Computational Prediction of GPCR Oligomerisation

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ABSTRACT

There has been a recent and prolific expansion in the number of GPCR crystal structures being solved: in both active and inactive forms and in complex with ligand, with G protein and with each other. Despite this, there is relatively little experimental information about the precise configuration of GPCR oligomers during these different biologically-relevant states. Whilst it may be possible to identify the experimental conditions necessary to crystallize a GPCR preferentially in a specific structural conformation, computational approaches afford a potentially more tractable means of describing the probability of formation of receptor dimers and higher order oligomers. Ensemble-based computational methods based on structurally-determined dimers, coupled with a computational workflow that uses quantum mechanical methods to analyse the chemical nature of the molecular interactions at a GPCR dimer interface, will generate the reproducible and accurate predictions needed to predict previously unidentified GPCR dimers and to inform future advances in our ability to understand and begin to precisely manipulate GPCR oligomers in biological systems. It may also provide information needed to achieve an increase in the number of available GPCR oligomeric crystal structures.

Introduction

GPCRs are "proteins with the patterns of design and malleability of structure required for discriminating between an extraordinary variety of chemical signals" [1]. GPCRs were believed for many years to function as monomeric proteins and it has only been through an increasing body of experimental evidence, demonstrating not only the existence but the physiological and functional relevance of GPCR oligomers, that both homo- and heterodimerisation and the formation of higher order oligomers has come to be (somewhat reluctantly) accepted by the GPCR field [2–5].

The absence of structural data may have contributed to the long-standing belief in the monomeric nature of these cell surface receptor proteins. GPCRs have proved refractory to crystallisation, relative to other protein classes, a difficulty that arises from the low conformational homogeneity of these signalling proteins and something that has only recently been resolved through the application of several innovative protein engineering techniques and crystallography methods [6–9]. As a consequence, there has been a recent and prolific increase in the number of the GPCR structures in the Protein Data Bank (PDB) [10] and structural evidence for GPCR oligomers is now being added to the weight of evidence obtained from biological methods of studying GPCR oligomers in native cells, in tissues or in recombinant mammalian expression systems [11] to inform a holistic understanding of the nature of these signalling proteins.

Experimentally-Determined Oligomeric GPCR Structures

Ironically, now that we have unequivocally demonstrated the biological existence of GPCR homo- and hetero-dimers and have successfully crystallised many members of this protein superfamily, it transpires that although there are over 300 solved GPCR structures [12], the overwhelming majority of these are, in fact, monomeric. Only 12 GPCR structures in PDB have a dimer present in the crystallographic asymmetric unit (i.e. dimers that were not generated by crystallographic symmetry) and possess a software-determined (PISA) quaternary structure that is also a dimer. GPCR dimer structures exist for the turkey β_1 adrenergic receptor (2VT4 and 4GPO), the CXCR4 chemokine receptor (3ODU and 3OE9), the N/OFQ opioid receptor (4EA3), the prostanoid EP3 receptor (6AK3), the C5a complement peptide receptor (5O9H), the platelet activating receptor (5ZKQ) and wild-type or mutated versions of rhodopsin (3CAP, 2PED and 2J4Y). The twelfth dimer is smoothened (4JKV), a Frizzled Class GPCR.

Two further Class A GPCR receptors have a dimer as the asymmetric unit: the A1 adenosine receptor (5UEN) and the kappa opioid receptor (4DJH). However, the dimer has not been confirmed by PISA for the former and the PDB record for the latter states that the dimeric quaternary structure has not been confirmed experimentally. The asymmetric unit for 2E4U, the Class C mGlu3 receptor, contains a biological homodimer but there is no PISA determination of the quaternary structure. There are additional GPCR dimers in the PDB database, but these are "inverted" and one or more of them contains explicit notes stating that they are an artefact of crystallisation and do not represent the natural oligomeric state of the protein. Examples of inverted GPCR dimers include the following PDB entries: 1F88, 1GZM, 1HZX, 2G87, 2HPY, 2J4Y, 4AIQ, 4N4W, 5DGY, 5UNF, 5UNH, 5V54, 5V56 and 5V57.

