REVIEW ARTICLE

Prediction and Targeting of Interaction Interfaces in G-Protein Coupled Receptor Oligomers

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Abstract: ***Background:*** Communication within a protein complex is mediated by physical interactions made among the protomers. Evidence for both the allosteric regulation present among the protomers of the protein oligomer and of the direct effect of membrane composition on this regulation has made it essential to investigate the underlying molecular mechanism that drives oligomerization, the type of interactions present within the complex, and to determine the identity of the interaction interface. This knowledge allows a holistic understanding of dynamics and also modulation of the function of the resulting oligomers/signalling complexes. G-protein-coupled receptors (GPCRs), which are targeted by 40% of currently prescribed drugs in the market, are widely involved in the formation of such physiological oligomers/signalling complexes.

Scope of the Review: This review highlights the importance of studying protein-protein interactions (PPI) by using a combination of data obtained from cutting-edge experimental and computational methods that were developed for this purpose. In particular, we focused on interaction interfaces found at GPCR oligomers as well as signalling complexes, since any problem associated with these interactions causes the onset of various crucial diseases.

Major Conclusions: In order to have a holistic mechanistic understanding of allosteric PPIs that drive the formation of GPCR oligomers and also to determine the composition of interaction interfaces with respect to different membrane compositions, it is essential to combine both relevant experimental and computational data. In this way, efficient and specific targeting of these interaction interfaces in oligomers/complexes can be achieved. Thus, effective therapeutic molecules with fewer side effects can be designed to modulate the function of these physiologically important receptor family.

1. INTRODUCTION

Determining key players that govern protein-protein interactions and also understanding the underlying molecular mechanism of oligomerization are essential for modulating various physiological functions in the cell such as signal transduction pathways, in which various proteins do function in coordination to respond to the stimulus reliably and timely. Evidences have shown that a protein, when is part of an oligomer, can modulate the function of the other members present in the complex. In this respect, G-Protein Coupled Receptors (GPCRs) constitute ideal systems for this phenomenon. According to the current knowledge, they are functional in monomeric and dimeric/oligomeric forms (either homo or hetero) [1] and also they form complexes with a wide array of signalling partners such as G-proteins [2], arrestins, GPCR-kinases, PDZ-domain [3] containing proteins to function properly. As to the GPCR oligomerization, it has been shown that protomers within the oligomer can allosterically cross-talk to each other either to alter the ligand binding affinity or efficacy of the other members present in the complex [4]. Considering the fact that GPCRs are targeted by approximately 40% of currently prescribed drugs in the market and also oligomers modulate the function of individual GPCRs it is crucial to understand the molecular mechanism of oligomer formation and also to determine interaction interfaces that emerge under different environmental conditions, *e.g.* membrane composition.

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The first step before determining the interaction interface and studying PPIs is the identification of the constituents of the complex/oligomer. There are a variety of experimental methods which are developed for this purpose. Among many others, proteomics approaches have been widely used despite the inherent problems in studying membrane proteins due to complex biochemical properties associated with these systems. Nevertheless, the cell-based and genetic assays have been found successful for identifying numerous interaction partners of GPCRs [5–13].

Once the partners and interaction interfaces are determined, computational methods can be used to complement experimental data as they provide atomistic information regarding both the structure dynamics of these physiological complexes/oligomers [14]. In particular, one can determine the set of residues involved in interaction interfaces and also have an insight on the molecular mechanism of allosteric interactions present among the protomers [15]. Moreover, one can also achieve a molecular level understanding of the effect that the membrane composition elicits on the dynamics and the identity of the resulting interfaces. Here, it is important to emphasize that since the relaxation times of such systems are large it is crucial to test if the results obtained from *in silico* calculations are statistically reliable and comparable to experimental data.

In spite of existing experimentally determined structures of GPCR oligomers (in particular, dimers) and signalling complexes (with either G-protein or arrestin) they are scarce. These structures reveal that some GPCR interfaces are favoured over the others, in particular, those that are formed by either transmembrane (TM) TM4-TM5 or TM1, TM2 and TM8 suggesting that similar mechanisms might mediate the oligomer formation in this receptor family [16,17]. Considering the fact that GPCR oligomers are involved in various pathophysiological pathways, in particular, neurological disorders, cancer, an atomistic level knowledge regarding these interfaces can lead to breakthroughs in the field of neurology and also oncology.

In this review, we aim to make an extensive review on recent experimental and computational methods that have been widely used to determine interaction partners in GPCR oligomers/signalling complexes and also those that are developed to investigate the identity and dynamics of the interaction interfaces. In addition, we present several examples of software that are widely used for hot-spot prediction, inhibitor design that target interaction interfaces in GPCRs. Lastly, we finish by giving an example of one of the GPCRs that has been known to form oligomers, namely Ghrelin receptor. We also discussed the methods that have been used to target dimers formed by this receptor.

2. *IN SILICO* APPROACHES APPLIED TO THE STUDY OF GPCR DIMERIZATION

2.1. Structural Determination and Characterization of the Dimerization Interface

If any experimental data regarding the interaction interface is available then it can be used to guide molecular docking calculations, instead of performing blind docking whose success has been shown to be far below than that of the guided one. Alternatively, coarse-grained molecular dynamics (CGMD) simulations can also be used to determine the most probable interface. However, such calculations may end up with more than one interface each of which having a similar frequency. Under such circumstances, the stability of each of these interfaces can be determined by using umbrella sampling [18] or steered molecular dynamic (MD) simulations [19–21]. These methods can also be used to discriminate between the native oligomer and other oligomers that might be present in crystal structures of GPCR complexes as a result of crystallization artefacts. Below, we discuss above-mentioned computational techniques in the context of identification and assessment of the stability of protein-protein interface(s) in GPCR oligomers.

2.1.1. Coarse-grained Molecular Dynamics Simulations: A Computational Tool for Estimating Interaction Interface(s) in GPCR Oligomers

Coarse-grain modelling can be used to represent a given atomistic system by a reduced number of degrees of freedom. As a result of the reduction in the degrees of freedom and elimination of fine details, one can simulate systems with larger length scales and can access longer time scales at the expense of losing atomistic details. Martini force field [22] has been widely used for performing CGMD simulations of GPCRs in an explicit membrane environment. According to the force field, each residue is represented by one backbone bead and zero or more side-chains beads depending on the type of the amino acid. The protein in question is allowed to change its tertiary arrangement; however, the local secondary structure, which has an effect on the bead type and also on the bonded parameters, is pre-defined and so it is fixed throughout the simulation. Therefore, for instance, one cannot study ligand-induced conformational changes in the GPCR using CGMD simulations. Instead, the exact conformational state of the receptor (active or inactive) must be defined and assigned *a-priori* to each residue of the receptor. The Martini force field allows [22] usage of a time step in the range of 20-40 fs depending on the system properties. In particular, a four-to-one mapping is used where four heavy atoms and associated hydrogens are on average represented by a single interaction center. As a result, a standard conversion factor of 4, which corresponds to the effective speed-up factor in Martini water diffusion dynamics, is used. For modelling non-bonded interactions, standard cutoff schemes are used where Lennard Jones interactions are shifted to zero in the range of 0.9-1.2 nm whereas electrostatic interactions in the range of 0.0-1.2 nm. The studies on test systems have shown that while the translational and rotational diffusion of a Class A GPCR, namely Rhodopsin, have been shown to be in good agreement with experimental data [23] the sampling of the local configurational space of a lipid molecule [24] and the aggregation rates of lipids into bilayers however have [25] been accelerated. Before performing CGMD simulation of any GPCR-membrane system, corresponding Martini time-scales of the system components, protein, water, lipid, should be compared to available experimental data to have an insight on the speed-up factor.

The self-assembly of GPCRs involves the slow diffusion of lipid and receptor molecules, which may lead to problems in achieving convergence due to lack of binding/unbinding

events [26]. This can be partially overcome by simulating different replicas of the same system in parallel, in each of which individual GPCRs are placed differently with respect to each other. A recently developed high-throughput simulation method, namely, docking assay for transmembrane components (DAFT) [27,28], provides an automated extensive sampling of different GPCR dimerization interfaces, which is shown to be in excellent agreement with experiments [28,29]. According to the method, multiple CG simulations of the GPCR dimer, which is embedded in an explicit membrane environment, are performed simultaneously. The two GPCRs are initially placed at a fixed distance but at different starting orientations. By means of this ensemble simulation setup, one can achieve statistically meaningful results on the dimerization interface. Once the convergence issue has been fixed in order to discriminate between random contacts and recurrent interfaces root-mean-square-difference-based clustering can be used [30]. According to the method, first, the dimer pairs are fitted and then matrix of positional root-mean-square-difference of the backbone beads of the dimers is calculated. Subsequently, the number of neighbouring dimers in the set is counted for each dimer conformation. The dimer with the highest number of neighbours is removed from the system together with its neighbours. The process is repeated until the pool is empty.

2.1.2. In Silico Determination of Potential of Mean Force (PMF) to Measure the Strength of Interaction interface(s) in GPCR Oligomers

CGMD simulations of self-assembly of GPCRs may end up with more than one oligomerization interface as mentioned above. In order to determine the relative stability of these interaction interfaces, one can calculate the potential of mean force (PMF) between corresponding GPCR monomer or oligomer pairs. In addition, PMF can also be used to discriminate between the native oligomer and the others present in the crystal, which might be formed artificially because of the crystallization conditions. In principle, PMF can be computed from probability distribution functions of conformations that are sampled in unbiased simulations; however, the lack of binding/unbinding events, even in CGMD simulations, prevents one to compute statistically meaningful PMF. In such circumstances, umbrella sampling [18] or steered MD simulations [19,20] can be used together with Martini force field [22], which has been shown to reproduce reasonable protein-protein interaction energies upon a reduction in Lennard Jones interaction term in the force field [31].

To perform an umbrella sampling, first, a series of initial configurations of the GPCR dimer is generated along an appropriate reaction coordinate, which is usually taken as the distance between the pair of the receptor. In a study by Johnston *et al.* [32], the authors carried out metadynamics simulations to generate starting configurations for using in umbrella sampling. In each of these configurations, one of the protomers in the GPCR dimer is harmonically restrained with respect to the other at increasing center-of-mass distance from a reference starting point. In this way, the GPCR dimer is allowed to sample a defined region of the configurational space along the selected reaction coordinate. After preparation of initial configurations in each window, simulations are started in parallel. Until achieving a good overlap

between neighbouring windows, which is important for the proper reconstruction of the PMF, the simulations are performed. In a recent study, it has been shown that replica-exchange between windows can be used for a better convergence [33]. Finally, the change in free energy in each window can be calculated by means of sampled distributions along the reaction coordinate. The windows can be combined by using weighted histogram analysis method (WHAM) [34]. However, in order to estimate errors bootstrap method can be preferably used [35].

Steered MD simulations, in contrast to umbrella sampling, are performed under non-equilibrium conditions, where the motion is guided continuously along the reaction coordinate by an external potential function. This is done to drive the system from state A to B (in the case of GPCR dimer, bound to unbound state). In this technique, the pulling of molecules is usually done by applying a force on one single atom. Alternatively, it can also be done by applying a force between the center of mass (CM) of the protomers in the GPCR dimer. The latter approach, which corresponds to applying a force uniformly to each atom in the given molecule in proportion with its mass, is not appropriate for big protein complexes such as GPCR, in which the protomers are bound to each other by a strong interaction. The method for such systems can induce distortion of the tertiary structure or partial unfolding before unbinding occurs. Moreover, if the interaction between the protomers is spread over a large surface, which is perpendicular to the pulling direction, the applied force may cause rotation of the two protomers with respect to each other. In order to overcome either possible distortions or rotation artefacts an alternative scheme can be used [36]. According to the method, the reference position of an atom is determined with respect to CM of the unit to which it belongs. A harmonic potential is applied only to the Z coordinate of the atom, while the movements in either X or Y direction remain free. Finally, the positions of the restrained atoms in the two protomers are uniformly shifted in opposite directions only along the Z coordinate, which leads an increment in CM distance.

The free energy differences from steered MD simulations can be recovered using the Jarzynski identity [37]. According to the method, multiple simulations, each of which starts with different initial velocity, are performed and the work done in each of these trajectories are calculated, thus having independent canonical distributions. Subsequently, the free energy change can be estimated by taking the ensemble average of the exponential of the work, which can be calculated using the exponential average method, as shown in Eq.1:

$$e^{-\beta \Delta G} = \left\langle e^{-\beta W} \right\rangle_0 \qquad \text{Eq.1}$$

The initial conformations used in each steered MD run can be obtained either from a long reference run at equilibrium or from different replicas each of which started with different initial velocity. The latter approach can provide a better convergence over the other because the conformations coming from individual runs do not deviate much from the reference structure and also more structural diversity can be achieved at the end of independent runs. Finally, the bias and errors can be calculated using the scheme developed in Gore *et al.* [38] and used in Sensoy *et al.* [39] for systems having a

small number of pulling experiments as long as the collection of individual runs displays Gaussian-like distributions.

