

# Conference Call Participants

**Rod Hubbard**

**Fiona Marshall**

**Chris Tate**

## Presentation

**Rod Hubbard**

The purpose of this conference call is to discuss some of the background and context for the Neuropharmacology conference which is happening in San Diego in November, in which some of the impacts of the new crystal structures, particularly GPCRs and what impact that's going to have upon drug discovery and research in the particular area.

I am joined today by Chris Tate from the LMB-MRC in Cambridge and Fiona Marshall of Heptares. My name is Rod Hubbard from the University of York and Vernalis, and I am going to be moderating and running this discussion. To begin with, what I thought I would do is to just briefly review the different areas in which structural information has had an impact on the drug discovery process. It can be seen, I think, in three different areas. The first is understanding the structure of a therapeutic target, the interactions it makes, the conformational changes it undergoes and so on, and to understand its mechanism of action and, from that, you can translate to its function and perhaps have an understanding about how and where to modulate it.

Secondly, the conventional way in which structured information is being used is to look at the crystal structures of ligands bound to the target and, in that way, understand how to make changes to those ligands to improve their drug-like properties, be that affinity, selectivity, DMPK and so on. The third area is perhaps the most recent area of excitement and interest, and that is actually using the structures as a way of identifying new ideas and new compounds to start a drug discovery process with, and these perhaps started with the computational methods of virtual screening but more recently have been really great successes achieved with methods such as fragment-based screening.

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These three aspects of how structural methods have had an impact on drug discovery have very much centred on looking at globular soluble proteins, such as kinases, proteases and so on. I think the point of today's discussion is to review just what the current status is, but also what the prospects are for using the structural information in the, perhaps more important, more relevant targets for neurosciences and that is the particular class of the GPCRs.

Perhaps we could start, if Chris could just give us a brief overview of what the challenges have been in getting to the crystal structures and perhaps then ending up with what the prospects are for getting to even more structures in the future.

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### **Chris Tate**

Thanks Rod. The last three years have been really incredibly exciting in the GPCR structure field. We have got now structures of the Beta-1 adrenergic receptor; the Beta-2 adrenergic receptor and the Adenosine A2A receptor. These have been proteins people have worked on for many, many years. It is not as though these have suddenly just appeared out of nowhere. There has been a huge biochemical effort to get them into a state which is available for crystallisation.

What are the real challenges? One of the major problems with GPCRs is that they undergo conformational changes, not only in the presence of ligands but more particularly in the absence of them. Because they are very dynamic, this makes them very difficult to crystallise. Another problem associated with this is that the activated state (the R\* state) turns out to be remarkably unstable so, invariably, what is happening is that when you detergent solubilise your membranes, which of course you have to do to be able to purify the protein, the proteins just start denaturing. So it is even a real challenge just to purify your protein in a fully functional state. People have managed this for many years, using lots of tricks – using mild detergents like dodecylmaltoside with CHS [cholesteryl hemisuccinate], lipids and CHAPS, and things like that, to stabilise it, but the problem there has been that the stabilised protein then becomes very difficult to crystallise because the micelle surrounding the proteins is actually very large.

Over the past few years there have been a few key technologies which have emerged, which have allowed GPCRs to be crystallised, or some GPCRs, at this stage anyway. The first breakthrough really came in 2007 when Brian Kobilka's group crystallised the Beta-2 receptor with antibody fragment bound. In this case the protein was stabilised in a bicelle mixture and the antibody provided a large hydrophilic area which allowed the crystal contacts to be formed.

A second technology was developed at the same time, in Brian Kobilka's group again, which allowed Ray Stevens group to solve a structure of the Beta-2 receptor at high resolution (about 2.4Å resolution) and the trick here was to insert T4 lysozyme as a fusion in the cytoplasmic loop 3 of the GPCR. What this allowed was to again increase the hydrophilic area and then produce very nice crystals using Lipidic Cubic Phase, which is a new technology previously only being successful for the bacteriorhodopsin family.

The third technology, which is what we have been developing in Cambridge, is the thermal stabilisation of the GPCRs. This technology gets away from perturbing structures by potentially putting in these large proteins [i.e. T4 lysozyme] or using antibodies and it is basically saying: can we make the GPCR much more stable in detergents? This we successfully did for the Beta-1 receptor, which allowed the structure to be solved at 2.7Å. So, over the years, what we now have is a larger toolbox which allows us now to use these technologies to extend the structures of the GPCRs, but there are still problems. It is not as though you can start working on GPCR and guarantee a structure. What is very helpful is to have ligands which bind with very high affinity. So, for example, the A2A structure that has been solved with a ZM compound which binds with very high affinity and this stabilises the protein quite dramatically. I do not think we are at the stage where we can crystallise any GPCR with any low affinity ligand, for example.

