mutant in the β-arrestin-2 sensor allowed the detection of a partially active β-arrestin-2 conformation, which is not dependent on receptor phosphorylation, while the deletion of the β-arrestin-2 finger-loop region detected the "tail-conformation" corresponding to the interaction of β-arrestin with the carboxyl-terminal domain of GPCRs. The new sensors combine the advantages of the BRET technique in terms of sensitivity, robustness, and suitability for real-time measurements with a high responsiveness toward early conformational changes to help to elucidate the different conformational states of β-arrestins associated with GPCR activation in living cells.

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P1.5.4 Characterization of ligand binding to Muscarinic acetylcholine M_4 receptor with budded baculoviruses and live cells

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Muscarinic acetylcholine receptors (mAChR) have 5 subtypes ($M_{1\text{-}5}$), that are linked to several severe diseases, making them an attractive drug target. The characterization of ligand binding to GPCR by the fluorescence methods have become quite popular. However, untlil now only a limited number of fluorescence ligands are available. Recently, several novel low molecular weight fluorescently labelled ligands targeting mAChRs were developed [1]. While M_1 and M_2 subtypes have received more attention, the M_4subtype has not been so widely studied. Therefore, new fluorescent ligands could be beneficial for studying M_4R subtype. We developed two novel fluorescence methods to study the binding of UR-CG072, one of the new fluorescently labelled ligands, to M_4R. Firstly, we implemented fluorescence anisotropy assay using M_4 displaying budded baculovirus particles [2] and secondly a combination of epifluorescence and bright-filed microscopy with live M_4R expressing CHO cells [3]. Both methods allow to monitor the binding kinetics of UR-CG072 and measure the affinities of unlabelled ligands. Ligand binding parameters acquired with both assays were in an agreement with previously published data. Both assays complement each other as fluorescence anisotropy assay allows higher throughput, while the live-cell assay provides a more native system for gaining additional information.

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P2.1.1 Functional solubilisation of the β_2-adrenoceptor (β_2AR) using Diisobutylene maleic acid (DIBMA)

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The β2-adrenoceptor (β2AR) is a well-established target in asthma and a prototypical GPCR for biophysical studies. Solubilisation of membrane proteins has classically used detergents; however, detergents often destabilise membrane proteins. Here we show the isolation of the β2AR in the polymer Diisobutylene maleic acid (DIBMA). We demonstrate that β2AR remains functional inside the DIBMALP and shows improved stability compared to n-Dodecyl- β-D-Maltopyranoside (DDM) detergent solubilised β2AR and previous use of thermostabilising mutations to improve β2AR stability. This provides an improved method for β2AR extraction for biophysical studies.

DIBMALP- β2AR functionality was assed using TR-FRET ligand binding assays and fluorescent propranolol. These studies showed comparable pK_D values for binding β2AR in membranes (pK_D=7.5±0.08), DDM-β2AR (pK_D=7.2±0.03) and DIBMALP-β2AR (pK_D=7.0±0.07), n=3. Moreover, competition binding studies showed comparable pK_i values for propranolol, isoprenaline and ICI 118551 binding DIBMALP-β2AR, DDM-β2AR and membrane -β2AR (n=3). TR-FRET thermostability assays gave a melting temperature (Tm) of 46.5°C ±2.1 for DIBMALP β2AR compared to conventional DDM β2AR (Tm=35.9°C ±2.4), n=3.

Overall, these data show that the DIBMALP-β2AR is functional and its pharmacological properties are similar to β2AR in membranes. DIBMALP-β2AR shows improved stability compared to the DDM solubilised receptor opening possibilities for further biophysical characterisation of the receptor.

P2.1.2 Mechanistic insights on how arrestin recognizes the GPCR “phosphorylation barcode”

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The functionality of G protein-coupled receptors (GPCRs) is linked to their interaction with multiple intracellular coupling partners, among them multifunctional proteins called β-arrestins (βarrs). Coupling of βarrs has been initially associated only with desensitization and internalization of GPCRs, however subsequent experimental data demonstrated their involvement in multiple downstream signalling pathways[1]. Interestingly, the formation of the GPCR/βarr complex can lead to various functional outcomes. One of the factors that drives this outcome is the specific phosphorylation pattern (or “phosphorylation barcode”) of the GPCR[2,3], however details of how arrestin is able to recognize and react to this code have remained obscure. To shed light on this question, we investigated the impact of specific phosphorylation patterns on βarr functionality in a prototypical GPCR - the Vasopressin 2 receptors. Combining biochemical and biophysical experiments we were able to link specific GPCR/βarr interaction patterns with a functional response[4]. Extensive molecular dynamics simulations allowed us to gain a mechanistic understanding how those patterns are recognized by arrestin, and subsequently promote a specific conformation of this protein. Most importantly, we propose that these conformational changes are linked to specific downstream events. Further experiments confirmed that the observed mechanism is conserved in other GPCRs[5].