2.1.3. The Effect of Membrane Nano-domains and Lipid Composition on GPCR Oligomerization

GPCR-mediated signal transduction is mainly performed by specific interactions between the receptors, G-proteins, adenylyl cyclases, channel proteins, phospholipases or GTP exchange factors [40]. On the other hand, these components have been reported to be expressed at low concentrations in the cell which suggests the compartmentalization of the components of GPCR signalling for producing effective signalling and also for increasing the probability of oligomerization [41]. GPCRs, as well as above-mentioned signalling components, have been shown to co-localize in dynamic membrane nano-domains, namely, lipid rafts which are densely packed, and are rich in glycosphingolipids and cholesterol [42,43]. Caveolae are composed of similar lipid composition, but they also contain the protein caveoline on the inner leaflet of the bilayer [44]. As being one of the dominant components in nano-domains cholesterol can modulate GPCR oligomerization by: 1) introducing higher order, preferentially, to saturated lipid tails, thus increasing the membrane thickness, 2) directly binding to specific parts of the receptor surface, eg. CRAC motif [45], thus precluding some areas from being involved at the interface or 3) intercalating between GPCR protomers to stabilize specific quaternary structures [46]. In addition to cholesterol, polyunsaturated fatty acid chains and also palmitoyl groups also affect the oligomerization of GPCRs. In particular, polyunsaturated omega-3 fatty acid docosahexaenoic (DHA) causes low lipid order due to the high conformational flexibility of the molecule, which allows the membrane to adopt various conformational organizations without remarkable energetic penalty [47,48]. The palmitoyl group(s), which is added post-translationally to carboxyl-terminal cysteine residue(s) of GPCRs, triggers compartmentalization of receptors in membrane nano-domains. They also preferably interact with cholesterol molecules [3,49], thus adjusting the membrane insertion depth of Helix-8, which is one of the domains involved in interaction interfaces of GPCR oligomers [3,49]. In particular, the assembly of GPCRs in membrane nano-domains is mediated by hydrophobic mismatch, which is defined as the difference between the thickness of the lipid bilayer and the hydrophobic part of the transmembrane domain [50]. Using CGMD simulations on systems containing multiple copies of Rhodopsin it has been shown that shorter lipid tails cause more hydrophobic mismatch induced deformation of the lipid bilayer [23]. To alleviate hydrophobic mismatch, the GPCR can: 1) associate with another receptor, 2) translate into a membrane region with increased thickness or 3) do both simultaneously.

2.1.4. Molecular Docking Approaches

The number of experimentally determined structures of GPCR dimers is still low and homology modelling can be used as a reliable computational approach to feel this gap and build accurate models of GPCRs [51]. Template selection, the first step of homology modelling, is extremely important for the production of robust GPCR models [51]. The similarity between the template and the target protein sequence must be at least 30-40% in order to obtain accurate

models [52]. Low sequence identity leads to inaccuracies in the alignment of sequences that result in dislocation of residues and impairment of important contacts [51]. Additionally, the activation state of the receptor must also be considered [51]. However, there are few active or pre-active crystal structures [53]. Inactive structures instead could be used as templates for active models if the ECL2 is modelled in the presence of a ligand [15,51]. Also, constraints such as disulphide bonds and transmembrane domains should be assigned for the geometric optimization [51]. Ligand similarity can also be used for template selection. Lin *et al.* organized family A of GPCRs into dendrograms considering the similarity of ligands and of the ligand binding site of receptors. This organization demonstrated that GPCRs which seem to be distantly related with respect to sequence can become closely related if they are grouped with respect to ligand similarity [54].

Kaczor *et al.* reviewed several docking tools applied to modelling of GPCR complexes, most of which originally used rigid-body docking approach; however, most currently used tools incorporate also protein side-chain flexibility, which has been showed to increase the quality of the results [55].

2.1.5. Other Approaches

Sequence-based bioinformatics methods such as statistical coevolution analysis (SCA) can also be used to infer functional coupling between distant sites manifested by co-evolution, and to define networks, which are indirectly associated with allostery in all its aspects, including dynamic modulation [56,57]. Beyond the prediction of allosteric and dynamic coupling that define "sectors" within a single chain, the latter method has been also applied for identifying interaction interfaces through the co-evolution analysis of distinct interacting partners [58]. An example to the latter is the application done by McCammon's group on the human CXC chemokine receptor type 4 (CXCR4) [59]. The authors considered a number of crystallographic dimers emerging from experiments and analysed the co-evolution properties of their residues, in order to identify the so-called sectors. Here, the predominant coevolution sector which lies along the observed dimer interface, suggesting that the dimers are evolutionarily conserved because of their functional relevance. Furthermore, coevolution scoring also provided a basis for determining significant nodes in the network which are formed by residues found along the interface of the homodimer, namely hot-spots (HS).

Alternatively, methods which are based on machine learning (ML) techniques that benefit from the Big Data Era can also be used to predict interaction interfaces. The method can be applied to study membrane-proteins, in particular GPCRs. Indeed, several ML algorithms that are based on various system properties such as transmembrane helices, helix-helix contacts and burial propensity, have been developed to predict interaction interfaces [60]. For example, TMHindex is a method that predicts interacting helices by considering only the amino acid sequence [61] of transmembrane regions. A much more complex method, named WRF-TMH, uses singular value decomposition to combine amino acid composition as well as their relevant physicochemical properties to efficiently predict the TM segments [62]. Other

servers like TransMembrane eXposure (TMX) [63] and Protein Solvent Accessible Surface Area Predictor (ASAP) [64] focus on the accessibility of the amino acids found on the helices. The former is based on uses evolutionary conservation while the latter predicts accessible surface area (SASA) values using PSI-BLAST profile. Predicting accessibility is important to understand which transmembrane residues are most likely to establish contacts with the other receptors. A neural network, which is developed by Fuchs *et al.* [65], is shown to successfully predict helix-helix contacts. The dataset used not only included commonly used features like residue distance in the sequence but also membrane protein specific features like residue orientation towards the membrane. By combining all of these methods, Ahmad *et al.* [66] trained multiple structural features in an integrated model. This algorithm seems to be able to predict one-dimensional structural features like SASA, dihedral angles and amino-acids helical topology.

Once the interaction interface has been determined normal mode analysis [55] can be used to investigate the effect of oligomerization on the dynamics of GPCRs. The principle is that vibrational nodes exhibiting low frequencies describe the largest movements in the protein and are the ones relevant to function [67]. Niv *et al.* used elastic network model to compare dynamics of monomer, dimer and tetramer of Rhodopsin and they showed that oligomerization alters GPCR dynamics. They also identified which residues are important for dynamics and the stability of the dimer [68].

2.2. Conformational Modification Upon Dimerization

2.2.1. Dynamic Perspective

Protein function and activation are determined by the interplay between structure and dynamic modulation, which, in the case of GPCRs, can lead to a change in affinity favouring or impairing the binding of the effector. Such modulation is fundamentally allosteric in nature, as it is generated at the binding site of the ligand and propagated through the TM domains towards the intracellular side [69]. Allostery can have both a structural and a dynamical component. Besides ligand induced conformational changes, which can be identified by high-resolution structural information and predicted by computational methods, the rearrangements that underlie allosteric functional regulation often include dynamic modulation [69]. This includes increased or decreased fluctuations at the allosteric site, which can increase affinity for the binding partner.

The dynamic component of allostery can be addressed computationally through structural approaches based on elastic network models (ENM) [70] that predict the intrinsic, structure-driven fluctuations. A network model is a representation of a biological macromolecule as an elastic mass-and-spring network used to characterize its long-time and large-scale dynamics, which is encoded in the lowest frequency normal modes of the model. The springs are usually defined for residue pairs closer than a given cut-off [71] and full atom description is neglected, in favour of a coarse grained representation as function of $C\alpha$ or $C\alpha$-$C\beta$ atoms [72]. For instance, Kolan *et al.* [73] built an elastic network representation in a number of GPCR monomer molecules, including M_2 and M_3 muscarinic receptors, A_{2A} adenosine receptor,

beta2 adrenergic and CXCR4 chemokine receptors, and rhodopsin. The normal modes of the elastic network were used to highlight the determinants of the intrinsic dynamics of the receptors, which in this study were related to activation. The collective motions described by the lowest frequency modes highlight a modulation of the GPCR vestibule in terms of dilation and contraction which is associated with ligand passage, and activation, respectively. Contraction of the vestibule on the extracellular side is correlated with cavity formation of the G-protein binding pocket on the intracellular side, which is connected to the initiation of intracellular signalling.

More generally, albeit with a higher computational expense, Molecular Dynamics can virtually address any conformational evolution in the protein and specific dynamic response. Instead of focusing on the intrinsic dynamic properties that are encoded in the protein topology, Molecular Dynamics-based approaches can account for the effect of a chemical perturbation such as a mutation, or the binding of a small molecule or of an interacting partner, and predict both conformational and dynamic modulation. Molecular Dynamics was applied, for instance, in an attempt to describe the intra- and intermolecular communication between a GPCR, thromboxane A2 receptor (TXA2R), as induced by an activating ligand, and structure and dynamics properties of a GDP-bound heterotrimeric G protein in response to receptor binding. Here, the dynamic modulation of the complex is analysed by extracting the global motions through PCA of the MD trajectory to highlight the most significant collective motions [74]. Several studies have focused on GPCR monomers to help elucidate the mechanism of propagation from the binding site to the intracellular side upon activation, as shown in studies of Shan *et al*. [75] and Perez-Aguilar *et al*. [76]. This approach could, in principle, be transferred to oligomers, provided that the computational power is high enough to allow one to simulate a multi-molecular complex. Thereby, collective motions can help elucidate the long range dynamic modulation and cross-talk between the units. Moreover, local fluctuation analysis that focuses on the RMSF spectra or distance fluctuations can also be applied to identify local modulation of hotspots and predict mutation sites to alter the dimerization interface.

2.2.2. Allostery and Networks

One popular computational approach aimed at describing the propagation of allosteric signals from the orthosteric binding site to a distal region involves the construction of a network, describing the communication propensity among residue pairs. This can either be based on proximity criteria (i.e. interatomic distances) or on dynamical features, such as the mutual information content or generalized correlation emerging from the spatial fluctuations of each residue. The fluctuation pattern, in turn, can be obtained by Molecular Dynamics or by Gaussian Network Models [77–79]. Besides illuminating the global motions, the information derived from the elastic network approach can be used to map the allosteric communication pathways and identify the critical residues –hotspots- that are coordinated and involved in the signal propagation underlying activation. This approach combines dynamics and topological properties, hence investigating the intrinsic dynamics (structure-induced) of the

system. A higher resolution methodological approach has been proposed by Levine *et al.* [80], the N-body Information Theory (NbIT) analysis, which is based on information theory and uses measures of configurational entropy derived from MD simulations, to identify residues involved in the signal propagation. Originally applied to the Leucine transporter LeuT, the method relies on all atom MD simulations and can be generally used to highlight sets of amino acids collectively involved in the coordination process, and can be in principle used to analyze dynamic coordination underlying the stability of dimer interfaces as well.

2.2.3. Networks and Dimerization

The occurrence of multimeric GPCR complexes, including intracellular and extracellular proteins, might imply that the propagation of conformational and dynamic changes induced by the ligand is also affected by the other partners and specifically in the case of homodimers, by the cognate receptor [81]. Therefore, when applying the network approach to GPCR dimers, the aims of the network-based allosteric analysis are twofold: on one hand, one wants to validate the dimerization interface, by comparing the allosteric activation pathway in the monomer to the one in the dimer, in order to assess whether are both compatible with a functional network. On the other hand, the interface itself can affect the network, hence the function of the GPCR; the analysis can therefore provide insight into the biological role of the dimerization process in sustaining receptor activation. Fanelli *et al* [82]. applied the strategy of defining the network structure for different assemblies of A_{2A} dimers to predict their biological relevance. In this study, MD simulations on three selected dimers combined with protein structure network (PSN) analysis was aimed at predicting the effects of homodimerization on the structural network of the monomer that is underlying activation. The PSN method, introduced by Vishveshwara and co- workers [83] is based on a graph theory approach applied to protein structures. A graph is defined by a set of points (nodes) and connections (edges) between them [84]. In a protein structure graph (PSG), each amino acid is represented as a node and these nodes are connected by edges based on the strength of non- covalent interactions between residues [40], defined with a contact criterion among their atoms. Hubs are defined as highly connected residues, and connectivity clusters can be defined, as well as the shortest communication pathways. Such pathways are then interpreted in terms of allosterically connected units. Putative dimers, obtained by means of rigid docking [85] were subjected to 10 ns MD simulation in implicit solvent in order to relax the structure at equilibrium. Then, on the equilibrated snapshots of the trajectory, the PSN analysis was performed to identify allosteric pathways involved in the GPCR activation.