Computational Approaches to GPCR Oligomerisation

The paucity of GPCR dimers and higher order oligomers in the PDB has prompted the use of computational modelling methods for the prediction of GPCR oligomers (for examples, see [11,13–17][18][19]). There are several caveats that need to be applied when interpreting results obtained with these approaches. Firstly, very few of the published studies involve performing a substantial number of replicas for each set of simulation conditions (summarised in [11]). Whilst such studies can provide a snapshot of one possible outcome, single molecular dynamics simulations exhibit a random Gaussian behaviour and the accurate properties of the system under study cannot be determined by a single run. Ensemble-based averages, where independently performed replicas should reproduce identical results within error, are needed to obtain accurate computational results that can be compared with experimental data.

Secondly, given the mutations introduced to facilitate crystallisation, many of the computational methods for predicting GPCR dimers are based on non-native receptor

structures that have been obtained from an engineered GPCR whose atomic coordinates have undergone varying degrees of computational modification. For example, the thermostabilising mutations and exogenous stabilising domains introduced to facilitate crystallisation can be computationally-reversed by homology modelling. Where structures do not yet exist for a GPCR of interest, homology modelling of the nearest neighbour's structure can be used to produce a model for computational analysis. Structures are, occasionally, resolved with missing loops that can be replaced by modelling from a neighbouring structure. The quality of the computational predictions that can be obtained are dependent upon the accuracy of the structure being modelled.

There is a third consideration that is worth noting - that of the impact of constraints placed upon the computational model by experimental findings. The convergence of experimental and computational findings is of fundamental importance, however, it is very difficult to correlate experimental snapshots of receptor structure with the many different potential states and substates of a GPCR oligomer, making it challenging to know when experimental data may be reliably used to obtain accurate computational predictions. For example, modelling of the quaternary structure of a G protein-coupled receptor heterotetramer in complex with G_i and G_s [20] identified that a TM4:TM5 interface was likely to be the best fit for the A_{2A} adenosine receptor homodimer because this interface was observed in the β_1 adrenergic receptor structure used to build the model for the molecular dynamics simulation of A_{2A} and because TM4:TM5 was the only interface that favoured the experimentally-observed BRET signal. A possible TM4:TM4 interface was considered unlikely due to a clash with N-terminal helix of G_s. Does the TM4:TM4 interface exist in some A_{2A} configurations? Although the G protein was not present in the β_1 adrenergic receptor structure used to build the model, it was a feature of the experimental system and provided extra information with which to evaluate the computational analyses. In a separate study, it was noted that dimers predominant in

crystals of the CXCR4 receptor could not be reproduced in the membrane environment in a computational simulation due to the different orientation of TM5 in each case [21].

GPCR Dimer Interfaces

A number of computational studies have described GPCR dimer interfaces [18–30], many of these using inactive and active receptor models obtained from structurally-determined dimers. Comparisons between these interfaces and those obtained from experiment have been made (see [33–35]) and several different and, potentially conflicting, results have been obtained. Interestingly, whilst these conflicts could arise from the caveats mentioned in **Computational Approaches to GPCR Oligomerisation**, they may also reflect changes in dimer structure as a function of receptor activation or inactivation [18]. Several of the studies have explored dimer interfaces in relation to the activation state of the receptor, with one recent study proposing that a GPCR dimer may possess a "rolling" interface where the individual monomers sample different and interconverting configurations relative to one another [35]. This finding not only accommodates different configurations resulting from changes in receptor activation state, it provides scope for predicting the formation of higher-order GPCR oligomers.

Most GPCR dimers studied computationally are based either on a dimer formed from a a structurally-determined monomer or from modelling based on a related GPCR for which a dimeric structure exists. Until a greater number of cryo-EM GPCR structures or information from single molecule studies becomes available, the vast majority of these dimeric structures will have been obtained through X-ray crystallography. This introduces a potential bias, although the use of ensemble-based coarse-grained Molecular Dynamics will allow for proper sampling to achieve convergence of results. There is an alternative approach, however. Unlike Class C GPCRs, which dimerise through their large extracellular N terminal domain, dimerisation of Class A GPCRs is

widely accepted to take place through interactions between specific transmembrane helices of the individual monomers [4]. We have developed an ensemble-based coarse-grained molecular dynamics approach for the computational prediction of helix-helix interactions in G protein-coupled receptors [11]. This method allows the sampling of all computational space and potential conformations and orientations of interacting helices. This has allowed us to determine the specific points of interaction between GPCR dimers and has been validated using both experimental data and dimeric GPCR structures from PDB.