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P2.1.3 Calmodulin Calcium Sensors Regulates NMDAR Function in Alzheimer Disease

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N-methyl-D-aspartate receptors (NMDARs) respond to glutamate to allow the influx of calcium ions and the signaling to the mitogen-activated protein kinase (MAPK) cascade. Both MAPK- and Ca2+-mediated events are important for both neurotransmission and neural cell function and fate. Using a heterologous expression system, we demonstrate that NMDAR may interact with the EF-hand calcium-binding protein calmodulin, but not with caldendrin. NMDARs were present in primary cultures of both neurons and microglia from cortex and hippocampus. Calmodulin in microglia and neurons are necessary for NMDA-induced MAP kinase pathway activation. Remarkably, signaling to the MAP kinase pathway was blunted in primary cultures of cortical and hippocampal neurons and microglia from wild-type animals by proteins involved in neurodegenerative diseases: α-synuclein, Tau, and p-Tau. A similar blockade by pathogenic proteins was found using samples from the APPSw,Ind transgenic Alzheimer's disease model. Interestingly, a marked increase of NMDAR-CaM complexes was identified in microglia from the transgenic mice. The results show that α-synuclein, Tau, and p-Tau disrupt the signaling of NMDAR to the MAPK pathway and that calcium sensors are important for NMDAR function both in neurons and microglia.

P2.1.4 Conservative mutations of key lysines in the N-domain of arrestin inhibit C-tail release and thereby attenuate arrestin interactions with GPCRs

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Regulation of G protein-coupled receptor (GPCR) signalling requires receptor phosphorylation and subsequent arrestin binding. In this study, we examined the functional role of two lysine residues in the arrestin-1 N-domain, K14 and K15, which interact with arrestin's auto-inhibitory C-tail in the basal state and bind key phosphorylated sites on the receptor C-terminus in the high-affinity complex [1, 2]. Our analysis employed multiple biochemical and spectroscopic methods to assess arrestin activation and receptor binding. Intriguingly, conservative mutation of these two sites to arginine resulted in impaired interaction of arrestin-1 with certain functional forms of the GPCR rhodopsin. 'Pre-complex'-like interactions with inactive, phosphorylated receptors (i.e. dark-state RhoP and the aporeceptor OpsP) were hampered by the K14R,K15R mutations, while binding to light-activated, agonist-bound phosphorylated rhodopsin (Rho*P) was apparently not affected. Since pre-complex interactions are heavily dependent on receptor phosphorylation [3, 4], we compared the ability of the K14,15R mutant to bind distinctly phosphorylated rhodopsin peptide analogues. While wild-type arrestin-1 robustly associated with phosphopeptides containing at least three phosphorylated sites, the K14,15R mutant required more highly phosphorylated peptides for measureable interaction. Overall, our data suggest that conservative replacement of K14 and K15 with arginine strengthens association of the arrestin C-tail with the N-domain, thereby stabilising the basal state. Arrestin activation, which entails C-tail exchange with the phosphorylated receptor C-terminus, is not completely inhibited in the K14,15R mutant yet requires more energy in the form of more phosphorylated receptor residues and/or engagement of the active receptor core [5].

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P2.1.5 The Coupling Selectivity Landscape of 100 Therapeutically Relevant GPCRs

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The ability of individual G protein-coupled receptors (GPCRs) to engage multiple signaling pathways is a unique mechanism to respond and fine-tune all possible environmental stimuli. To gain insights into the molecular basis of selectivity do we require novel tools to determine the G protein subtypes and ß-arrestins engaged by a given receptor. Here, we analyse the selectivity landscape of 100 therapeutically relevant GPCRs based on data obtained through a new BRET-based effector membrane translocation assay (EMTA), which monitors the activation of each Gα protein through the recruitment of selective G protein effectors and βarrestins to the plasma membrane. Our analysis revealed a great diversity of coupling profiles with some receptors displaying exquisite selectivity, whereas others promiscuitly engage all four G protein families. We show that coupling profiles for individual subtypes largely cluster within evolutionary G protein family assignments. However, G15 displays a distinct profile from other members of the Gq/11 family. Comparison with existing datasets points to commonalities but also critical differences between studies. Overall, we describe a unique resource for studying GPCR selectivity, which opens opportunities for the development of better drugs.