As a reference, in the A_{2A} monomer, both in the presence and in the absence of the antagonist ZMA all possible shortest communication paths connecting extracellular and intracellular halves of the targeted monomer were searched by combining PSN data with cross-correlation of atomic fluctuations calculated by using the Linear Mutual Information (LMI) method [86]. The latter approach estimates allosteric connection between two sites by evaluating the quantity of coupled information, which is associated with allostery. The outcome of this mapping highlights a residue set involving mainly TM1, TM2, TM6-TM7, which is substantially conserved in the three dimer forms considered. Nevertheless, the path composition within each considered monomer in the context of the TM6–TM6/TM6–TM7 dimer differs from that of the same monomer simulated in isolation or in the TM1–TM1/TM2–TM2 and TM1–TM4/TM2–TM2 dimer architectures. In particular, the TM6–TM6/TM6–TM7 architecture relatively reduces the ZMA-mediated communications between ligand binding site and cytosolic region. TM1 turns out to play a significant role in mediating A2AR dimerization as two out of the three predicted dimers share TM1 at the inter-monomer interface. Moreover, these dimers retain the typology of the most frequent communication paths seen in the complexed form of the monomer, but increasing the overall coordination compared to the MONO form. In this respect, the TM1–TM4/TM2–TM2 architecture shows the most diffuse communication among all the ZMA- complexed forms. In contrast, the TM6–TM6/TM6–TM7 dimer is characterized by a dramatic reduction in the total number of paths compared to the MONO form, suggesting an impaired functionality. This analysis can therefore be used to validate the plausibility of the dimerization interface.

Another approach aimed at the validation of the dimerization surface in GPCRs and relying on a network approach was proposed by Nichols *et al.* [59] in the case of human CXC chemokine receptor type 4 (CXCR4). Here the network is built upon a sequence-based statistical method, the SCA analysis [87] coupled to MD simulations to detect the significant contacts. The network is used to highlight co-evolutionarily related residues acting as hubs, which are identified as hotspots stabilizing the interface, thereby validating the functional relevance of the experimentally observed dimer.

2.3. PPI Inhibition Through Hot-spot Targeting

Interfaces of protein-protein complexes consist of buried surface areas, which are mostly hydrophobic in nature [88]. These complexes are stable if the complex formation results in an increase in entropy, and a decrease in de-solvation energy [89,90]. The energetic contribution of individual residues at the interaction interface is not uniform and only a tiny fraction of these residues contributes to binding free-energy of complexes [91]. These key residues are known as hot-spots (HS) and are defined as sites where alanine mutations result with an increase of at least 2.0 kcal/mol in binding free energy [92]. The amino acid composition of hot-spots is very unique. The most representative residues that frequently act as hot-spots are tryptophan, arginine and tyrosine [93]. Bogan and Thorn hypothesized that they are sheltered from the solvent by surrounding residues, together which form an O-ring type packing structure [93].

Disease-causing non-synonymous single nucleotide polymorphism (nnSNPs) often occurs at protein-protein interfaces and is highly linked to hot-spots [94]. As such, identification of these residues is of utmost importance for investigating the molecular mechanism of various crucial diseases [95]. Various hot-spot databases have been constructed over the years. Among them are the alanine energetics database (ASEdb) [92], the binding interface database (BID) [96], the

protein-protein interactions thermodynamic database (PINT) [97] and structural, kinetic and energetic database of mutant proteins interactions (SKEMPI) [98] which have been widely used. Nevertheless, targeting hot-spots remains challenging as they are mostly "undruggable" due to their large surface areas and non-classical chemical/physical properties [95].

Computational methods can be used as alternatives for high-throughput hot-spot identification compared to more expensive experimental methods [99]. Molecular-dynamics (MD) simulations can be used to predict free energy changes occur upon complex formation by calculating the differences between the monomers and the complex [100,101]. However, these methods are computationally expensive due to large size of the systems studied [101]. Instead, rigid-body molecular docking, which uses physics-based models to search for binding poses having favourable energies and complementarity, can be used as alternative computational methods. However, the accuracy of the method is limited by the accuracy of the force field itself and the complexity of the search space [102].

Machine learning methods developed for prediction of hot-spots have been known for their computational efficiency [101,103–105]. These methods, which can be sequence- or structure-based, are very sensitive to the type of the features which are used to characterize the hot-spot residues [99,106]. Sequence-based methods explore the identity, physicochemical properties, and conservation and interface propensities of the amino acid residues. On the other hand, structure-based methods gather information about chemical composition, interface size and geometry, SASA and atomic interactions [99]. The latter has typically a better performance but is dependent on the knowledge of the three-dimensional structure of protein complexes, which are scarce for GPCRs. In addition, the structure of GPCR changes upon ligand binding but most of the crystal structures available are in the apo state raising the question that structural features of the unbound state may not represent the active structure [107]. Table **1** summarizes recently developed software/servers which are used for hot-spot prediction.

The occurrence of hot-spots at protein-protein interfaces provides the opportunity to inhibit complex/oligomer formation by targeting these residues by means of therapeutic agents. In this respect, computational methods are extremely valuable for drug-design since it helps filter most of the non-relevant compounds without a therapeutic value [123].

The workflow that can be used to develop therapeutic molecules is depicted in Fig. (**1**). Docking protocols are one the most widely used computational tools in the early stages of drug development. This technique provides a faster and cheaper way of screening a library of compounds [124]. Docking most recently has been used not only as a screening tool but also as a method for target identification. Hot-spot identification is a crucial step when designing inhibitors. The methods used for this purposed were discussed previously. Once the hot-spots are determined, structure- or ligand-based virtual screening can be done, along with protein-protein docking [125]. However, ligand-based screenings are rarely used for such purposes due to lack of significant numbers of known inhibitors [125].

Structure-based pharmacophore design can be done by using softwares, such as LigandScout [126] or Phase [127]. In addition, it can also be calculated by means of potential interaction sites which are derived by DSX [128] or Super-Star [129]. Alternatively, determination of pharmacophores can be based on hot-spots. Zerbe *et al.* compared hot-spots which are predicted by either alanine scanning mutagenesis or small molecule fragment screening. The authors showed that high correlation exists between the two groups while only a small subset of hot-spots, which are predicted by alanine mutagenesis, could be used for potential binding of inhibitors [130]. After achieving a pharmacophore model, various ligand databases can be searched for finding potential hits. The top poses can be identified by clustering the docking results according to their spatial arrangement and energy values. The inhibitors obtained in this way can be classified into three groups: antibodies, peptides and small molecules. Often the process starts with a peptide and then it is converted to a small molecule by incorporating important functional groups. Secondary structures like α-helices, β-sheets,β-turns, extended structures and proline-rich segments function as scaffolds for the design of inhibitors [123]. An example to such successful inhibitors is the one that can disrupt the interaction between the anti-apoptotic BCL-XL and its pro-apoptotic partners. Identification of such an inhibitor was done by using virtual screening which is based on structure-based pharmacophore modelling and sequential docking [131]. Mysinger *et al.* [132] were also able to identify 4 inhibitors which were developed against chemokine receptor CXCR4 using structure-based methods. Ligands retrieved showed high specificity towards the receptor. The same method was also used to develop ligands that can provide preferential coupling of the receptor to its cognate signalling partner such as G-protein or Arrestin by using biased ligands [21]. In (Fig. **2**) we illustrated the use of ours SpotOn software, which classifies interfacial residues as hot-spots and is able to highlight key binding determinants for the coupling of the 2 binding partners of a typical GPCR [99,101]. These type of information can also be used to develop new and more specfic ligands.

Consequently, preclinical and clinical studies have been initiated for development of effective biased agonists that target GPCRs, in particular, opioid receptors. Development of such specific ligands towards these receptors is necessary to overcome drug resistance and treat substance abuse [133].

3. EXPERIMENTAL APPROACHES APPLIED TO THE STUDY OF GPCR DIMERIZATION

Investigation of PPIs, in particular in GPCR complexes/oligomers, is a challenging task. In order to find the most appropriate method for the system the following points should be considered [10]:

- If the study is discovery-driven, then, a high-throughput-screening-suitable (HTS-suitable) method should be preferred to allow for exploring of interactomes or alternatively, screening of whole libraries;

- For targeted approaches with defined interaction partners' assays which use tagged proteins are desirable;

Table 1.   A list of software/servers that are currently used for prediction of hot-spots which is given along with the relevant features and algorithm/methods used. Adapted from Moreira et al. [108].

Name	Features Used	Method	Ref
Foldef	Free energy differences from 3D structure.	Energy based method	[109]
KFC2	47 features including SASA, neighbours amino-acid properties, local atomic density and π-π and cation-π interactions	Two knowledge-based methods using SVM: KFCa has the highest prediction accuracy for hot-spots but low accuracy for null-spots. KFCb has comparable predictive ability with other methods available.	[110]
HOTPOINT	Relative Accessible Surface Area: relative change in ASA upon complex formation, conservation, amino acid propensity and total contact potential.	Empirical model which is based on relative accessibility in complex and total pair potentials.	[111]
HOTREGION (FORMER HOTSPRINT)	Uses hot-spot residues predicted by HotPoint and structural properties such as ASA, relative ASA and pair potentials of interface residues.	Database of computational predicted hot-spots.	[112,113]
HOTSPOTEC	Combination of 83 independent physicochemical properties of amino acids and relative accessible surface area.	IBk algorithm, an algorithm that extends the K-nearest neighbour (KNN) algorithms with a reduced storage requirement.	[114]
ISIS	Sequence based method using features such as sequence environment, evolutionary profile, predicted SASA and conservation score	Neural Networks system	[115]
MAPPIS	Physicochemical interactions and binding properties in 3D.	Evolutionary conservation: the method performs multiple alignments to detect spatially conserved interaction patterns.	[116,117]
PCRPI	Three main sources of information: energetic, structural and evolution.	Probabilistic method using Bayesian Networks (BN)	[118]
POCKETQUERY	Mines structural data from PDB and uses third-party calculations for SASA, free energy differences and sequence conservations scores.	SVM algorithm.	[119]
PSIPRED (FORMER HSPRED)	Uses energy terms like Van der Waals potentials, solvation energy, hydrogen bonds and Coulomb electrostatics with data from Arg and Glu residues mutations.	Combination of energy terms and SVM algorithms.	[120]
PREDHS	108 structural and energetic features, including local structural entropy, side chain energy score, four-body pseudo-potential and topographical score.	Integrates Euclidian and Voronoi neighbourhoods with sequence- and structure-based data to construct an SVM predictor.	[121]
ROBETTA		Energy based method that scores protein-protein interfaces residues by individually replacing them with alanine. Binding energy is calculated.	[122]
SBHD	Combination of sequence and structural features, focusing on several SASA features and genetic conversation at protein interfaces.	ML method using BN for PPI and generic algorithm-SVM-full (GA-SVM-Full) for Protein-Nucleic acid interaction.	[104]
SPOTON	881 features divided structure, sequence and evolutionary-based. On the structural perspective focus on SASA, type of residues in the interface and intermolecular interactions.	ML ensemble combining random-forest, svmPoly and pda methods	[99,101]

- The sensitivity of the assay is important: for weak, transient interactions only very few assays are suitable, if stable/strong interaction will be studied, most assays can be used;

- Determination of the stoichiometry of the complex- that is to say- if consideration of binary PPIs is enough or the whole protein complex is of interest should be considered as well;

- The dependence of the results on the type of the medium in which the sample is preserved should be checked. For instance, experiments will be done in cells or native tissues, or can the cells be lysed and proteins solubilized?

