Orchestrators of signalling

G protein-coupled receptors mediate a wide range of physiological processes and are important therapeutic targets. Here, **Dr Alexandre Fürstenberg** describes his passion for these molecules and his efforts to enhance understanding of them using innovative imaging techniques

Can you provide an introduction to G protein-coupled receptors (GPCRs)?

GPCRs represent the largest family of membrane proteins in humans. They mediate most of our physiological responses to hormones, neurotransmitters and environmental stimulants, and are responsible for olfaction, taste and vision. They respond to chemical stimulation by subtly changing their conformation (3D shape), this induces the dissociation of an intracellular bound G protein and initiates a cascade of signalling events inside the target cell, which in turn leads to a physiological response.

Because of their position in the cell membrane and control of such a broad range of physiological effects, GPCRs have potential as therapeutic targets for a wide spectrum of diseases, the most prominent being cardiovascular disease, cancer, AIDS and obesity. In fact, 30-40 per cent of currently marketed medicines target GPCRs. However, these are mostly not developed through rational drug design but by standard screening technologies.

What attracted you to the field of molecular biophysics?

Since my first chemistry classes, I have been fascinated by the molecules of life; particularly proteins, which perform a tremendously diverse range of actions even though they are all made from the same 20 amino acids – just like how all English words are made out of the same 26 letters. Life can be seen as the consequence of an orchestrated interaction of molecules with each other, which induce certain actions.

Trying to understand the molecular mechanisms underlying biology has been a constant goal for me. GPCRs are fascinating molecules because they differ from classical textbook proteins: they do not have just two states – active or inactive; they can exist in many different conformations, each of which induces a different panel of physiological effects. They can be compared to microprocessors of information, with different inputs leading to many different possible outputs. Trying to decipher how such microprocessors work has been challenging but a lot of progress has been made in the field, as the Nobel Prize committee acknowledged in 2012.

The key objectives of your research are to create a dynamic and structural model of GPCR activation, and relate the pharmacological activity of ligands to receptor conformation. Why have you chosen to focus on these areas?

Molecules that bind GPCRs (ligands) can be structurally very similar yet induce very different physiological outcomes. GPCRs are dynamic molecules, in equilibrium between various conformations, some of which might be active with respect to a certain physiological effect, some of which are not. I like to compare them to trees in a light wind; you still recognise the tree under the wind, but the branches and leaves are moving and the tree constantly changes shape.

The ligand changes the equilibrium, making certain rare conformations more frequent, others impossible to reach, or enabling conformations which were inaccessible before. We think that understanding the dynamics of the interchange of conformations, and identifying the most relevant forms, is key to deciphering the action of ligands or drugs on GPCRs.

C-C chemokine receptor type 5 (CCR5), a GPCR, is the principle co-receptor used by HIV to infect target cells. How is your laboratory targeting CCR5?

My research has been hosted by the laboratory of Professor Oliver Hartley at the University of Geneva, Switzerland. His team is developing topical agents designed to block HIV entry into target cells. They have come up with tens of molecules that very efficiently block HIV infection *in vitro* and their lead compound, 5P12-RANTES, will enter phase I clinical trials later this year. Key to the development of CCR5 inhibitors was the realisation that individuals with a mutated version of CCR5 were immune to HIV infection, and that natural chemokine ligands, such as RANTES/CCL5, had anti-HIV activity.

What do you envision for the future of your research?

In the long term, I dream of being able to routinely perform single-molecule experiments in a high-throughput screening platform and of getting near-molecular insight into the 3D organisation and dynamics of proteins in living cells. Only then will we get closer to understanding the mysteries of life from a molecular perspective.

Distribution of CCR5 in a cell by super-resolution imaging. The membrane receptor is especially concentrated in the filopodia.

Super-resolution imaging

Researchers from the University of Geneva, Switzerland, are developing novel, high-resolution imaging techniques to better understand the function of a dynamic, multifaceted class of proteins

G PROTEIN-COUPLED RECEPTORS (GPCRS) are a remarkable class of molecules that have played a crucial role in modern medicine. Expressed on the surface of cells, they internalise a range of extracellular cues into signals that regulate bodily functions, including sensation, growth, blood clotting and nerve transmission.

Despite their vital physiological role and importance to therapeutics, the molecular mechanisms underlying the activation and downstream signalling of GPCRs remain poorly understood. Dr Alexandre Fürstenberg, of the Department of Human Protein Sciences at the University of Geneva, hopes to change this. By developing novel tools based on single-molecule and super-resolution imaging, he aims to better understand the function of these dynamic proteins. "We work at the cellular level to characterise the physiological effects of a ligand, identify the signalling cascade it initiates upon binding to its receptor, and determine whether the GPCR gets internalised after stimulation," he outlines. "Then, we work at the molecular level, and attempt to watch GPCRs change their conformation in real time by following their dynamics using fluorescence spectroscopy and single-molecule imaging."