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P2.2.1 Novel, non-apoptotic functions of FasL in bone development and maintenance

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FasL/Fas system is a well-known regulator of apoptotic cell death, particularly in the immune system. More recent studies, however, show this signaling also in non-apoptotic events, including bone formation. From our observation, FasL was detected in different cells in prenatal bone, with the most abundant expression in osteoblasts. Analysis of osteogenic genes in the mandible of FasL-deficient (gld) mice showed decreased expression of two osteogenic markers, Sost and Mmp2. This regulatory pathway was confirmed in MC3T3-E1 cells, a lineage widely used in osteoblastic research. Addition of recombinant mouse FasL resulted in increased Mmp2 expression, and inversely, treatment by anti-FasL molecule decreased expression of Mmp2. These changes in osteogenic pathways might be connected with increased bone density observed in the adult gld mice. Notably, an age specific phenotype was detected in gld mice when the bone density showed a decrease in early postnatal development when compared to normal mice of the same stage. These pilot findings point to specific functions of FasL in different bone cells and indicate novel roles of FasL/Fas signaling in osteogenesis.

Further research and international networking have been supported by the Inter-COST project LTC20048 (www.msmt.cz)

P2.2.2 C5aR$_1$ stimulates the onset of the osteoclastogenesis process

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Recent insights have indicated an active role of the complex complement system not only in immunity, but also in bone remodeling[1]. Evidence from knockout mice and observations from skeletal diseases have drawn attention to the C5a/C5aR$_1$ axis of the complement cascade in the modulation of osteoclast functions[2]. With the aim to identify novel C5aR$_1$ regulators, a medicinal chemistry program was initiated, driven by structural information on a minor pocket of C5aR$_1$ that has been proposed to be a key motif for C5aR$_1$ intracellular activation. The impact of the peptidomimetic orthosteric C5aR$_1$ antagonist (PMX-53), of two newly synthesized allosteric C5aR$_1$ antagonists (DF2593A, DF3016A), and of specific siRNA downregulation of C5aR$_1$, were examined for regulation of osteoclastogenesis, using a wellvalidated in-vitro model starting from RAW264.7 precursor cells. These pharmacological and molecular approaches both reduced the osteoclast maturation of RAW264.7 cells, induced by the cytokine RANKL, to limit the transcription of the main differentiation markers (NFATc1, Cathepsin-k, MMP-9 and TRAP), as evaluated by real-time PCR. These treatments were ineffective on the subsequent step of osteoclast syncytium formation. Among the C5aR$_1$ antagonists analyzed, DF3016A inhibited the osteoclast degradation activity through the inhibition of C5aR$_1$ signal transduction and transcription. This study was extended to a human osteoclastogenesis model, using as osteoclast precursors monocytes purified by negative depletion of peripheral blood mononuclear cells of healthy donors. In this system, DF2593A was significantly efficient in reducing the osteoclast functional activity. Altogether, these results indicate the therapeutic potential of C5aR$_1$ allosteric modulators for the treatment of osteoclast-related pathologies.

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P2.2.3 Myb transcription factor – investigation of novel roles related to osteogenesis

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Based on the origin, there are two types of osteogenesis (bone formation): intramembranous and endochondral, when the latter one contains a chondrogenic step prior to osteoblastic differentiation. Myb transcription factor is a well characterized oncogene with long term known essential engagement in hematopoiesis. Notably, recent reports indicate novel associations with osteogenesis. We have shown that during the prenatal development of mandibular (intramembranous) bone, MYB protein was detected in the osteoblasts and osteoclasts of the tooth surrounding bone. In postnatal tooth and bone development, the MYB protein was observed especially along the outer surface of the alveolar bone ridge. The osteogenic potential was later confirmed and further specified also by other teams and Myb was demonstrated to enhance new bone formation in case of dental implants. Regarding long (endochondral) bones, chondrogenic and osteogenic potential of Myb was analysed using micromasss cultures. Despite several indications of novel functions of Myb in osteogenesis, the mechanism has not been clarified yet.