A second real challenge is to get agonist structures. As I mentioned earlier, the agonist state is much less stable than the antagonist-bound state, so that's also going to be quite tricky to actually solve the structures of, especially if the ligands are also very low affinity, but now we are in a fantastic position because, from the rhodopsin field, they have managed to solve the structure of opsin, so this is in an activated state and now, for the first time, we can see these conformational changes occurring; the movement of helix 6 out from the centre of the protein on the cytoplasmic side by about 6 Ångstroms or so, opening up this cleft which allows the C-terminal peptide of the G protein to fit in there and to eventually activate the G protein.

So we are getting a very, very good idea now of the biology of how these things are working, but we need a lot more structures to allow us to be able to do [drug] development and also to define other aspects of GPCR biology, like partial agonists and the like.

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### **Rod Hubbard**

I think there have been fantastic technological advances that have opened up this whole field and I think provided some quite startling insights into the structure and perhaps towards the functional mechanism of some of these proteins. What do you think realistically is going to be the next big

advance – let's say the next two years as a horizon in terms of, if you like, the next functional level of understanding that we ought to be able to achieve from these structures?

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### **Chris Tate**

In the next few years I think we are going to have a far better idea of why some compounds when they bind work as a partial agonist, rather than a full agonist, and this will come from the technology we have now which will allow co-crystallisation of these receptors with a variety of different compounds. In our lab we have managed to solve the structure with four different agonists and about five different antagonists. So these structures will give us initial insight into how these actually happen, but the next big challenge is to get the full structural change as well, so the full agonist activated stage. Then, in the long term, what we really want are the complexes, with not only the agonist-bound but then with G-protein coupled complexes, arrestin complexes and the like, because there are some fascinating facts which are coming out with agonists; you know, one receptor can bind two different agonists and then signal through different pathways – so how can we explain that in terms of just one single conformation? That cannot be – there must be more conformations of agonist activated states and that is going to be a real challenge to do, because I think these complexes are going to be quite unstable for crystallography.

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### **Rod Hubbard**

Thanks so much, Chris, and perhaps we can move across now to Fiona, and get the discussion a bit more towards the actual issues for drug discovery. Perhaps, Fiona, you could start by perhaps just reviewing: what are the problems that are facing the field at the moment?

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### **Fiona Marshall**

Traditionally people think of GPCRs as being quite tractable targets and there are over 50 GPCRs that have drugs on the market, but most of these have small molecule natural ligands and in fact many of the drugs on the market are actually simple analogs of the ligands, so the beta blockers, for example, are analogs of adrenaline, essentially, and the sort of GPCRs that people are interested in now tend to have much more complicated ligands, large peptides or protein ligands, sort of metabolites like lipids and you cannot just simply make analogs of these, so the standard route is by high-throughput screening in the absence of structures and really the high-throughput screening efficiency rate is quite low, so generally you only get about 50% of

GPCRs that you can even get any hits out of a high-throughput screen. That is much worse for, for example, the family B class of receptors.

Many of the compounds that you get out of high-throughput screening have quite high molecular weight. They are not ideal in terms of ligand efficiency, so they do not bind tightly into the binding site. They don't have very good drug-like properties, so the attrition rate during lead optimisation is very high and, even worse, when you actually get through to clinical trials, so out of all the GPCRs screened at HTS, only about one in ten actually ever gets a compound into the clinic, so I think it is clear that we need new approaches to actually finding different chemo types that can be built into more effective drugs and clearly the sorts of strategies that have been used for soluble targets, that I know you are very familiar with, Rod; hopefully we can now apply some of these to GPCR targets.

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**Rod Hubbard**

So which of the targets do you think we are going to see these structures having the biggest impact, first of all?

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**Fiona Marshall**

At the moment, again, most of the structures we have got are with very small ligands so the adrenergic receptors, the Adenosine receptors, where we really need structures now is for the peptide receptors. There are many important neuropeptides so the opioids – we have drugs for those, but they are mostly derived from plants and natural products and the same with the cannabinoid receptors. Where we would like to find drugs is for things like the orexin receptor, which is a very interesting CNS target for sleep; Ghrelin, the peptides that regulate appetite through CNS through the hypothalamus; those have been very difficult traditionally to actually get good small molecule drugs and people are actually using the natural peptides as drugs instead, so they are clinically validated, many of these targets with the peptides and biologicals, but trying to find good small molecules for these targets is proving to be very difficult.