THE CHALLENGES OF DYNAMICITY

GPCRs can exist in multiple conformations. As a result of their dynamic nature, it is very difficult to capture their structure using standard techniques such as X-ray crystallography and nuclear magnetic resonance (NMR), which yield averaged static structures. "To identify intermediate, short-lived conformations, one must use single-molecule fluorescence spectroscopy," Fürstenberg explains. Advanced fluorescence imaging techniques allow the detection and localisation of individual molecules, thereby imaging different populations of the same molecular species with an almost molecular spatial resolution. Indeed, modern fluorescence microscopy, alongside novel imaging, analysis and labelling techniques, has great potential to reveal the mechanisms underlying the activation and dynamics of GPCRs.

The absence of structural information represents a barrier to rational drug design, a barrier Fürstenberg hopes to remove by developing a dynamic structural model of GPCR activation. Looking to the future, he is striving to develop cutting-edge biophysical techniques based on single-molecule fluorescence imaging to relate ligand pharmacological activity to receptor conformation.

A PROTOTYPE GPCR

In order to achieve this, Fürstenberg is using the C-C chemokine receptor 5 (CCR5) as an archetypal GPCR. CCR5 is the chemokine co-receptor used by HIV to gain entry into macrophages, and it has been successfully targeted to inhibit HIV infection by Fürstenberg's colleague and close collaborator, Professor Oliver Hartley. He has developed modified analogues of the natural CCR5 ligand (RANTES/CCL5) to block HIV infection, however, the molecular mechanisms by which these chemokine analogues function remain unknown. The analogues have very different pharmacological profiles, likely because each ligand stabilises a different subset of CCR5 conformations.

In order to explain how the chemokine analogues are working, Fürstenberg turned to other partners of GPCRs that may be differentially recruited. It is crucial that cells are able to stop the signalling cascades started by ligand binding so that they can respond to future stimuli. This is called desensitisation, and arrestin proteins are vital to this process. They block the interaction between GPCRs and G proteins, and recruit adaptor proteins that cause the internalisation of GPCRs. Fürstenberg thus decided to investigate the effect of the chemokine analogues on arrestin recruitment: "We observed that ligands responsible for strongly inducing G protein signalling caused the formation of arrestin clusters in cells overexpressing fluorescent versions of these proteins. We thought that characterising cluster formation might be a way to quantify arrestin recruitment to CCR5 using microscopy, instead of biochemical techniques," he explains.

ASSAY FORWARD

In his efforts to observe the different receptor conformations, Fürstenberg is also developing an assay based on purified, site-specifically labelled CCR5. The advent of an assay to detect different CCR5 conformations would help explain the molecular basis of the discrete pharmacological profiles of the analogues and, in turn, allow exploration of the relationship between chemokine sequence and CCR5 conformation.

Such an assay first requires purifying the receptor and then placing a small fluorescent label in a well-defined position, allowing changes to the fluorescence intensity of the label to reflect conformational changes in the GPCR. "Luckily, we were able to team up with the group of Professors Thomas Sakmar and Thomas Huber from Rockefeller University, USA," enthuses Fürstenberg. "Not only had they been developing cutting-edge technology to site-specifically label GPCRs, but they had also successfully applied it to CCR5. Our goals were very similar and a very fruitful collaboration was initiated."

FLUORESCENT LABELLING

In order for observations to be meaningful, the individual CCR5 molecules need to be in a setting that is as close to their natural environment as possible. This means they must be in solution and in a membrane-like environment. Furthermore, in order to observe single receptors under a microscope for a sufficient length of time, they must be attached to a surface in such a way that they are prevented from diffusing away, but also so their function is preserved. To meet these needs, the team developed a self-assembled surface coating which enabled them to specifically capture CCR5 in detergent

MEMBRANE DYNAMICS AND CONFORMATIONAL DIVERSITY OF G PROTEIN-COUPLED RECEPTORS

OBJECTIVES

To develop and apply molecular tools, fluorescence microscopy and spectroscopy techniques with high spatial and temporal resolution; with a special interest in the organisation and dynamics of proteins – G protein coupled receptors (GPCRs) in particular – both *in vitro* and in living cells. These tools will enable researchers to give quantitative answers to biological questions.

KEY COLLABORATORS

Professor Dr Oliver Hartley, University of Geneva, Switzerland

Professor Dr Thomas P Sakmar; **Professor Dr Thomas Huber**, Rockefeller University, USA

Professor Dr Mike Heilemann, Goethe University Frankfurt, Germany

FUNDING

Swiss National Science Foundation (SNSF)

Fondation Dormeur

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ALEXANDRE FÜRSTENBERG has been a junior group leader at the University of Geneva since 2010 and is currently a visiting scholar at the Goethe University Frankfurt, Germany. He received the PhD prize of the European Photochemistry Association (2008) and was awarded an Ambizione fellowhip of the SNSF (2010). His research focuses on the development and application of advanced fluorescence imaging techniques and molecular tools for biology.

micelles or nanodiscs, while preventing the unspecific sticking of chemokines. "A big issue when working with RANTES analogues is that these highly positively charged proteins stick unspecifically to glass, whose surface is negatively charged. The positively charged polymer we are using to coat the surface prevents this," Fürstenberg explains.