Myb knock-out is prenatally lethal (by day 15 in the mouse) thus simple bone analyses are hindered. Nevertheless, the mandibular bone at day 15 reaches a stage when osteoblasts, osteoclasts as well as osteocytes are already present. We aim to use this model for following investigations with focus on Rank-Rankl-Opg signalling where the preliminary data point to Rank decrease and Rankl/Opg increase in Myb deficient bones. For this purpose, cooperation with prof. Frampton (University of Birmingham) has been established.

Further research and international networking have been supported by the Inter-COST project LTC20048 (www.msmt.cz)

P2.2.4 Selective Desensitization of the M_3 Receptor: toward improvement of Asthma treatment

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BACKGROUND: M_3 receptor (M_3R) antagonists are efficient in the short-term treatment of asthma mainly by limiting bronchoconstriction. Nevertheless, they can aggravate respiratory symptoms over time by upregulating M_3R. Inversely, M_3R can be desensitized by mechanisms of uncoupling and/or downregulation in the case of prolonged activation. In this study, we tested the hypothesis that selective M_3R desensitization could be a good approach to treat asthma. Furthermore, we characterized the molecular mechanisms behind M3R desensitization by using tissue and cell models of M_3R desensitization.

METHODS: An HDM murine model of asthma was exposed to an aerosolized M_3R agonist, methacholine (MCh), for 4.5 consecutive days. *In vivo* airway responsiveness and the contractility of isolated tracheas were then assessed by using the FlexiVent system and in organ baths, respectively. The tissue model of M3R desensitization consists of exposing the tracheas to MCh and evaluating their contractility. The cell-based model of desensitization consists to monitor receptor internalization in response to M_3R agonist in HEK293SL cells by using the ebBRET technology.

RESULTS: Repeated *in vivo* exposures of 'asthmatic' mice to MCh decreased their airway hyperresponsiveness and tracheal contractility. The tissue and cellular model of desensitization was abolished by an inhibitor of internalization (hypertonic sucrose). Gene knockout in the cellular model demonstrated that M_3R internalization is dependent on b-arrestins and GRKs and, in contrast, independent of G_q proteins associated with bronchoconstriction.

CONCLUSION: This study suggests that a compound that selectively promotes the M_3R desensitization is conceivable and could have long-term clinical benefit in the context of asthma.

P2.2.5 Human NPY and its conservation in C. elegans challenges phylogenetic views on the FLP/NPR system

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Neuropeptide Y (NPY) and its receptors affect various processes, among others food intake, and therefore, it is a potential target for obesity therapy[1]. As the NPY system is an enlarged multi-ligand/ multi-receptor network, studies in model organisms are indispensable to assess molecular properties in a physiological context.

With the FMRFamide-like peptide (FLP)/Neuropeptide receptor-Resemblance (NPR) system as main candidate to be the NPY orthologous system, *C. elegans* could be an excellent model for such studies. However, although the NPY system is evolutionary conserved in all bilaterians, there is no clear evidence that the FLP/NPR system is indeed a true orthologous system in *C. elegans*.

To address this issue, we performed a comprehensive pharmacological study of the FLP/NPR system proving that G-protein coupling and ligand requirements for receptor activation are similar to the human NPY system. *In vitro* and *in vivo* analyses showed cross-reactivity of human NPY with the FLP/NPR system culminating in the ability of the human receptors to functionally substitute FLP/NPR signalling *in vivo*. Further, an exchange of conserved amino acids in the neuropeptides of both systems led to a strong shift of receptor binding and activation *in vitro*.

These data demonstrate the pharmacological and functional similarities of human and *C. elegans* NPY systems, expanding our understanding of the evolution of the neuropeptide Y system and its function beyond phylogenetic reconstructions.

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P2.3.1 Sweet chirality – the taste of L- and D-glucose stereoisomers

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Chirality plays a key role in biomolecular recognition. Typically, a change in chirality will dramatically affect ligand–receptor binding. However, both D-glucose and its enantiomer L-glucose elicit sweet taste in humans. We show that L- and D-glucose are perceived as similarly sweet by humans, and that in cell-based functional assays, both enantiomers activate the human sweet taste receptor TaS1R2/TaS1R3. We hypothesize that both L- and D-glucose occupy the orthosteric binding site in the VFT domain of TAS1R2 [1]. Using induced-fit docking to a homology model of this domain, we identify two subpockets in this binding site [2]. The model suggests that glucose molecules can bind in either of these subpockets, which overlap with the predicted positions of monosaccharide units of sucrose. One subpocket is close to the hinge between the two VFT domain lobes, and overlaps with aspartame and neotame site, the second subpocket overlaps with a sweetness enhancers site. These findings suggest a framework for rational design of sweeteners combinations.