- The necessity of certain (co-)factors, auxiliary proteins or micro-environments for interactions to occur should also be determined;

- Does the whole protein need to be analysed or is a part of it (either short peptides or domains that represent the whole protein's properties) sufficient?
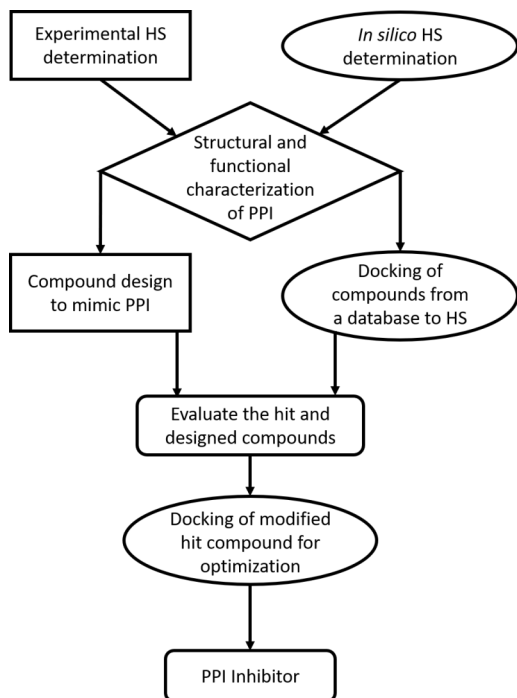

Fig. (1). Workflow used for computational design of PPI inhibitors. Adapted from Sable *et al.* [123].

First indications of PPIs can be achieved by using biochemical (co-) immunoprecipitation or pull-down experiments. When working with recombinant proteins mostly tags are used, such as glutathione-S-transferase (GST), human influenza hemagglutinin (HA) or myc tags [10,134,135]. To further characterize true interactions mostly fluorescence-based methods are applied, such as FRET (fluorescence resonance energy transfer), BRET (bioluminescense resonance energy transfer), BiFC (biomolecular fluorescence complementation assays) or more recently developed methods which emerged from the standard methods, like time-resolved FRET (Tr-FRET). However, all these methods have in common that they do not address the questions about the interfaces involved in the oligomers/complexes, but rather they only confirm the interaction itself. In addition, these methods are not suitable for analysing interactions in native tissues or those that are being transient. For dynamic monitoring of transient interactions a novel technique, namely total internal reflection fluorescence microscopy (TIRFM), which can be used with the SNAP-tag technology can be used to label GPCRs at the cell surface of living cells [136]. Alternatively, BioID [137] can also be used for detecting transient interactions; however, it has not been used for the study of GPCRs yet. For deciphering the interaction sites experimentally, cleverly designed mutagenesis studies are essential. In some cases, especially for interactions between receptors and specific domains, microarrays can be well suited to decipher such interaction sites.

Especially for receptors activated by peptides the development of PPI inhibitors interfering or preventing ligand binding can be of high interest for the treatment of several diseases or to reduce side effects by tailoring the drug responses to selective pathways. For example, for ghrelin receptors different heterodimers have been described, such as GHS-R1a-SST5, which are involved in controlling the glucose homeostasis [138] or GHSR-MC3R heterodimers, which are important for hypothalamic weight regulation [139] (check Table **2** and section C).

For the design of inhibitors, the nature of the interaction as well as the type of modulation of PPIs must be considered and the type of assay should be chosen accordingly. The
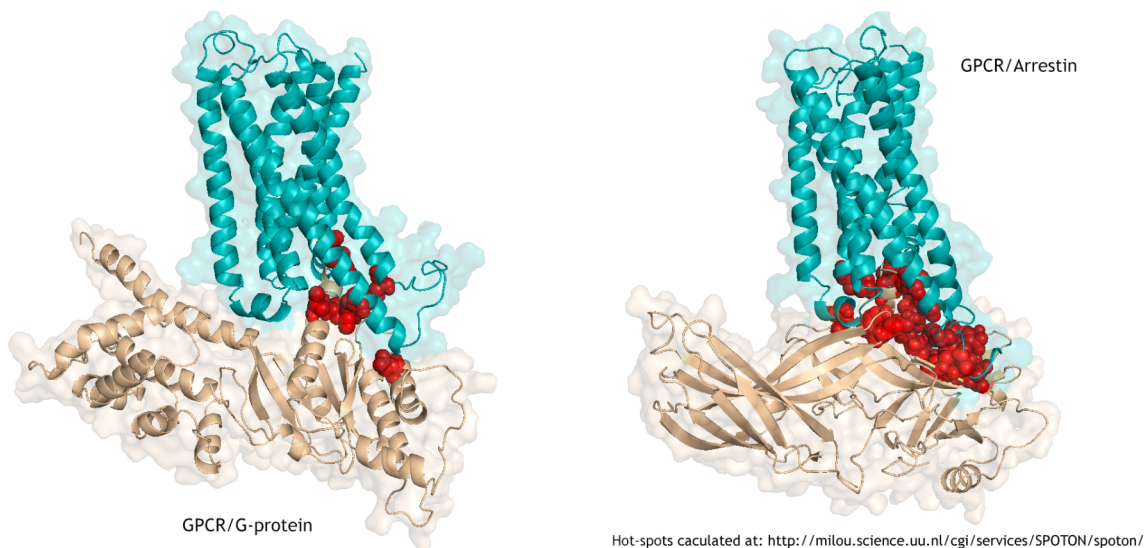

Fig. (2). Binding hot-spots at the interface of a typical GPCR with the two intracellular partners: G-protein and arrestin.

Table 2.   **A list of protein-protein interactions taken from GPCR oligomers and GPCR signalling complexes.**

Receptor	Interaction partner	Method Used	Biological System	Associated Disease/ Relevance	Refs.
CLASS A GPCRS					
	Peptide Receptors				
	Growth hormone secretagogue receptor (Ghrelin receptor, GHSR)				
GHSR	Melanocortin receptor 3 (MC3R)	cAMP/IP$_3$ assay	COS7 cells, HEK293 cells	Body weight regulation; obesity	[139]
GHSR1A	Somatostatin receptor type 5 (SS5R)	Tr-FRET, BRET	*ghrelin$^-$/* and *ghsr$^-$/* mice, HEK293 cells	Inhibition of insulin secretion	[138]
	GHSR1a	FRET	-	-	[141]
	GHSR1b	FRET	-	-	[141]
	GPR83	BiFC,sandwich (ELISA), YFP-based PCA	COS7 cells, HEK293 cells	Obesity	[142, 143]
	D(2) dopamine receptor	FRET, Tr-FRET	*ghrelin$^-$/* and *ghsr$^-$/* mice, hypothalamic neurons	Inhibition of food intake; anorexia	[144-146]
	D(1A) dopamine receptor	FRET, Tr-FRET	*ghsr$^{-/-}$* mice, *ghsr$^{+/+}$* mice, hippocampal neurons, HEK293 cells	Parkinson's Disease, drug addiction, food reward, cognition, memory	[147, 148]
	Melanocortin receptor accessory protein 2 (MRAP2)	Co-IP, NanoBit protein-protein interaction assay	HEK293T cells	Obesity	[149]
	Opioid receptors				
M-OPIOID RECEPTOR (MU-TYPE OPIOID RECEPTOR, MOR)	Delta-type opioid receptor(DOR-1)	Co-IP, BRET	COS7 cells, CHO-K1 cells	Chronic and/or neuropathic pain	[150-153]
	chemokine receptor CCR5	Co-IP	human CEM ×174 and monkey lymphocytes, CHO cells	AIDS (inhibition of viral entry)	[154, 155]
	Sst$_{2A}$ somatostatin receptor	Co-IP	HEK293 cells	Pancreatic cancer	[156, 157]
	Neurokinin-1 receptor (substance P receptor, NK1)	Co-IP, BRET	HEK293 cells	Pain modulation	[158, 159]
	Nociceptin receptor (NOR)	Co-IP	HEK293 cells	Pain modulation	[160]
	κ-opioid receptor (KOR)	BRET	HEK293 cells	Pain modulation	[161, 162]
	Cannabinoid CB$_1$ receptor	Co-IP, BRET, FRET	Neuro2A cells, HEK293 cells,BHK cells	Chronic and/or neuropathic pain	[163-165]
	α$_{2A}$-adrenoceptor	Co-IP, BRET, FRET	HEK-293 cells, MDCK cells, rat primary hippocampal neurons	Pain modulation	[166-168]
	Metabotropic glutamate receptor-5 (mGluR$_5$)	Co-IP	HEK293 cells	Pain modulation	[169, 152]

(Table 2) contd….

Receptor	Interaction partner	Method Used	Biological System	Associated Disease/ Relevance	Refs.
			CLASS A GPCRS		
	Gastrin-releasing peptidereceptor (GRPR)	Co-IP	HEK 293 cells,Mice spinal cord	Morphine-induced scratching (MIS)	[170]
	$5HT_{1A}$	Co-IP, BRET	HEK 293 cells, COS7 cells	Pain modulation	[171]
	Galanin receptor subtype Gal1 (Gal1R)	BiFC, BRET	HEK293T cells, rat ventral tegmental area	Opioid use disorders	[172]
	Negative elongation factor A	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
Δ-OPIOID RECEP-TOR(DELTA-TYPE OPIOID RECEPTOR, DOR)	α_{2A}-adrenoceptor	Co-IP, BRET	HEK293 cells,rat spinal cord	Pain modulation	[173, 174]
	β2-adrenoceptor	Co-IP, BRET	HEK293 cells, CHO cells	Alteration of β2-adrenoceptor internalization	[175, 176]
	chemokine receptor CCR5	Co-IP	human CEM ×174 and monkey lymphocytes	AIDS	[154]
	Sensory Neuron-Specific Receptor-4 (SNSR-4)	BRET	HEK293 cells	Pain modulation	[177]
	Cannabinoid CB_1 receptor	Co-IP, BRET	Neuro2A cells, HEK293 cells	Altered subcellular localiza-tion of CB_1 receptor, enhanced CB_1 receptor desensitization	[163, 178]
	CXCR4 chemokine receptor	Co-IP, FRET	MM-1 cells, HEK293 cells	Inflammation, Pain, sensing HIV-infection	[179]
	κ-opioid receptor (KOR)	Co-IP, BRET	peripheral sensory neurons, HEK293 cells	Pain modulation, allodynia	[180, 176, 151]
K-OPIOID RECEPTOR (KAPPA-TYPE OPIOID RECEPTOR, KOR)	β2-adrenoceptor	Co-IP, BRET	HEK293 cells, CHO cells	-	[175, 176]
	chemokine receptor CCR5	Co-IP	human CEM ×174 and monkey lymphocytes	AIDS	[154]
	Apelin receptor (APJ)	Co-IP, BRET	HEK293 cells	Increase in cell proliferation	[181]
	Bradykinin B_2 re-ceptor	BRET, PLA	HEK293 cells	Increase in cell proliferation	[182]
NOCICEPTIN RE-CEPTOR (NOR, KAPPA-TYPE 3 OPIOID RECEP-TOR,(KOR-3), OPIOID RECEPTOR-LIKE 1 RECEPTOR (ORL1))	Ceramide synthase 6 (CerS6)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
			Protease activated receptor 2 (PAR-2, also known as thrombin receptor-like 1)		
PAR-2	Regulator of G-protein signalling 8 (RGS8)	GST pull-down, BRET	HEK293 cells, Neuro2a cells	-	[183]
	Major prion protein (PrP)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]

(Table 2) contd….

Receptor	Interaction partner	Method Used	Biological System	Associated Disease/ Relevance	Refs.
CLASS A GPCRS					
	Sarcoplasmic/ endo-plasmic reticulum calcium ATPase 2 (SERCA2)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Heat shock 70 kDa protein 1B (HSP70-2)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
Type-1 angiotensin II receptor (AT1R)					
AT1R	Bradykinin B$_2$ re-ceptor	Co-IP	HEK293 cells, mesangial cells (rat)	Hypertension	[184, 185]
	Cannabinoid CB$_1$ receptor	Co-IP, BRET	HEK293 cells,Neuro2A cells,HSCs	Fibrosis	[186, 151]
	α$_{2C}$- adrenoceptor	BRET, FRET	HEK293 cells	Hypertension, heart failure	[187]
	Sodium/potassium-transporting ATPase subunit beta-1	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	DnaJ homolog subfamily C member 8	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Ceramide synthase 6 (CerS6)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Ornithine decar-boxylase antizyme 1 (ODC-Az)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
5a anaphylatoxin chemotactic receptor 2 (C5a-R, GPR77)					
C5A-R	Calmodulin-1, 2, 3	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	uncharacterized protein C4orf3 (Hepatitis C virus F protein-transactivated pro-tein 1)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Mitochondrial 2-oxoglutarate/malate carrier protein (OGCP)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Synaptogyrin-2	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
Oxytocin receptor (OTR)					
OXYTOCIN RECEP-TORS	Oxytocin receptor	Co-IP, BRET, Tr-FRET	COS7 cells , rat mammary glands	-	[188, 189]
	Vasopressin V1 receptor (V1R)	Co-IP, BRET, tr-FRET	HEK293T cells, CHO cells, COS7 cells, rat mammary glands	-	[190, 189]
	Vasopressin V2 receptor (V2R)	Co-IP, BRET, tr-FRET	HEK293T cells, COS7 cells, rat mammary glands	-	[190, 189]

(Table 2) contd….