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**Rod Hubbard**

So perhaps coming onto that next step then, How effective do you think virtual screening is going to be against these structures?

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**Fiona Marshall**

Well I think you have to look at what's happened before, for the enzyme targets and I think you can probably comment on how effective it's been for that, for kinases and proteases. For GPCRs at the moment, it's still very difficult, because we still only have four different structures, to actually efficiently do virtual screening. I think we're going to need a lot more structures. We're going to need co-structures of diverse chemo types and probably also going to need to combine that with mutagenesis studies to really understand where ligands are binding.

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**Rod Hubbard**

Because I think the...certainly the experience with the, if you like, the more classical targets, the kinases, the proteases, the soluble, domain proteins, is that virtual screening can work if you really understand the structure of your target very well and you can then use that knowledge and that insight to help pare down the list of compounds that the computer suggests to you, down to the ones that are really going to bind. I think a lot of people have found that if they just do virtual screening without that in-depth knowledge, then they end up with almost random hit rates. But it can be an effective way of finding things, if you really understand the target well. So I think you're right, you're going to need quite a lot of crystal structures to do that. What about some of these other newer techniques; do you think there is a prospect of doing fragment based screening, for example, against these sorts of targets?

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**Chris Tate**

I think it's a real possibility. The key there is really to be able to crystallise your receptor in the absence of ligand. I think the technology is now there. So for example, we've stabilised the beta one receptor and you can nice crystals in the presence of ligand. But you can actually then do another round, you can re-stabilise it, make it even more stable. And we now have a receptor that essentially is stable in SDS, it's actually quite remarkable. Then, what we're hoping, is that now this is a more stable version, you can then actually crystallise it in the absence of any ligands, or at the very least in the presence of fragments. I think that technology is not going to be simple, but I think once you've invested so much time and effort into getting crystals in the first place, it's not such a big step, to actually go on and to do fragment based screening.

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**Fiona Marshall**

One of the by-products of doing the structural studies of course, is that you generate large quantities of pure protein. Those in themselves make very useful reagents for running other types

of fragment screening approaches that don't necessarily, need structures. So for example, you can do other biophysical] studies such as surface plasmon resonance or NMR, Mass-spec, all the techniques that have been developed for fragment screening, for soluble targets, they're going to be more difficult for GPCRs, you're going to have to do them in detergent and you're still going to need some level, way of stabilising the protein, to be able to put them into the detergent. But many of these techniques look like they could now be applied to GPCRs to give you the starting point of the fragments.

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### **Rod Hubbard**

An exciting prospect, although as you say, I think there is going to be some real challenges in there. Just to give us an idea, one of the things that you need to back up a structured base programme, is quite rapid turnaround on the structures, particularly as you are trying to support a team of chemists that's eagerly awaiting that next round of insight to design the compounds. What do you think is a realistic prospect over the next year or two as to how fast one is going to be able to turn out such structures?

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### **Chris Tate**

That's a very difficult question and I think it depends so much on the target, it really does. The key issue is really the stability of the target in detergent. If you have something that is ridiculously stable in detergent, then actually it's doable within a year if you like. For example, with the beta receptor structure that we did, Tony had spent eight years trying to crystallise it without any joy at all, because it was very unstable. But putting in six thermo stabilising mutations within two months he had crystals, within two months they'd diffracted to beyond 3 Å resolution and eight months later you have a structure. So there it makes a key difference. But of course, stability is not the only issue, you also have to remove all the flexible sites in the protein and so there's biochemical issues there as well. But I think in the year time scale, one, two, maybe three years. Some are going to take longer than others.

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### **Rod Hubbard**

But once you've got the system established, that you are able to produce crystals, how rapidly, how many liganded crystal structures can be determined. Presumably you don't have to go through all the process for every ligand?

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**Chris Tate**

Absolutely not. So we've now got structures with nine different ligands and that's been essentially about, I don't know, about a year and half work.

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**Rod Hubbard**

So perhaps moving on from the crystallography itself. Perhaps just talk a bit more about what the structures actually open up in terms of the wider perspective on GPCRs and so on. So far, there's been the structure of rhodopsin, like bacteriorhodopsin structures, there's then the beta adrenergic and then more recently the A2a receptor. So I think there is one or two bits of history there that we could look back at and ask the question. Firstly, how translatable are those experiences, how close are those structures and how good have models been, built upon that various succession of structures been? Secondly, how well do those structures provide you with models that can be used to rationalise and guide SAR in other families and in closer family members?