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P2.3.2 Sweet taste of heavy water is mediated by taste GPCR receptor

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Work group section: Macromolecular Interactions in Signalling Pathways; Molecular Modulators of Signal Transduction

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Hydrogen to deuterium isotopic substitution has only a minor effect on physical and chemical properties of water and, as such, is not supposed to influence its neutral taste. Here, we conclusively demonstrate that humans are, nevertheless, able to distinguish D_2O from H_2O by taste. Indeed, highly purified heavy water has a distinctly sweeter taste than same-purity normal water and adds to perceived sweetness of sweeteners. In contrast, mice do not prefer D_2O over H_2O, indicating that they are not likely to perceive heavy water as sweet. For humans, the sweet taste of D_2O is suppressed by lactisole, which is an inhibitor known to act via the TAS1R3 monomer of the TAS1R2/TAS1R3 sweet taste receptor. HEK 293T cells transfected with the TAS1R2/TAS1R3 heterodimer and the chimeric Gα16gust44 G-protein are activated by D_2O but not by H_2O. The present study resolves a long-time controversy about the taste of heavy water, confirms that its sweet taste is mediated by the human TAS1R2/TAS1R3 sweet taste receptor, and opens way to future studies of potential sites and modes of action.

P2.3.3 Growth inhibition and apoptosis induction of fibrosarcoma cells via GPR18-dependent and independent pathways

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G protein-coupled receptor 18 (GPR18) is considered an orphan receptor and may represent a novel therapeutic target. There is an urgent need to develop truly useful tools to study GPR18 signalling and pharmacology, both antagonists and agonists. In our previous studies, bicyclic imidazole-4-one derivatives were discovered as the first synthetic scaffolds that block GPR18 receptor function as detected in β-arrestin assays [1,2]. Among them, two compounds showed the best potency and selectivity profile: PSB-CB-5 (IC_{50}=0.279 µM) and PSB-CB-27 (IC_{50}=0.650 µM). In the further course of our research small-peptide like compounds were developed which represent a new class of GPR18 agonists: KS4 and KS5. In the present study, we investigated the GPR18-dependent signalling pathway that is involved in cell survival and apoptosis induction in human HT-1080 fibrosarcoma cells.

Activation of GPR18 with the selective agonists KS4 and KS5 inhibited HT-1080 cells growth and induced apoptosis. The presence of GPR18 antagonists (PSB-CB-5 or PSB-CB-27) restored cell viability to control levels, but did not protect cells against the proapoptotic effects of the agonists. These results suggest that cell growth inhibition is directly GPR18-dependent as opposed to the induction of apoptosis. HT-1080 cells death was found to be caspase-dependent but not induced by GPR18 activation.

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P2.3.4 Biased Agonism and the Phosphorylation Barcode at CXCR3

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G protein-coupled receptors (GPCRs), the largest class of receptors in the human genome, are implicated in a vast array of pathologies and physiologic processes. Following ligand stimulation, GPCRs interact with G proteins and β-arrestins, which have distinct signalling profiles. The ability of ligands or receptors to preferentially activate one signalling pathway over the other, a phenomenon known as biased agonism, illustrates the high degree of specificity in GPCR signalling and presents exciting opportunities for drug discovery[1]. One mechanism underlying this phenomenon is the phosphorylation barcode hypothesis which states that the phosphorylation pattern of the receptor's C-terminal tail promotes distinct interactions with certain transducer elements over others[2]. The chemokine system, a subfamily of GPCRs primarily present on leukocytes, serves as an endogenous example of biased agonism due to the significant promiscuity between ligands and receptors in this system[3]. The chemokine receptor CXCR3 and its ligands CXCL9, CXCL10, and CXCL11 have clearly demonstrated bias in vivo and in vitro[4, 5]. To investigate how the phosphorylation pattern of CXCR3 impacts its signalling, we created phosphodeficient CXCR3 mutants and probed their interactions with different isoforms of GPCR kinases (GRKs) upon stimulation with the endogenous ligands using a split nano-luciferase assay. Our data reveals significant signalling bias, at the ligand, receptor, and GRK level. The mutants demonstrate clear preference for certain isoforms of GRKs over others upon ligand stimulation, and this preference depends on the identity of the ligand-receptor combination. These results support the hypothesis that specific GPCR phosphorylation patterns may promote certain signalling pathways over others.