Receptor	Interaction partner	Method Used	Biological System	Associated Disease/ Relevance	Refs.
CLASS A GPCRS					
Thyrotropin-releasing hormone receptor (TRHR)					
TRHR	TRHR	BRET	HEK293 cells, COS1 cells	-	[191]
Gonadotrophin-releasing hormone receptors (GnRHR)					
GNRHR	GnRHR	BRET	HEK293 cells, COS1 cells	-	[191]
Protein receptors					
Thyrotropin receptor (Thyroid-stimulating hormone receptor) (TSH-R)					
TSH-R	TSH-R	BRET	HEK293T cells	-	[192]
	Mid1-interacting protein 1	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Synaptotagmin-1	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
C-C chemokine receptors					
CCR1	Myelin basic protein (MBP)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Major prion protein (PrP)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
Lipid receptors					
Platelet-activating factor receptor (PAF-R) Lipid					
PAF-R	Myelin basic protein (MBP)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Major prion protein (PrP)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Plasmolipin	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Rhomboid domain-containing protein 2	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Transmembrane protein 120A	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
Thromboxane A2 receptor (TXA2-R), also known as Prostanoid TP receptor					
TXA2-R	G-protein coupled receptor-associated sorting protein 1-3, 7 (GASP-1-3, 7)	GST-pull down experiments, Co-IP	HEK293 cells	-	[9]
Aminergic receptors					
Dopamine receptors					
D(1) DOPAMINE RE-CEPTOR	D(2) dopamine receptor	Co-IP, FRET, BRET	Rat striatal neurons, HEK293 cells,striatal post-mortem brain samples	Depression, schizophrenia, addiction	[193, 194, 151, 195]
	D(3) dopamine receptor	BRET, FRET, Tr-FRET	HEK293T cells, rat brain striatum	Basal-ganglia disorders	[196, 197]

Receptor	Interaction partner	Method Used	Biological System	Associated Disease/ Relevance	Refs.
CLASS A GPCRS					
	Corticotropin-releasing factor receptor 2α (CRFR-2α)	Co-IP, BRET, FRET	HEK293T cells	Addiction	[198]
	NR1A/NR2B N-methyl-D-aspartate (NMDA) glutamate receptor subunits	Co-IP, PLA, BRET	HEK293T cells, rat and mouse cortical brain sections	Schizophrenia	[199, 195]
	Mu-type opioid receptor (MOR-1)	Co-IP, BRET	HEK293 cells, mouse brain striatum	Addiction	[200]
D(2) DOPAMINE RE-CEPTOR	D(2) dopamine receptor	BiFC, BiLC, BRET, tr-FRET	HEK293 cells, COS7 cells	Parkinson's Disease, Schizophrenia	[201, 189, 202]
	D(3) dopamine receptor	Co-IP	COS7 cells	Hypothermia, Schizophrenia	[203, 195]
	D(4) dopamine receptor	Co-IP, BRET	HEK293T cells, rodent (rat and mouse) brain striatum	ADHD	[204, 195]
	Cannabinoid CB1 receptor	Co-IP	HEK 293 cells	Possible role in determining the responses of neurons to neurotrans-mitter	[205]
	Somatostatin receptor type 5 (SS5R)	Immunohisto-chemical colocaliza-tion in rat brain cortex and striatum, FRET	CHO-K1 cells	Cancer	[206, 207]
	5-hydroxy-tryptamine 5-HT$_{(2A)}$ receptors	BRET	HEK293T cells	Schizophrenia	[208, 151, 195]
	Neurotensin NTS1 receptor	Co-IP, BRET	HEK293T cells	Parkinson's Disease	[209, 210]
	Angiotensin II type 1 receptor	PLA, BRET	HEK293T cells, primary cultures of neurons (rat), rat striatal slices	Parkinson's Disease and/or dyskinesia	[211]
D(3) DOPAMINE RE-CEPTOR	Nicotinic acetycho-line receptor (nAChR)	BRET, PLA	HEK293T cells, primary cultures of midbrain dopa-mine neurons (mice)	Nicotine addiction	[212]
Serotonin receptor (5-HT)					
5HT$_{1A}$	Galanin receptor (GalR)	FRET	HEK293 cells	Depression	[213, 214]
	5HT$_7$	Co-IP, FRET	N1E-115 neuroblastoma cells, mouse brain	Depression, Anxiety	[215]
	D(2) dopamine receptor	Homogenous time-resolved FRET, FLIM-FRET	HEK293 cells, mouse corti-cal neurons	Schizophrenia	[216]
5-HT$_{4D}$	GPR37	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]

(Table 2) contd….

Receptor	Interaction partner	Method Used	Biological System	Associated Disease/ Relevance	Refs.
CLASS A GPCRS					
	G protein-regulated inducer of neurite outgrowth 2 (GRIN2)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
Beta adrenergic receptors					
B₁₋ ADRENOCEPTOR	G-protein coupled receptor-associated sorting protein 1-3, 7 (GASP-1-3, 7)	GST-pull down experiments, Co-IP	HEK293 cells	-	[9]
B₂₋ ADRENOCEPTOR	Major prion protein (PrP)	MYTH screen, BRET	yeast, HEK293 cells	-	[11]
	G-protein coupled receptor-associated sorting protein (GASP)	Co-IP, MYTH screen, BRET	yeast, HEK293 cells	-	[9, 11]
	Myelin basic protein (MBP)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Heterogeneous nuclear ribonucleo-protein K (hnRNP K)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Monoglyceride lipase (MGL)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
Muscarinic acetylcholine receptors (MR)					
M₁ MUSCARINIC ACETYLCHOLINE RECEPTOR (MR₁)	G-protein coupled receptor-associated sorting protein 7 (GASP-7)	GST-pull down experiment, Co-IP	HEK293 cells	-	[9]
Histamine receptors					
HISTAMINE H3 RE-CEPTOR	NR1A/NR2B N-methyl-D-aspartate (NMDA) glutamate receptor subunits	Co-IP, PLA, BRET	HEK293T cells, rat and mouse cortical brain sections	Prevention of neurodege-neration	[199]
Purinergic receptors					
Adenosine receptors					
ADENOSINE RECEP-TOR A₁ (A₁AR)	Adenosine receptor A₂ₐ (A₂ₐAR)	Co-IP, BRET, tr-FRET	HEK293 cells, rat striatal synaptosomes	Fine-tuning modulation of glutamatergic neurotrans-mission	[217]
	D(1) dopamine receptor	Immunoprecipita-tion, double immu-nolabeling	Mouse fibroblasts Ltk⁻ cells	Addiction	[218, 195]
	PY1 receptor	Co-IP	HEK293T cells,rat brain (primary cultures)	-	[219, 220]
	Metabotropic glu-tamate recptor-1α (mGlu1αR)	Co-IP	HEK293 cells	Neuro-psychiatric disorders	[221]

Receptor	Interaction partner	Method Used	Biological System	Associated Disease/ Relevance	Refs.
CLASS A GPCRS					
	β_1-adrenergic receptor	Co-IP	HEK293 cells	Modulation of βR-induced positive inotropy in cardiac ventricular myocytes	[222]
	β_2-adrenergic receptor	Co-IP	HEK293 cells	Modulation of βR-induced positive inotropy in cardiac ventricular myocytes	[222]
	Thyrotropin receptor (thyroid-stimulating hormone receptor, (TSH-R)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
ADENOSINE RECEPTOR A_{2A} (A_{2A}AR)	Adenosine receptor A_{2A} (A_{2A}AR)	FRET, BRET	HEK293T cells	Basal ganglia disorders such as Parkinson´s disease	[223]
	Adenosine receptor A_{2B} (A_{2B}AR)	PLA, BRET, BiFC, FRET	CHO-K1 cells, dorsal hippocampus of the rat brain	Cancer	[224]
	D(2) dopamine receptor	BiFC, PLA, FRET, BRET	HEK293T cells,Mice striatal sections	Parkinson's Disease, Addiction, Schizophrenia	[225, 223] [226-228, 151, 195]
	D(3) dopamine receptor	FRET	HeLa cells	Schizophrenia	[229]
	Metabotropic glutamate receptor-5 (mGlu$_5$R)	Co-IP	HEK293 cells, rat striatum	Schizophrenia	[230, 231]
	GPR37	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Histamin H$_3$ receptors	Co-IP	HEK-293T cells, rat striatal synaptosomes	ADHD, autism, OCD	[232]
	Cannabinoid CB$_1$ receptor	Co-IP, PLA	Mice dorsal-striatum sections	Neurodege-nerative diseases	[233]
ADENOSINE RECEPTOR A_3 (A_3AR)	Adenosine receptor A_3 (A_3AR)	BiFC	CHO-K1 cells	Cancer	[234, 235]
Nucleotide receptors					
P2Y1	P2Y11	Co-IP, co-pulldown, FRET	HEK293 cells, 1321N1 astrocytoma cells	Cardiovascular diseases	[236, 237]
Class A orphan					
GPR37	Fatty acid 2-hydroxylase	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	4F2 cell-surface antigen heavy chain (solute carrier family 3, member 2)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Transmembrane protein 161A	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Protein tweety homolog 1	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
	Protein YIF1A	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]

(Table 2) contd….

Receptor	Interaction partner	Method Used	Biological System	Associated Disease/ Relevance	Refs.
CLASS A GPCRS					
	Protein YIF1A	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
GPR50	Melatonin MT$_1$ receptor	Co-IP, BRET	HEK293 cells	Inhibition of MT$_1$ function	[238, 239]
GPR83	Melanocortin receptor 4 (MC4-R)	Co-IP	yeast, HEK293 cells	-	[9]
	Melanocortin receptor 3 (MC3-R)	Sandwich ELISA, YFP-based PCA	COS7 cells, HEK293 cells	-	[142]
	GPR171	BiFC, Co-IP, PLA	COS7 cells, HEK293 cells,CHO cells,rat brain	-	[143, 240]
GPR143	Tyrosinase	Co-IP, FRET	COS7 cells, melanocytes	Ocular Albinism Type I	[118]
GPR179	Metabotropic glutamate receptor-6 (mGlu$_6$R)	Co-IP, PLA	HEK293T cells, mouse retina	-	[241]
	Regulator of G-protein signaling (RGS) protein	Co-IP, PLA	HEK293T cells, mouse retina	-	[241]
	Transient receptor potential cation channel subfamily M member 1 (TRPM1)	Co-IP, PLA	HEK293T cells, mouse retina	-	[241]
MAS-RELATED RECEPTOR MRGD	Mas-related receptor MrgE	Co-IP, Tr-FRET	HEK293 cells	Pain	[242]
CLASS B1 (SECRETIN) GPCR					
HUMAN VASOACTIVE INTESTINAL POLYPEPTIDE RECEPTOR (VPACR)	VPACR	BRET	COS cells	-	[243]
	Secretin receptor (SCTR)	BRET	COS cells	-	[243]
SECRETIN RECEPTOR (SCTR)	Secretin receptor (SCTR)	BRET	COS cells	-	[243]
	Parathyroid hormone receptor (PTHR)	BRET, FRET	COS cells	-	[244]
	Glucagon-like peptide receptor (GLPR)	BRET, FRET	COS cells	-	[244]
	Growth hormone-releasing hormone receptor (GHRHR)	BRET, FRET	COS cells	-	[244]
	Type-1 angiotensin II receptor (AT1aR)	BRET, FRET	CHO cells,COS cells	Osmoregulation	[245]
GASTRIC INHIBITORY POLYPEPTIDE RECEPTOR (GIPR)	Opsin receptor	BRET	COS7 cells, HEK293 cells	-	[246]
	β$_2$-adrenoceptor	BRET	COS7 cells, HEK293 cells	-	[246]

(Table 2) contd….