With this method, we are able to discriminate between residues that form specific interactions and residues that are in close proximity but do not interact. A distinct advantage to the method is that the structures of the helices can be extracted from monomeric GPCR structures, thereby increasing the available number of receptors that can be studied with minimal modelling required. We have used this methodology to confirm the experimental finding of interaction between TM5:TM5 of the A_{2A} adenosine receptor [36] and were able to identify that the CXCR4 receptor dimer (3ODU) also possessed interacting residues in TM5:TM5. We were also able to show that the interacting helices for the two other GPCR dimers available at that time, the β_1 adrenergic receptor and rhodopsin, interacted through TM4:TM5. Whilst these receptors were also believed to interact through TM1:TM1, we were able to demonstrate that this was only true for the adrenergic receptor and that rhodopsin's TM1 helices were in close proximity to each other, but the interaction was identified as being between TM1:TM2 [11].

By using only transmembrane domains for these analyses, this method specifically interrogates interactions formed between residues in each monomer, providing a starting point to the high-throughput identification of potential dimer partners whilst avoiding issues of starting bias inherent in the use of full GPCR structures that contain

extracellular and intracellular loops and other features such as lipids that can influence, but don't necessarily define, the dimerization interface between monomers. This approach accurately identified contact interfaces of the wild type helices of TM5:TM5 of the A$_{2A}$ adenosine receptor that corresponded to residues identified experimentally and provided a molecular explanation for the experimental finding that the M193$^{5.54}$A mutation alters the monomer:dimer ratio at a level of detail that could not be determined biophysically and would require structural biology studies to confirm experimentally. It is of interest to note that the use of helical pairs will not only identify the intermolecular interactions between dimers, it will enable the detection of the intramolecular (inward facing interactions) network interactions within an individual monomer.