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P2.3.5 The orphan GPR50 inhibits neurite outgrowth and cell migration by constitutively activating the G12/13 -RhoA pathway

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Variants of the orphan G protein-coupled receptor GPR50 have been identified as risk factors for neuropsychiatric disorders. However, the role of GPR50 in the central nervous system (CNS) and its link to CNS disorders remain elusive. By using cells from GPR50 knockout mice we define GPR50 as a novel inhibitor of neurite outgrowth and neuronal migration by constitutively activating G12 / 13 proteins and the RhoA pathway. GPR50 appears to be an important new regulator of neurite formation via a pathway independent of NogoA, one of the best characterized myelin-associated neurite growth inhibitors.

P2.4.1 Allosteric Regulation of GPCR Rhodopsin by Soft Matter

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G-protein-coupled receptors (GPCRs) are allosteric membrane proteins that mediate cellular signal transduction. They exist as dynamic conformational ensembles with multiple inactive and active conformations. Here we use the visual receptor rhodopsin as an archetypal GPCR to investigate how soft matter (lipid membrane and cellular water) modulate the conformational dynamics of the GPCR activation, hypothesizing a flexible surface model. By using different polyethylene glycol (PEG) solutions, the osmotic pressure on rhodopsin in native membranes and POPC recombinant membranes was varied. Shifting of the metarhodopsin equilibrium due to the changing the lipid environment and osmotic pressure was probed using UV-Visible spectroscopy. The metarhodopsin equilibrium was shifted towards the inactive Meta-I state in POPC recombinant membranes compared to the native membrane environment. Furthermore, the analysis of transducin peptide-binding isotherms reveals that the binding affinity of the peptide is significantly decreased when the lipid environment is changed from the native lipids to POPC lipids. We further performed a series of experiments to study how the binding affinity of the transducin C-terminal peptide to the Meta-II state is affected by the osmotic pressure in recombinant membranes. The POPC lipid membrane has a zero-spontaneous curvature that shifts the equilibrium towards the more compact, inactive Meta-I state compared to the native lipid membrane environment that has a negative spontaneous curvature that favors the more expanded state of Meta-II. Our results delineate the crucial role of soft matter (lipids and water) in regulating the metarhodopsin equilibrium.

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P2.4.2 The pharmacology of CXCR2 intracellular NAMs assessed using novel TR-FRET ligand binding and real time receptor signalling assays

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CXCR2 receptor signalling is involved in chronic inflammation and cancer progression[1].
Here we investigated the mechanism of action of structurally distinct intracellular negative allosteric modulators (NAMs) of CXCR2 using time-resolved Förster resonance energy transfer (TR-FRET) and split luciferase complementation (NanoBiT, Promega) assays.

NanoBit assays were conducted in HEK293T cells transfected with the human CXCR2 modified with a LgBit fragment and either β-arrestin2 or miniGo modified with a SmBit fragment. Cells were pre-treated with NAMs and basal and CXCL8-stimulated luminescence was measured kinetically.

TR-FRET assays were conducted in Tb-labelled[2] membranes prepared from HEK293T cells expressing the N-terminally SNAP-modified CXCR2. Membranes were pre-treated with NAMs and the binding of AF647-CXCL8 (10nM) was measured at 1-5-hour endpoints.

In arrestin and miniGo NanoBit assays, SB2656510 primarily decreased the potency of CXCL8, whereas navarixin and AZ10397767 decreased the maximal response of the chemokine. Navarixin and SB265610 decreased the basal effector recruitment, whilst AZ10397767 increased it. Cold CXCL8 displaced tracer binding with a pKi of 8.16 ± 0.28, whereas SB265610, navarixin and AZ10397767 only caused partial tracer displacement relative to CXCL8 (35.30 ± 7.76 %; 36.33 ± 2.41; 30.32 ± 8.30 respectively).

SB265610, navarixin and AZ10397767 produced equivalent NAM effects on CXCR2 recruitment of different effector proteins and were each partial inhibitors of chemokine binding in the TR-FRET assay. However, they displayed distinct effects on basal effector recruitment and CXCL8 concentration-response relationships despite similarities in the proposed intracellular target binding site. The high affinity and slow kinetic properties of navarixin may underpin this functional variation.