Receptor	Interaction partner	Method Used	Biological System	Associated Disease/ Relevance	Refs.
CLASS B1 (SECRETIN) GPCR					
CALCITONIN RE-CEPTOR (CT-R) (PEP-TIDE RECEPTOR)	G-protein coupled receptor-associated sorting protein 1-3 and 7 (GASP-1-3, 7)	GST-pull down experiments, Co-IP	HEK293 cells	-	[117]
CORTICOTROPIN-RELEASING FACTOR RECEPTOR 1 (CRFR-1)	Vasopressin V_{1b} receptor	Co-IP, BRET	CHO cells	-	[247]
CLASS C GPCR					
CALCIUM-SENSING RECEPTOR (CAR)	Calcium-sensing receptor (CaR)	Co-IP	HEK293 cells	Familial hypocalciuric hyper-calcemia	[248]
GAMMA-AMINOBUTYRIC ACID TYPE B RE-CEPTOR (GABA-B RECEPTOR)	Gamma-aminobutyric acid receptor (GABA-B receptor), splice variants $GABA_BR1$ and $GABA_BR2$)	IF	COS7 cells	Only $GABA_BR1$/ $GABA_BR2$ heterodimers are functional	[249]
	GPR37	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]
CLASS F (FRIZZLED) GPCR					
ATYPICAL FRIZZLED 4 RECEPTOR(FZ-4)	G-protein coupled receptor-associated sorting protein 1, 3 and 7 (GASP-1, 3, 7)	GST-pull down experiments, Co-IP	HEK293 cells	-	[9]
FRIZZLED-7 RECEP-TOR (FZ-7)	Major prion protein (PrP)	MYTH screen, Co-IP, BRET	yeast, HEK293 cells	-	[11]

Abbr.: ADHD, attention-deficit hyperactivity disorder (ADHD); BHK cells, baby hamster kidney cells; BiFC, biomolecular fluorescence complementation; BRET, bioluminescence resonance energy transfer; Co-IP, coimmunoprecipitation; ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer; GST, Glutathione-S-transferase; HEK293 cells, human embryonic kidney cells; HSCs, hepatic stellate cells; MDCK cells, Madin-Darby canine kidney cells; MM-1 cells, human monocytic cell line Mono-Mac-1; MYTH, membrane yeast two-hybrid; OCD, obsessive and compulsive disorder; PCA, protein complementation assay; PLA, proximity ligation assay; Tr-FRET, time resolved-FRET.

types of interactions can be permanent or transient, weak or strong interaction. In addition, they may depend on the topological or kinetic changes and also on the expression levels of the interacting proteins. Commonly, PPIs are only considered true if the interfaces do not include the catalytic sites or the binding pockets to which small molecule/ligand can bind [140]. The types of modulation may be either orthosteric or allosteric. The former will directly bind to the interface between the protein partners while the latter can bind anywhere on the protein other than the orthosteric binding site. One of the common "problems" with allosteric inhibitors is that the exact mechanism is often not clear and therefore the rational design is challenging [140].

3.1. Affinity-based Methods

3.1.1. Co-immunoprecipitation (Co-IP)

The classical biochemical approach for the identification of PPIs is co-immunoprecipitation (Co-IP) which is followed by Western blot analysis to detect co-precipitated proteins as shown in Fig. (**3**). This method relies on the availability of either highly selective antibodies or the modification of the proteins by adding tags, which might influence the original PPIs, and it is often used for hypothesis-driven approaches as a first indication of the interaction of the two proteins [12] (for more examples see Table 2). Nevertheless, the disadvantage of this method is that the cells have to be lysed and membrane proteins have to be solubilized which may influence or destroy the interactions. Another limitation of the method is that the detection can only be measured for protein partners having strong interactions. Additionally, high abundant proteins can be often co-purified thus leading to false-positive interaction partners. The method is suitable for native proteins when effective antibodies are available. If not, it can also be done with epitope-tagged proteins (Fig. **2B**).

3.1.2. Tandem Affinity Purification (TAP)

The tandem affinity purification (TAP) is a method, which allows the isolation of protein complexes through a double purification process, thus removing more background
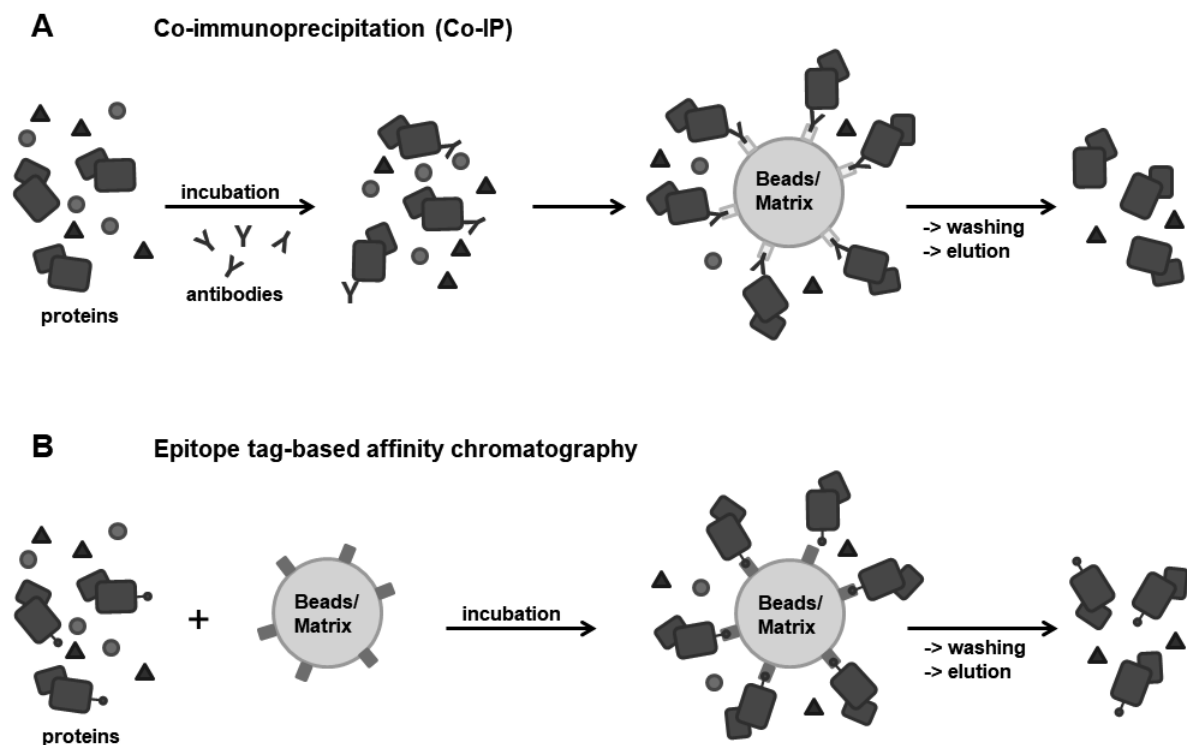
Fig. (3). Co-immunoprecipitation (Co-IP). (**A**) In the classical Co-IP approach proteins are incubated with specific antibodies against one of the proteins of interest and then captured by beads or a matrix. After washing and elution proteins are separated and Western blot analysis is performed to detect co-precipitated proteins. (**B**) If no antibodies against the native proteins are available the same method can be applied by using epitope-tagged proteins.

than that present in the classical AP methods (see Fig. **4**) [250]. Originally, the protein of interest is tagged with the TAP tag, consisting of a calmodulin binding protein, a TEV protease cleavage site and protein A. In the first step the protein is bound to IgG via the protein A tag, then after washing it is cleaved by the TEV protease and bound to calmodulin beads. After the second washing step the complexes can be eluted by EGTA [7]. The protein complexes can then be analysed by Western blotting or proteomics methods.

3.2. Proteomics-based Methods

3.2.1. Pull-down- and Affinity Purification-linked Mass Spectroscopy (AP-MS)

Affinity Purification-Mass Spectrometry (AP-MS) is a powerful method for the analysis and identification of interactomes [13]. The first step of the method is similar to the biochemical IP, but involves the immobilization of the "bait" protein on the beads *e.g.* agarose, then binding partners are captured from a soluble phase, making this method also more difficult for the analysis of membrane proteins, since cell lysis and solubilisation of the proteins are needed. After affinity purification the captured proteins are digested with trypsin or other proteases to generate peptides, which can be fractioned by high-pressure liquid chromatography (HPLC) and detected by a mass spectrometer. AP-MS is applicable for native proteins or recombinant proteins bearing epitope tags and suitable for HTS approaches while not suited for the identification of dynamic, transient interactions and some-

times also difficult for endogenous proteins having low expression levels. As stated above, it is also possible that highly abundant proteins are co-purified using this method [13]. Several strategies are established which can overcome some of the limitations, such as tandem affinity purification (TAP) and quantification approaches either with labelling (*e.g.* stable isotope labelling with amino acids and cell culture, SILAC) or label-free [13,250,251]. It is also possible to identify contaminants via comparison with databases [10]. MS-based proteomics has been applied to gain insights into the mechanisms involved in β-arrestin-biased agonism of GPCR [50,52,252].

3.2.2. BioID

The proximity labelling strategy, namely BioID, relys on the expression of the protein of interest, which is fused to a mutant form of the biotin ligase BirA (BirA*) [137]. When BirA* is present lysine residues which are in close proximity (<10 nm) can be biotinylated [253]. This method allows the detection of interaction partners and also proteins which are present in close proximity of the protein of interest, but do not directly interact with it. Biotinylated proteins can be affinity-purified using streptavidin and subsequently analysed by mass spectrometry. BioID is suited for the detection of transient interactions in whole cells. Recently, the method has also been modified to split-BioID. In this method BirA* is split in two halves, which are fused to the proteins of interest. Only after dimerization the two halves can complement each other and subsequently biotinylate proteins which
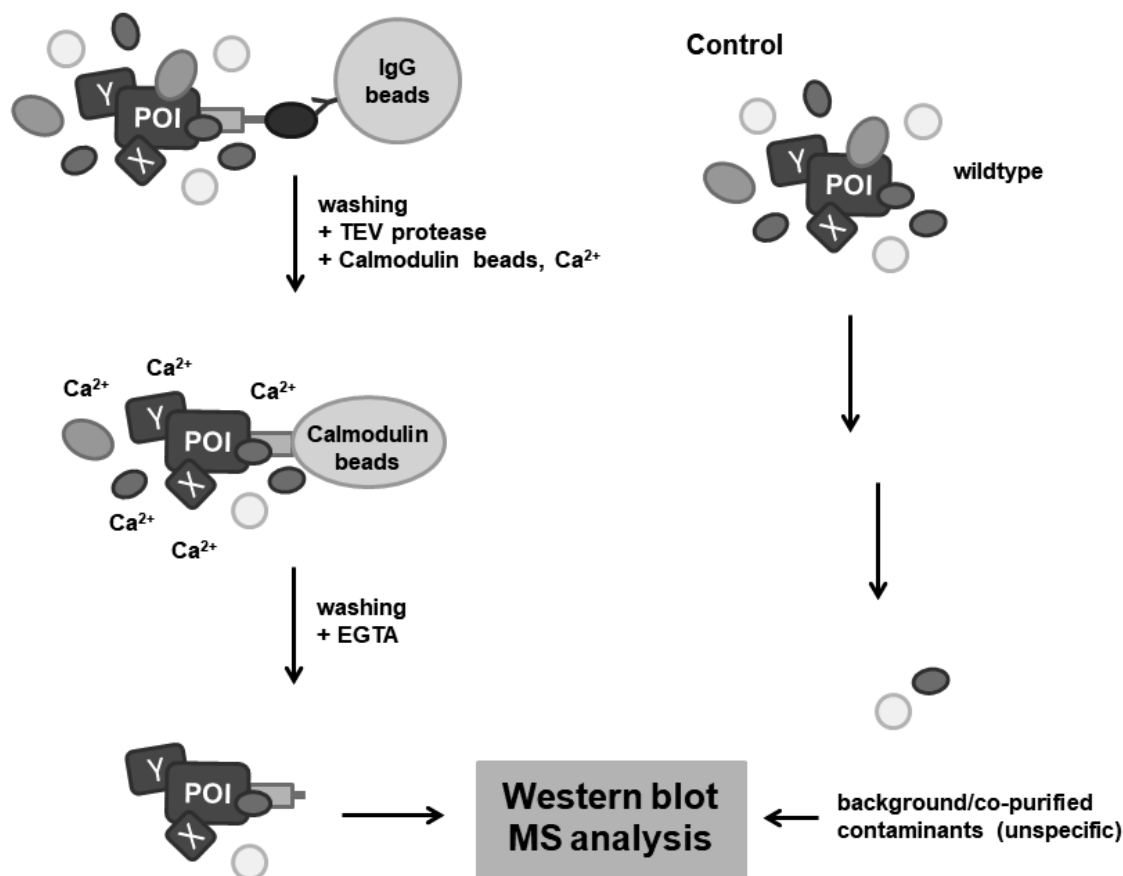
Fig. (4). Tandem affinity purification (TAP). Protein complexes are purified by a double purification process. First, the protein of interest (POI) is tagged with a TAP tag consisting of a calmodulin binding protein, a TEV protease cleavage site and protein A. In the first purification step the complexes are captured by IgG beads via protein A, after washing TEV protease is added cleaving the tag. Remaining complexes are re-purified again by using calmodulin beads. After washing and elution steps EGTA complexes are analysed using Western blotting and mass spectrometry (MS). For the detection of the background and co-purified contaminants usage of the untagged wild-type protein as a control is essential.

are in close proximity. In comparison to the classical BioID, the split-BioID method allows for identification of protein dimers [254].