Identifying the Molecular Signature of GPCR Dimer Interfaces

In light of the increasing interest in identifying GPCR dimer interfaces, we have extended our previous studies to explore all pairwise combinations of A$_{2A}$ adenosine receptor TM helices and have identified interactions between TM1:TM2, TM4:TM4, the previously identified TM5:TM5 and TM6:TM6. TM1:TM2 is one of the dimer interfaces identified in Class A GPCRs and Figure 1 shows the TM1:TM2 interaction we have identified in the A$_{2A}$ receptor. There are 11 specific TM1:TM2 interactions identified for the A$_{2A}$ receptor (Figure 1 panels a, b) compared with the previously identified 8 interactions for rhodopsin [11], with interactions involving residues 1.30, 1.43 and 2.44 (labelled according to the Ballesteros-Weinstein nomenclature; [37]) conserved between the two receptors. 1.39 is involved in three separate interactions in the A$_{2A}$ receptor and mutation of this residue from glutamic acid to alanine causes all 11 TM1:TM2 interactions to be lost and two new interactions to be formed (Figure 1 panels c, d).
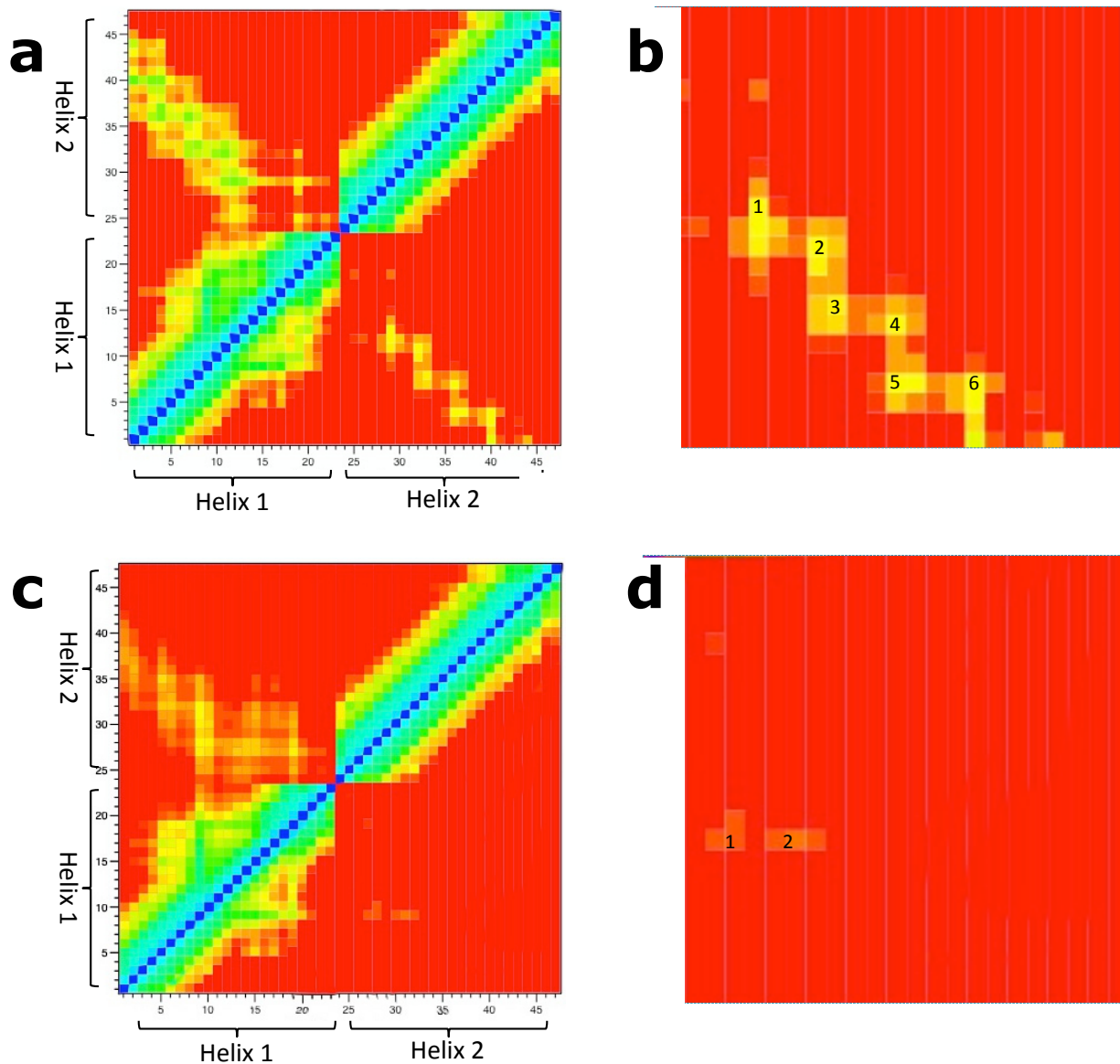
Figure 1: Contact matrices showing specific pairwise interactions between TM2 and wild type TM1 (panels a, b) or E13A-mutated TM1 (panels c, d) of the A_{2A} adenosine receptor. Interhelical distances at the 15Å cutoff are shown in the top left quarter of panels and interhelical distances at the 12Å cutoff are shown in the bottom right corner. The color scale indicates distance between helices: blue corresponds to 0Å (superposition of the two helical backbones at all cutoffs); green corresponds to 6Å (12Å cutoff), 7.5Å (15Å cutoff); yellow corresponds to 8Å (12Å cutoff), 12Å (15Å cutoff); red corresponds to the cutoff distances applied (12Å or 15Å). The region shown in the black rectangle in panels a and c is magnified in panels b and d, respectively. The numbered interactions in panel b correspond to: 1) $E13^{1.39}$-$A54^{2.52}$, $E13^{1.39}$-$V55^{2.53}$ and $E13^{1.39}$-$G56^{2.54}$; 2) $A17^{1.43}$-$A54^{2.52}$; 3) $A17^{1.43}$-$A51^{2.49}$; 4) $L19^{1.45}$-$A50^{2.48}$; 5) $L19^{1.45}$-$S47^{2.45}$ and $A20^{1.46}$-$S47^{2.45}$; 6) $G23^{1.49}$-$F44^{2.42}$, $G23^{1.49}$-$V46^{2.44}$ and $G23^{1.49}$-$S47^{2.45}$. The numbered interactions in panel d corresponde to: $T11^{1.37}$-$D52^{2.50}$ and $A15^{1.41}$-$D52^{2.50}$.

The interaction between TM4:TM4 in the A_{2A} adenosine receptor is of particular interest as this is the A_{2A} adenosine receptor dimer interface identified through a different