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P2.4.3 Dissecting the roles of GRK2 and GRK3 in μ-opioid receptor internalization and b-arrestin2 recruitment using CRISPR/Cas9-edited HEK293 cells

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Most G protein-coupled receptors (GPCRs) recruit arrestins and internalize upon agonist stimulation. For the μ-opioid receptor (μ-OR), this process has been linked to development of opioid tolerance. GPCR kinases (GRKs), particularly GRK2 and GRK3, have been shown to be important for μ-OR recruitment of arrestins and internalization. However, the contribution of GRK2 and GRK3 to arrestin recruitment and receptor internalization, remain to be determined in their complete absence. Using CRISPR/Cas9-mediated genome editing we established HEK293 cells with knockout of GRK2, GRK3 or both to dissect their individual contributions in b-arrestin2 (arrestin3) recruitment and μ-OR internalization upon stimulation with four different agonists. We showed that GRK2/3 removal reduced agonist-induced μ-OR internalization and b-arrestin2 recruitment substantially and we found GRK2 to be more important for these processes than GRK3. Furthermore, we observed a sustained and GRK2/3 independent component of b-arrestin2 recruitment to the plasma membrane upon μ-OR activation. Rescue expression experiments restored GRK2/3 functions. Inhibition of GRK2/3 using the small molecule inhibitor CMPD101 showed a high similarity between the genetic and pharmacological approaches, cross-validating the specificity of both. However, off-target effects were observed at high CMPD101 concentrations. These GRK2/3 KO cell lines should prove useful for a wide range of studies on GPCR function.

P2.4.4 Ligand-Specific Allosteric Coupling Controls GProtein-Coupled Receptor Signaling

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Allosteric coupling describes a reciprocal process whereby G-protein-coupled receptors (GPCRs) relay ligand-induced conformational changes from the extracellular binding pocket to the intracellular signaling surface. Therefore, GPCR activation is sensitive to both the type of extracellular ligand and intracellular signaling protein. We hypothesized that ligand-specific allosteric coupling may result in preferential (i.e., biased) engagement of downstream effectors. However, the structural basis underlying ligand-dependent control of this essential allosteric mechanism is poorly understood. Here, we show that two sets of extended muscarinic acetylcholine receptor M_1 agonists, which only differ in linker length, progressively constrain receptor signaling. We demonstrate that stepwise shortening of their chemical linker gradually hampers binding pocket closure, resulting in divergent coupling to distinct G-protein families. Our data provide an experimental strategy for the design of ligands with selective G-protein recognition and reveal a potentially general mechanism of ligand-specific allosteric coupling.

P2.4.5 Discovery of κ-opioid receptor peptide ligands in plants

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G protein coupled-receptors (GPCRs) protoypes, that have been attracting the great attention in discovery and development of painrelivers throughout the past decades are opioid receptors. To this date, μ-opioid receptor (MOR) remains, undeniably, the most valuable GPCR target in terms of effective treatment of clinical pain. However, the resulting side effects are highly severe, even fatal. For instance, opioid addiction occurring as the result of opioid overuse is the main cause for the ongoing 'opioid crisis' in the USA (1). In this context the κ- opioid receptor (KOR) may be considered as a valuable alternative pain target since it is far less associated with MOR-based adverse reactions (2). Hence, the demand to develop KOR-targeting therapeutics with favorable side-effect profile is of great importance. Historically, natural products have been an invaluable source of novel molecules for drug discovery. For instance, cysteine-rich peptides found in plants represent promising starting points for development of novel therapeutics. Previous studies have demonstrated the potential of the so-called cyclotides as pharmacological modulators of GPCRs (3). Accordingly, the aim of this project was to identify novel plant peptides and investigate their ability to modulate KOR signaling. This project contributes to the idea of utilizing the potential of plant-derived peptides to serve as ligands of KOR, and other GPCRs. More generally, our findings could aid the discovery and development of novel and safer KOR-based drug candidates.