3.2.3. Protein/Peptide Microarrays

Functional protein microarrays are well suited for high-throughput screening approaches for detection of PPIs [255]. Protein microarrays consist of purified proteins or peptides, *e.g.* contain specific domains [134] or modifications, such as phosphorylation sites, which allow capturing proteins which interact with the domains or proteins of the interest [256,257]. Recently biased agonism has become a focus in the field and several groups investigated different phosphorylation "bar codes" which are introduced by certain GRKs and may stabilize distinct active conformations [258].

3.2.4. AlphaScreen Technology in HTS

The Alpha Technology (Amplified Luminescent Proximity Homogeneous Assay) was developed by Perkin-Elmer and is a flexible bead-based proximity assay which is suitable compared to other applications that are used for measuring PPIs (Fig. **5**) [259,260]. The assay is based on two types

of beads, namely donor and acceptor, which are linked to the proteins of interest using streptavidin or anti-GST. If two proteins are in close proximity, the donor beads containing a photosensitizer are excited and oxygen is converted into singlets upon irradiation at 680 nm. The oxygen singlets can then diffuse for up to 200 nm and can cause a chemiluminescent signal in the acceptor beads, which can be measured. Alpha-screen assays are well suited for HTS approaches for identifying PPI inhibitors from large libraries [261].

3.3. Fluorescence-based Assays

3.3.1. Fluorescence Resonance Energy Transfer (FRET)

One of the advantages of FRET over classical biochemical methods is that the method allows the detection of dynamic, transient interactions and is therefore well-suited for investigating events along the signal transduction cascades, even in real-time measurements. For instance, PPIs involved in desensitization/internalization of GPCRs can be studied in detail by using FRET or BRET assays. The FRET method is based on the energy transfer from a donor to an acceptor fluorophore, where the distance between the fluorophores
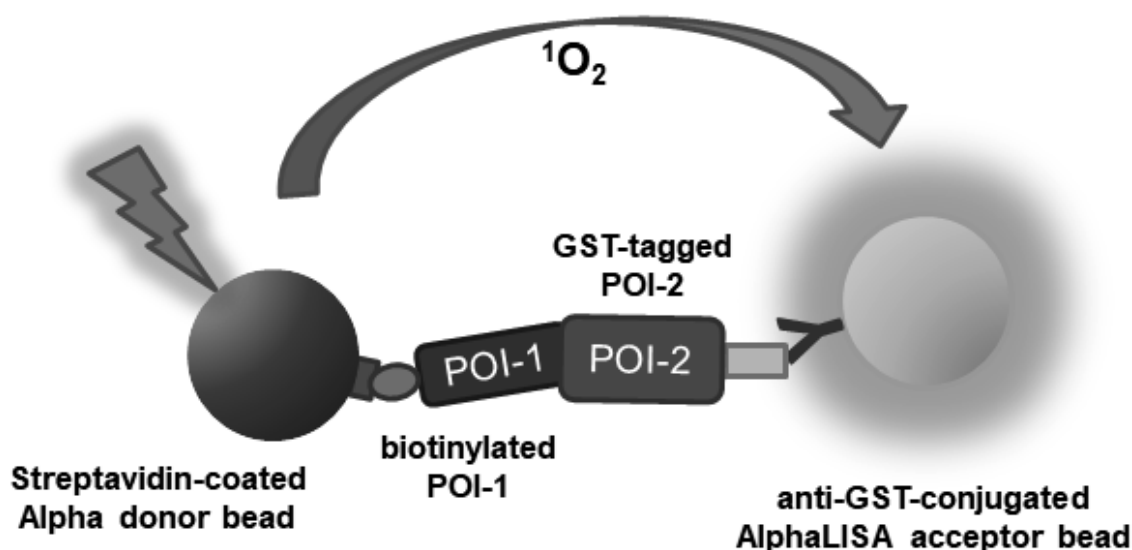
Fig. (5). Amplified Luminescent Proximity Homogeneous Assay (Alpha screen). Proteins of interest (POI) are tagged with donor and acceptor beads, respectively. When proteins are in close proximity donor beads containing a photosensitizer are excited and oxygen is converted into singlets upon irradiation at 680 nm. The oxygen singlets can then diffuse for up to 200 nm and can cause a chemiluminescent signal in the acceptor beads which can be measured (modified after [262]).

has to be no longer than 10 nm. Most commonly, fluorescent proteins are expressed as fusion proteins with the proteins of interest (Fig. **6A**). The cyan and yellow fluorescent protein pairs are widely used. For labelling smaller proteins, FLAsH-based tags are often suitable [263]. Commonly used probes for detecting GPCR interactions are either ligand-based or protein fluorescent probes. The latter is composed of either modified peptides or, in some cases, small molecules associated with fluorophores [8]. If tagged proteins are not available, *e.g.* for analysis in native tissues, fluorescent-conjugated antibodies can be used instead. Fluorescent-based methods are suitable for detection of either ligand-receptor or protein-protein interactions, both at the single-cell or tissue level. In some cases, such interactions can also be analysed even at the molecular level, *e.g.* studying conformational changes of receptors/proteins [8]. For examples see Table **2**.

3.3.2. Time Resolved-FRET (Tr-FRET)

Time resolved FRET is an enhancement of the classic FRET method which is suitable for high-throughput screening assays [138,145,147]. The emitted light will be measured with a slight delay of a few milliseconds to reduce or eliminate the crosstalk between excitation and emission signals to remove the background fluorescence [146]. Leyris *et al.* developed an assay which uses the so-called tag-lite technology, in which TrFRET measurements are combined with a covalently bound terbium cryptate, that was attached to the ghrelin receptor via a SNAP tag and a high-affinity red fluorescent ghrelin ligand [264].

3.3.4. Bioluminescence Resonance Energy Transfer (BRET)

In BRET assays, the donor is a luciferase, most commonly Renilla luciferase (RLUC) is used, which shows a similar emission spectrum as the CFP and can therefore be combined with Enhanced Yellow Fluorescent Protein

(EYFP) as the acceptor fluorophore [265]. For BRET assays it is required that both proteins of interest are genetically modified to express either RLUC or EYFP as fusion proteins. The luciferase substrate, namely, coelenterazine, is membrane permeable, so the method is suitable for intact cells as depicted in Fig. (**6B**). BRET assays have some advantages over FRET assays because there is no need for excitation or photo-bleaching, which can damage the photo-responsive cells. It is also well-suited for cells or tissue having high auto-fluorescence. Another advantage of BRET compared to FRET is that in the former the detection of the expression levels of donor and acceptor is less complicated, since they can be quantified independently from each other, while for FRET pairs often an excitation overlap exists (bleedthrough) which necessitates a correction for the measurements done. Compared to FRET, BRET is the most common method used to investigate GPCR oligomers (see table 2 for examples).

3.3.5. Biomolecular Fluorescence Complementation (BiFC)

Biomolecular fluorescence complementation assays rely on the ability of certain proteins, such as GFP, CFP, YFP, venus, cerulean, to reconstitute into functional fluorescent proteins when expressed as non-fluorescent fragments (see Table 3) [266–269]. According to the BiFC the C- and N-terminal fragments of the fluorescent proteins are fused to the C-termini of the GPCRs or proteins of interest and co-expressed in the same cells [142,143,172,224,234,270]. Upon close interaction of the receptors, the fragments reconstitute into a functional protein and PPIs are then monitored as increasing fluorescent intensities (Fig. **6C**). To analyse multiple proteins involved in PPIs *e.g.* during signal transduction pathways simultaneously, a multi-colour fluorescence complementation approach can be applied by coupling BRET or BiFC experiments to FRET analysis [114,270]. The same technique can also be applied to study interactions

Table 3. **Common proteins used for Biomolecular Fluorescence Complementation (BiFC)**

Protein	Amino acid position of split	Excitation/Emission wavelength	Refs.
CERULEAN	172-173	434/475	[267]
CYAN FLUORESCENT PROTEIN (CFP)	154-155	452/478	[268]
GREEN FLUORESCENT PROTEIN (GFP)	157-158	485/500	[269]
VENUS	172-173	515/528	[266]
YELLOW FLUORESCENT PROTEIN (YFP)	154-155	515/528	[266]

regarding GPCR dimers or even higher order oligomers [271,272].

3.3.6. Total Internal Reflection Fluorescence Microscopy (TIRFM)

As mentioned above monitoring of transient interactions became possible with total internal reflection fluorescence microscopy (TIRFM). With this method it is possible to analyse receptor monomers, which are localized in the plasma membrane. Fluorophores can simply be added to the receptors via SNAP-tags [136]. TIRF microscopy is especially interesting to investigate processes occurring directly at the plasma membrane, such as receptor trafficking, i.e. internalization, etc [273]. Suitable probes are the pH sensitive eGFP variant super ecliptic phluorin (SEP) or antibody-labeled quantum dots or SNAP tagged fusion proteins [8,273–275]. While for most fluorescent-based methods receptors are tagged at the cytosolic site it is much better to tag the receptors at their extracellular domains using TIRFM [273].

3.3.7. Fluorescence Fluctuation Spectroscopy (FCS)

Fluorescence fluctuation spectroscopy (FCS) is a comparably novel fluorescent method which allows studying mobility and oligomerization dynamics of GPCRs as well as individually labelled proteins [136,276]. By measuring the molecular brightness of the tagged protein the number of fluorescent molecules can be estimated [136].
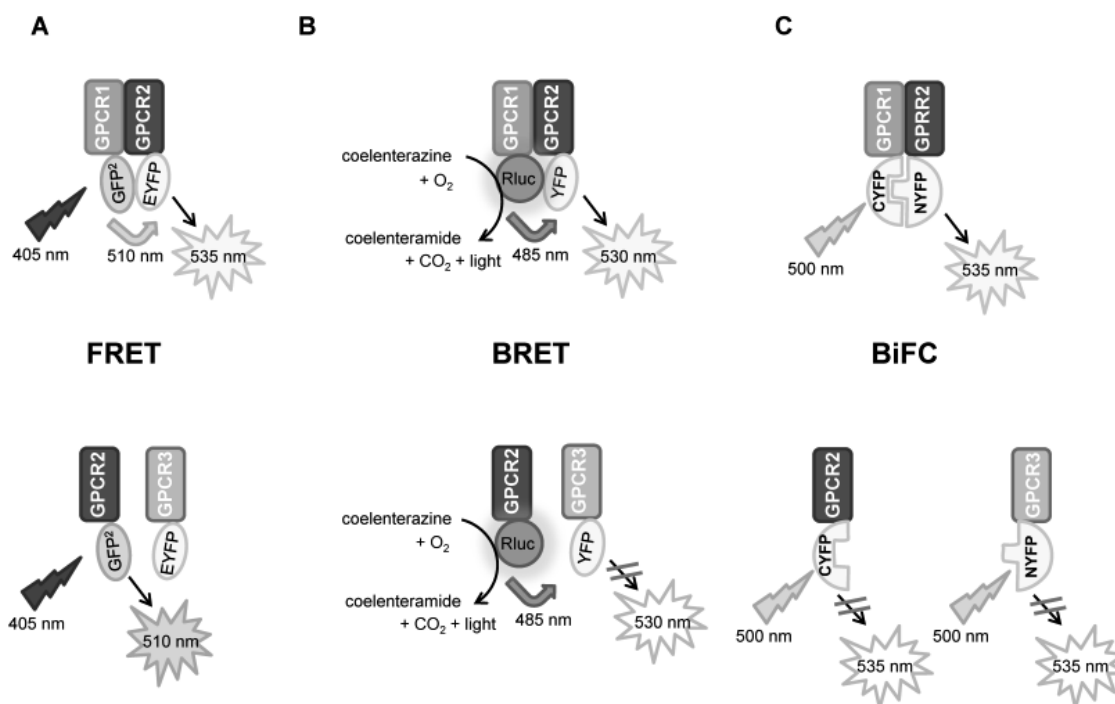