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**Chris Tate**

Well I suppose from an historical perspective, everybody started off with bacteriorhodopsin for building models and clearly that was not the way to go, as the packing of the transmembrane domains is completely different. But once the Rhodopsin was solved in 2000, then again everybody was very keen on building models. But the real surprise came with the beta structure, is that basically the extra cellular side is completely different. That was not predicted at all, because the top of external loop two is involved in binding of some of the ligands, again that was not predicted. So with the RMSD being about 2.4 Ångströms between the beta receptors and the Rhodopsin, the models were not particularly predictive. You know, it's the same problem, when you go the A2A receptor, so this is coming back to a question of how many structures do you want. I think that really we need one for each basically class of GPCRs and then once you've got that structure, then it will be far easier to predict and to model effectively, other members of that class.

So with the Beta 1 and Beta 2 [receptors] the RMSD is about 0.6-0.7 Ångströms. So then you're in the real right area to be able to model effectively.

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**Rod Hubbard**

I think we're going to see, hopefully in the time leading up to this conference and perhaps the conference itself, there's quite a few people have picked up on the structures that have become available and started to do some of these retrospective analysis and ask the question, just how much more of GPCR space do these structures actually open up, to give you a firm model on which you can actually do design and rationalise SAR. Fascinating time coming up.

Okay. Fiona, any more comments?

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**Fiona Marshall**

Well I think another interesting area from the structures and also using these new types of approaches based on pure proteins, is that we can start to look at some of the other binding sites, other than the orthosteric binding site on the protein. So allosteric regulators is an area of considerable interest, but nobody really understands what impact those have on the orthosteric binding site, how they're actually modulating ligand activation. So I think one of the exciting new areas coming through is potentially how allosteric modulators are binding to the receptor, how they're changing the function. Those probably bind mainly to the extra cellular regions, the extracellular loops, but also there are some very novel structures coming through actually, from high throughput screening of compounds that seem to bind to the intracellular parts of receptors. Again, sort of act in an allosteric way to regulate G-protein activation. So I think this is another new area that's really emerging in the GPCR space, that again having structures is really going to help. Because again, trying to do the SAR on allosteric regulators without really understanding their mechanism of action, or how they interact with the receptor, is very, very difficult and it's very empirical approach.

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**Rod Hubbard**

Okay, thanks very much for that. Chris, any other comments you'd like to make?

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**Chris Tate**

Well that does remind me...Fiona's comments just reminded me, that you were asking previously about challenges. Well obviously, one of the real challenges is now looking at dimerization of GPCRs and how that really affects, both from a structural perspective, but also from a drug design perspective. That is going to be a real cracking challenge.

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**Rod Hubbard**

Is there any evidence from the crystal structures or the different crystal forms you've had so far, of seeing dimerization?

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**Chris Tate**

Well you have to be really very careful of when you look at a crystal structure of saying, is this a physiological dimer and I work on the assumption that basically, everything you see in a crystal is actually a crystallographic dimer until proven otherwise. Same with, for example, cholesterol binding sites and things like that. We're seeing CHS bound all over the place on these structures. So, it's very difficult to say. Certainly from the 2D crystals of Rhodopsin, there were suggestions of potential dimers, but I think there's a lot more work needs to be done on that, to be able to say that they really are physiological dimers rather than just crystallographic dimers.

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**Fiona Marshall**

Similarly, obviously complexes with other proteins that GPCRs interact with is very important. So they're not just sitting there in the membrane on their own, they're part of multi protein signalling complexes. So, understanding how they interact with the G-protein which I think is at the top of everybody's list of the next interesting structure we'd like to see, if not the whole G-protein a peptide from the G-protein. Then of course the other main signalling pathway of GPCRs is through beta-arrestin. And as Chris mentioned earlier, we know now that there are ligands that direct signalling through G-proteins or through a different confirmation, probably through beta-arrestin signalling. So if we can start to get complexes with these other signalling proteins, then I think that's going to be really informative to how the receptors are working and also to drug design.

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**Rod Hubbard**

It's going to be a challenge.

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**Chris Tate**

It certainly is.

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**Rod Hubbard**

Okay, well thanks ever so much for those comments. Perhaps just in concluding, we've clearly during this discussion, focussed in on the GPCRs because it is for those structures that we're very fortunate to have Chris and Fiona with us and that's their field and their area. I think we should bear it in mind that there have been similarly advances in other membrane protein structure determinations and in other protein classes that are important in neuro pharmacology. And in particular point out some of the transporter structures that have started to emerge from one or two groups round the world. I think it will be interesting to see whether they will actually give us any insights into the mechanism for some of the current drugs that are in the clinic, which allegedly operate through these transporter mechanisms. Thanks so much for your time and looking forward to seeing you in San Diego.