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P2.4.6 Novel off-target effects of anti-cancer drugs on cardiovascular gap-junctional proteins

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Doxorubicin is widely used as a chemotherapeutic agent however, patients display dose-dependent cardiotoxicity that manifest as arrhythmias, ischemia, cardiomyopathy, systolic dysfunction and heart failure [1]. Circulating doxorubicin impacts human coronary microvascular function [2] and increases permeability of the endothelium and impacts cell to cell communication [3]**.** Our studies have focused on the impact of doxorubicin on a gap-junction protein, connexin-43. Connexin-43 is important to maintain normal cardiac function and has also been found to play a role in cancer and metastasis formation. Our studies conducted in human coronary artery endothelial cells, revealed connexin-43 re-localisation in response to doxorubicin. Normally, connexin-43 is mainly expressed on the cell membrane, however doxorubicin induced higher cytosolic expression which coincided with a reduction of total connexin-43 levels. Current ongoing research is investigating the role of extracellular vesicles in connexin-43 release in triple negative breast cancer cells in response to doxorubicin, introducing a potential indirect effect of the drug on the vascular system.

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P.2.5.1 ATOLL: A visualization tool to compare transmembrane domains structures

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The 3D structure of a transmembrane protein can be represented in a 2D frame in various ways, with more or less structural information. The top-viewed helix box diagram, as in GPCRdb [1], simply shows which helices are in contact and the putative contact residues. The screenshot of a crystal structure or a modelled conformation can provide atomic details, yet it can also be difficult to interpret. If the protein is described by multiple conformations (e.g. molecular dynamics), the representation has to be simplified. Usually dimensionality reduction is applied. For example, a distance-based heatmap that shows distance pair frequencies, or, the root mean square deviation of atomic coordinates over time. In these cases, global view of the receptor is lost.

Here we propose ATOLL (Aligned Transmembrane dOmains Layout fLattening), a tool to visualize the multiple layouts of the transmembrane domains of a protein. It is based on the 3D-aligment of the domains followed by a projection of atomic coordinates onto the plane of the membrane.

We describe here two possible applications of ATOLL: the characterization of ADRB2 deactivation as simulated by molecular dynamics [2] and the comparison of 265 PDB structures describing 53 GPCRs in active, intermediate or inactive states [3]. The pictures well show that the difference of structural characteristics between active and inactive states, within the same receptor or shared by the class A GPCRs.

ATOLL is applicable not only to GPCRs but to any transmembrane proteins. It produces self-explanatory, user-customizable and high quality plots (coming soon on github).

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P2.5.2 New applications in GPCRmd: from pharmacophores to channels

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GPCRmd (http://gpcrmd.org/) is an online, public platform dedicated to the storage and analysis of G protein -coupled receptors (GPCR) dynamic simulations1. As such, it provides access to more than 500 simulations for most GPCR families. It also provides a comprehensive set of interactive online tools aimed to facilitate the analysis of simulation data. Such tools are meant to be intuitive and user-friendly, allowing researchers from different fields of expertise (e.g. biologists, medicinal chemists, pharmacologists) to browse and exploit simulation data by using them.

In this poster, we aim to present the main features of GPCRmd. Among them we include some of the newest updates, such as the addition of tools to visualize pharmacophores and tunnels or channels obtained from the simulation data. Our ultimate objective is to provide simulation data for every GPCR with an available structure, as well as tools and resources for the GPCR community to take advantage of this data.

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P2.5.3 TIRF microscopy based ligand binding assay with immobilized budded baculovirus particles as a receptor source

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The novel total internal reflection fluorescence (TIRF) microscopy-based method was developed to study mechanisms of receptor-ligand interactions. This method maintains the right balance between retaining the receptors in the lipid environment, sufficient throughput for ligand screening, and high sensitivity of microscopy, offering a more detailed view into the ligand-binding process. The novel method combines G protein-coupled receptor display in budded baculovirus particles and the immobilization of these particles to a functionalized coverslip. The immobilization selectivity was determined and ligand-binding assay was validated using budded baculovirus particles displaying neuropeptide Y Y_1 receptors and high-affinity TAMRA labeled fluorescent ligand UR-MC026. To scale the system for ligand binding assays, we developed both open-source multiwell systems and image analysis software SPOTNIC (http://gpcr.ut.ee/aparecium.html) for flexible assay design. The affinities of the fluorescent ligand and the competitive unlabeled ligands studied (BIBO3304, UR-MK299, PYY, pNPY) obtained with the developed method were in good agreement with both the parallel measurements using the fluorescence anisotropy method [1] and the data published earlier [2]. This novel system could be extended to various advanced assays involving super-resolution methods, enabling the investigation and description of the ligand-binding processes of membrane proteins at the single-molecule level.

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