computational approach by Navarro *et al*.[20]. The TM4:TM4 interaction is also found in another class A GPCR, having been shown in the active form of the dopamine D2 receptor to involve the crosslinking of residues that would not have been proximal in the AMF model of inactive receptor [38]. This finding reveals that conformational changes at the dimer interface are an important part of receptor activation.
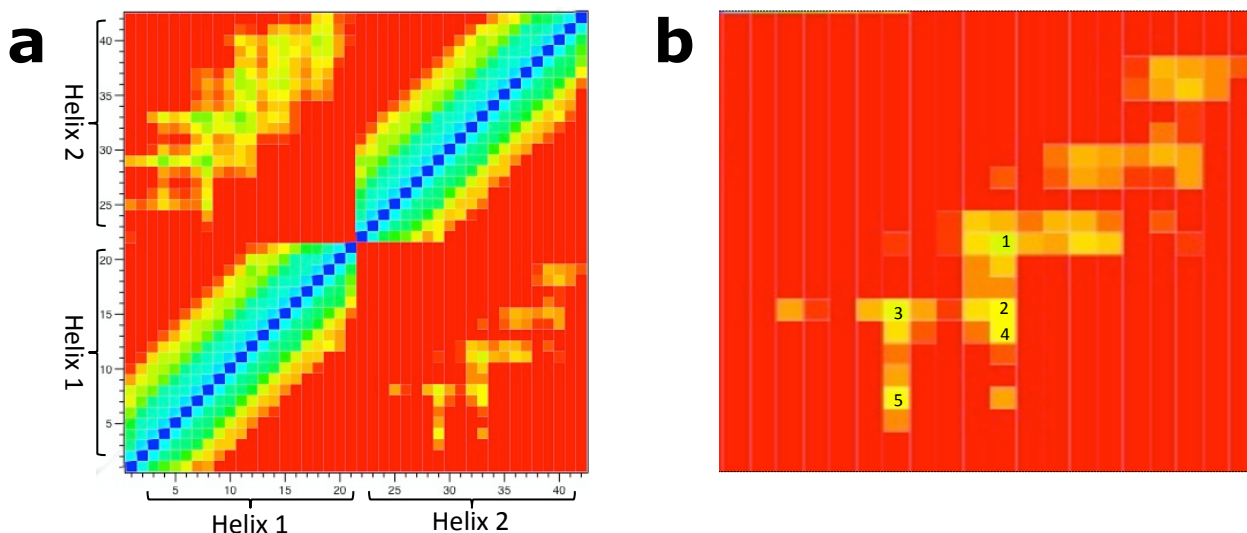

Figure 2: Contact matrices showing specific pairwise interactions between TM4-TM4 of the A_{2A} adenosine receptor. Interhelical distances at the 15Å cutoff are shown in the top left quarter of panel a and interhelical distances at the 12Å cutoff are shown in the bottom right corner. The color scale is as indicated in Figure 1. The region shown in the black rectangle in panel a is magnified in panel b. Specific interactions numbered 1-5 took place between residues found in the $I125^{4.46}xxCWxxS132^{4.53}$ motif on the first TM4 helix with the residues found in the $W129^{4.50}xxxxA134^{4.55}$ motif on the second TM4 helix.
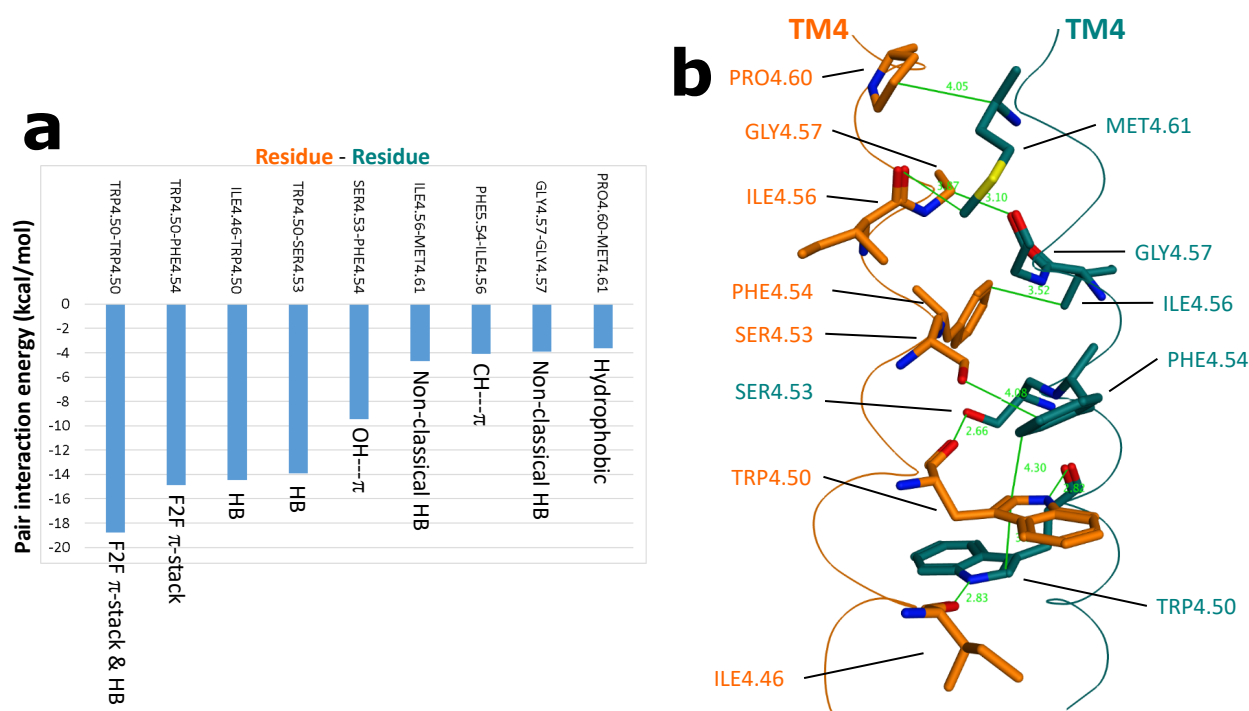
Figure 3: FMO calculations for the two interacting TM4 helices shown in Figure 2. Residues numbering follows the Ballesteros-Weinstein indexing scheme. Significant residue-residue pair interaction energy (PIE) is shown in panel a, with any interaction with an absolute PIE greater than or equal to 3.0 kcal/mol considered to be significant. HB = hydrogen bond. The interactions between the two TM4 helices are shown in panel b. The carbon atoms of the first TM4 helix are shown in light orange and the TM4 residues of the second TM 4 helix are shown in dark green. Nitrogen atoms are shown in blue, oxygen in red, sulfur in yellow and chlorine in light green. The interactions detected by FMO are marked with a light green line and the distances provided in Å.