Continuing its partnership with Hartley, the team has also devised a scheme to fluorescently label chemokine analogues using click chemistry (a method of generating substances quickly by joining small units together). Combining these technologies, the team was able to observe – for the first time – the specific and long-term binding of fluorescent 5P12-RANTES to immobilised, fluorescent CCR5 using single-molecule imaging.

RATIONAL DRUG DESIGN

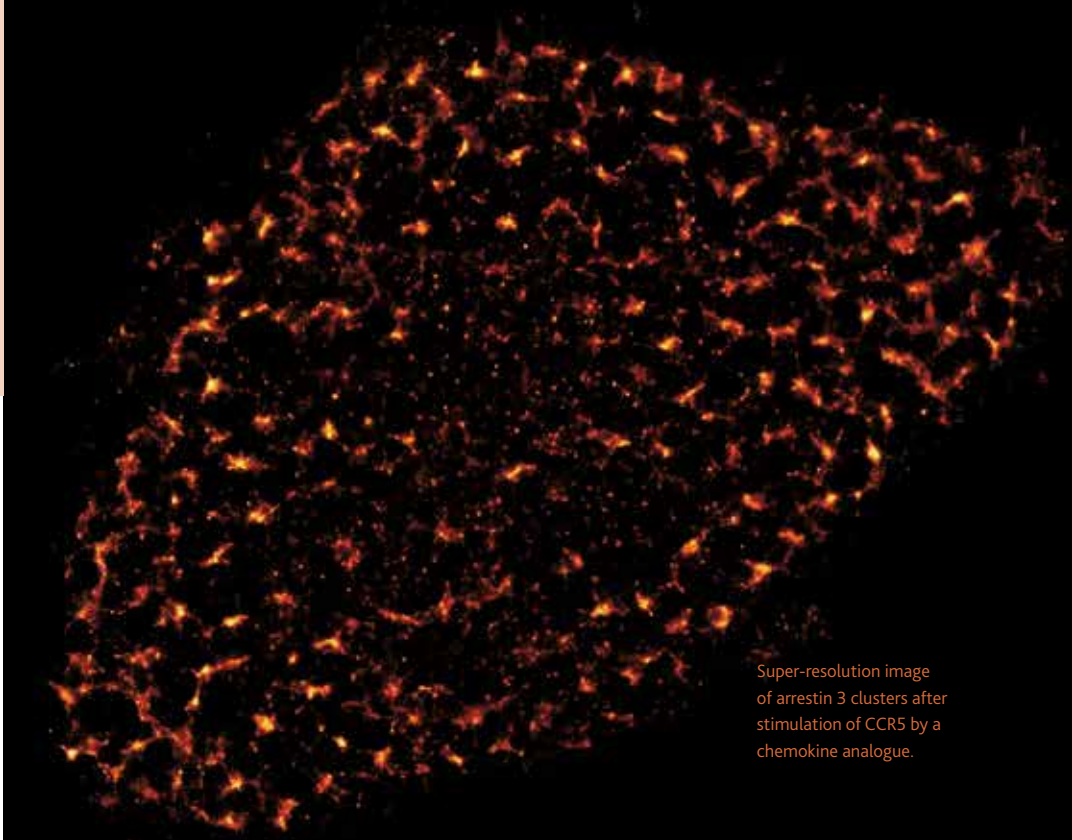
This team now has all the tools necessary to achieve their goals of elucidating the binding and dissociation of chemokine analogues and developing a conformational assay for CCR5 – all that remains are the experiments.

Fürstenberg is making great strides in understanding the diffusion dynamics of CCR5 and has already demonstrated the differential mobilisation of arrestin by RANTES analogues. Through their pioneering research and novel techniques, the Geneva group's work has been key to a number of important breakthroughs in their field, including the development of technologies such as fluorescent chemokines, site-specific CCR5 labelling and self-assembly of a surface coating for single-molecule CCR5 studies; the latter of which will allow the researchers to follow the binding and dissociation events of chemokines, G proteins and other binding partners of CCR5, and will be key to the development of a conformational assay for CCR5. "Such an assay would not only help us understand how changes in the structure of ligands can control CCR5 conformations, but ultimately lead to more rational design of drugs targeting CCR5, and perhaps even GPCRs in general," Fürstenberg concludes.

GPCRs: versatile proteins

GPCRs bind a broad range of external signals to activate nearby G proteins, leading to the production of second messengers that regulate diverse cellular functions

1. The GPCR binds an external signalling molecule (ligand)
2. Binding causes a change to the GPCR's conformation
3. This leads to activation of a nearby G protein
4. Activation of the G protein triggers the production of a second messenger – a small molecule that initiates and coordinates intracellular signalling pathways



Super-resolution image of arrestin 3 clusters after stimulation of CCR5 by a chemokine analogue.

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