We have previously refined and applied the Fragment Molecular Orbital Method (FMO) to characterise GPCR-Ligand interactions [39–47](see related review by Heifetz *et al.,* in this issue), but have used it here to characterise the protein-protein interactions of the two interacting TM4 helices in the A_{2A} receptor (see Figure 3). FMO is a quantum mechanically-informed computational approach used to analyse the chemical nature of the molecular interactions between two systems that can provide insight into the nature of the TM4-TM4 interactions. From these analyses, it can be seen that three hydrogen bonds are involved, two of them non-classical, and a π stack at the canonical residue 4.50 is observed. This computationally-derived insight can be used to inform mutagenesis of the residues involved and provide the opportunity to obtain experimental evidence with which to evaluate the computational findings.

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Conclusions

GPCR dimers are a dynamic species with multiple forms and a changing dimerization interface that shifts during receptor activation and inactivation. The changes in the structure network and molecular signature of GPCRs during these processes are now beginning to be elucidated [48,49]. The computational characterisation of TM helices allows the greatest flexibility in identifying all potential interfaces, providing rich information with which to interrogate experimental findings to identify GPCR states and substates. Ensemble-based computational simulations of TM helices that include FMO can be used effectively for the accurate prediction of the molecular nature of interactions at GPCR dimer interfaces, providing reproducible, reliable and precise results that can be compared with experimental data obtained from structural studies, techniques such as super-resolved microscopy [50] and pharmacological data obtained from site-directed mutagenesis studies and naturally-occurring genetic variations. FMO will also be effective in identifying the molecular nature of the interactions found in structurally-determined GPCR dimers and higher order oligomers.

The current lack of structural dimer information makes computational methodologies a valuable means of extrapolating information from those few dimer structures that exist. The results from these computational approaches can be used to inform a revisitation of previously-obtained experimental results, enabling a reinterpretation of these using dimer receptor models [51], something that will enhance the richness of the existing experimental data and guide the generation and analysis of new experimental data. It may even become possible to use this information to engineer GPCRs in such a way as to favour the experimental determination of oligomeric receptor structures at the atomic level of resolution for each of the many different receptor states we know these proteins transition through in the execution of their biological functions. "In fact, receptor-

coupled signalling processes in general now seem more Buddha-like in their structures, both in their stationary setting and in the multi-component structure which appear to interact in a flickering fashion, more in keeping with the ephemeral relationship between action and inaction…"[1].

DECLARATION OF INTEREST

Declarations of interest: none

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●…….of special interest
●●…..of outstanding interest

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