Fig. (6). Fluorescence-based assays. (**A**) The Fluorescence resonance energy transfer (FRET) method is based on the energy transfer from a donor fluorophore to an acceptor fluorophore both fused to the proteins of interest, which come into close proximity when proteins interact *e.g.* by forming a complex. (**B**) In Bioluminescence resonance energy transfer (BRET) assays the donor is a luciferase which is combined with EYFP as acceptor. When two proteins are in close proximity luciferase substrate coelenterazine leads to chemiluminescence which excites the acceptor and emission can be measured. (**C**) In Bimolecular fluorescence complementation (BiFC) assays a C terminal and N terminal half of a fluorescence protein, *e.g.* YFP are fused the the proteins of interest. Upon close interaction emission of the reconstituted fluorescence protein can be measured. GPCR, G protein-coupled protein; GFP, green fluorescent protein; (E)YFP, (enhanced) yellow fluorescent protein; Rluc, Renilla luciferase (modified from Hinz *et al.)* [224].
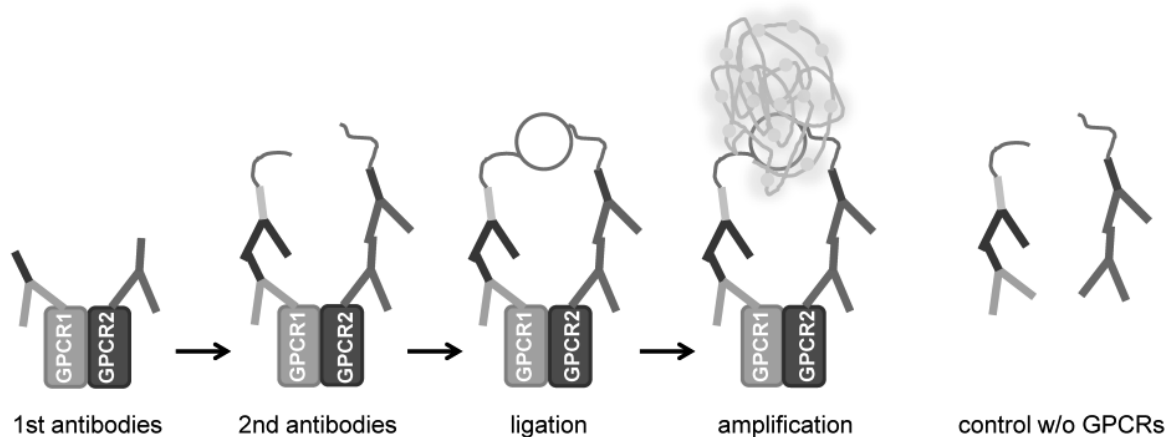
Fig. (7). Selective antibodies directed against the proteins of interest are needed for the proximity ligation assay (PLA). Either primary or secondary antibodies are conjugated to oligonucleotides, which can be ligated when they are in close proximity, amplified and visualized by a fluorescent probe. Several controls, especially one without the proteins of interest are necessary (adapted from [224]).

3.3.8. Proximity Ligation Assay (PLA)

The proximity ligation assay (PLA) is an antibody-based method, which is also suitable for native tissues, since no modifications of the proteins are necessary [136,199,211,224,233]. However, highly selective antibodies are crucial for the two target receptors. Either the primary or the secondary antibodies are conjugated to oligonucleotides, which can be ligated when they are in close proximity, amplified and visualized by a fluorescent probe (Fig. **7**). The distance between the two antibodies can be as far as 16 nm, which is larger than used for FRET fluorophores (10 nm). Therefore two GPCRs might not form heteromers but might just be in close proximity [136]. The method is highly sensitive and can detect interactions at the molecular level and can also capture transient interactions [10]. The disadvantages associated with the method are high costs of the assays and that PLA is not suitable to be used in HTS.

3.4. Genetic Assays

3.4.1. Yeast Two Hybrid Systems (YTH)

Yeast two hybrid (YTH) assays are suitable for the detection of PPIs based on the complementation of the two halves of a transcription factor, which are fused to the potential interaction partners. Commonly, the bait is screened against a library of pray proteins as shown in Fig. (**8**). The major disadvantage of the method for membrane proteins is that both proteins have to be in the nucleus and the assay is only able to detect one binding partner at a time and is not suitable for detection of oligomeric structures or transient interactions [10].

3.4.2. Membrane Yeast Two Hybrid (MYTH, Split-ubiquitin System)

Based on the classical YTH assays, a novel method has been established to identify interactions between membrane proteins, which is known as the membrane yeast two hybrid (MYTH) assay (Fig. **9**). This assay is also based on protein complementation of a C-terminal fragment fused to the membrane protein of interest and an N-terminal fragment fused to the potential prey proteins, which can either be

membrane proteins or cytosolic proteins. The two halves constitute a pseudoubiquitin, which is cleaved in a way that a transcription factor is released and can activate a reporter gene expression system. This assay is also limited to the detection of binary interactions and can lead to artefacts because mostly non-native proteins are expressed in yeast host cells [10]. However, this method has successfully been used by Sokolina *et al.* who recently reported the first systematic interactome analysis of 48 human GPCRs by using modified MYTH approach [11].

4. GHRELIN RECEPTOR AS AN EXAMPLE SYSTEM

4.1. Ghrelin and its Receptor

Ghrelin is a 28-amino acid peptide secreted by X/A-like cells of the oxyntic glands, located in the gastric fundus, and then transported to the brain. Ghrelin circulates in two forms: acylated (-5%) and desacylated (95%) [277]. It acts directly in the hypophysis by stimulating the release of growth hormone. Also it can have a homeostatic role in other parts of the brain and the rest of the body. The most prominent of these roles is the appetite stimulatory action of ghrelin, from which it takes its name: "the hunger hormone" [277]. Ghrelin stimulates feeding by activating orexigenic neurons and suppressing neurons containing anorexigenic peptides. In addition, ghrelin has been implicated in other physiologic processes in the central nervous system like neuroprotection, neurogenesis, anti-anxiety effects and some higher functions like memory and cognition regulation [278]. Ghrelin is an endogenous ligand of the growth hormone receptor 1 (GHS-R), a GPCR, which can dimerize to form homodimers [279] or can dimerize with other GPCRs thus forming heterodimers. The most common heterodimers are formed with melanocortin receptor 3 (MC3), GPR83, dopamine receptor 1 (D_1 receptor), dopamine receptor 2 (D_2 receptor) and serotonin receptor 2c (5-HT_{2c} receptor) (see Table 2) [280]. Heterodimer interactions can result in altered trafficking and signalling [279]. The activation of GHS-R1a upon ligand coupling leads to conformational changes thus providing a surface to heterotrimeric guanine nucleotide-binding proteins (G proteins) and β-arrestin [279,281] for coupling to the re-
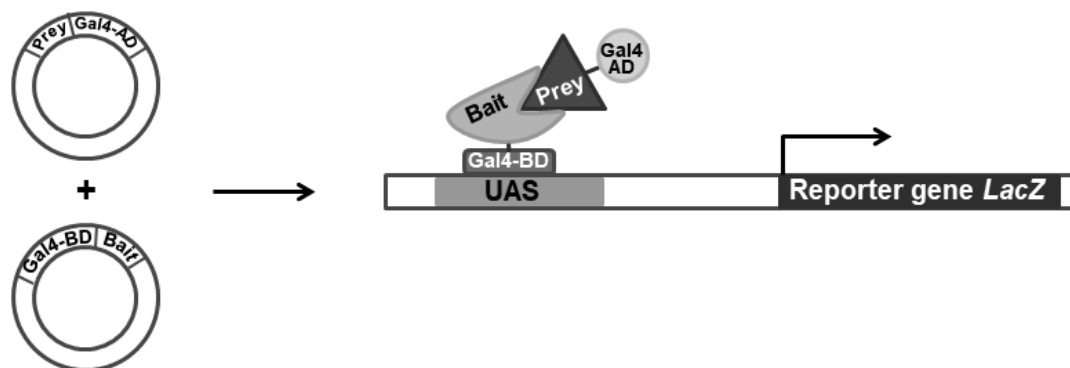
Fig. (8). Yeast two hybrid (YTH) assays are based on the complementation of two halves of a transcription factor (Gal4-AD and Gal4-BD) which are fused to the proteins of interest (bait and pray). Upon protein-protein interaction a reporter gene is expressed and yeast clones can be detected.
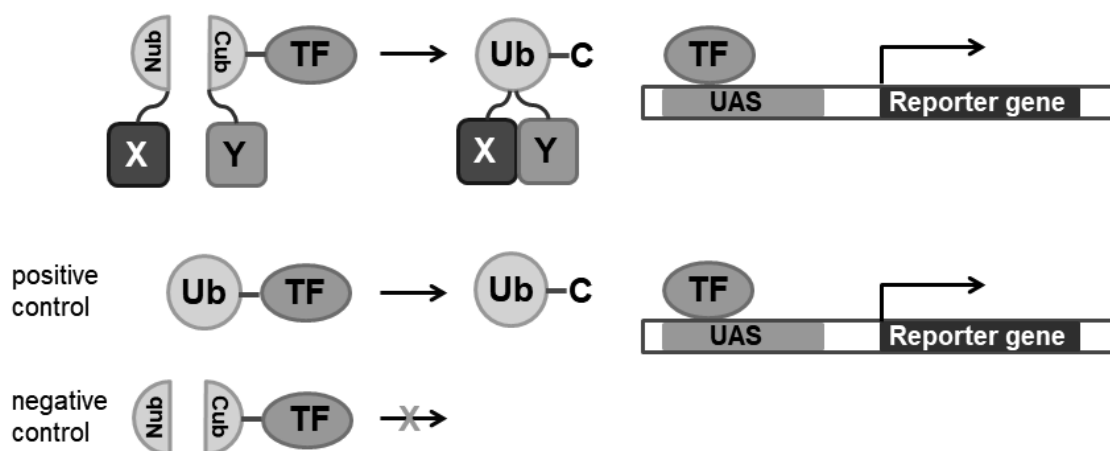

Fig. (9). The membrane yeast two hybrid (MYTH) assay is based on the complementation of two halves Nub and Cub of an ubiquitin (Ub) where the C terminal half is conjugated to a transcription factor (TF). Upon protein protein interaction ubiquitin is complemented and the transcription factor is cleaved by ubiquitin-specific proteases and reporter gene expression is triggered which can be measured. It is essential to include positive and negative controls.

ceptor. In addition, GHS-R1a is a constitutively highly active GPCR [279], which means that GHS-R1a is able to adopt an active conformation in the absence of agonists, thus increasing basal activity of G-protein- and effector system [282].

4.2. GHSR1a Dimers

4.2.1. D1R-GHSR1a Heterodimers

Jiang *et al.* demonstrated that ghrelin receptor amplifies dopamine-induced cAMP accumulation via D1R [283]. The coexpression of GHSR1a and D1R has been reported in the cortex, substantia nigra, midbrain, hippocampus and ventral tegmental areas [283]. Treatment with dopamine and ghrelin in cells expressing the two receptors revealed an amplification of D1R-associated cAMP signalling. This increase requires both dopamine and ghrelin, since the treatment with only ghrelin did not increase cAMP accumulation [283] and is usually seen in brain areas involved in mood, learning and memory.

The synergy between GHSR1a and D1R is due to switching of the ghrelin receptor signalling from $G\alpha_{q11}$-mediated to $G\alpha_{i/o}$-mediated, which is a G-protein that is not normally coupled to neither of these receptors. Additionally, GHSR1a

as well as D1R agonists can induce co-internalization, terminating the effect of the partner [284]. The molecular mechanism of this synergy was proposed by *Jiang et al.* which is based on G-Protein activation by D1R that involves dissociation of $G\alpha_s$ and $G\beta/\gamma$ subunits. The latter only plays a stimulatory role in the presence of $G\alpha_s$. As stated before the formation of D1R-GHSR1a heterodimer causes GHSR1a to switch from G_{q11} to $G_{i/o}$. The dissociation of the $G\beta/\gamma$ subunit from the $G\alpha_i$, which would inhibit cAMP accumulation, switches to a stimulatory to $G\alpha_s$ [283].

4.2.2. D2R-GHSR1a Heterodimers

FRET experiments demonstrated dimerization between D2 and GHSR1a in hippocampal cultures [145], which alters intracellular signalling, resulting in a rapid increase of Ca^{2+} levels upon dopamine agonist administration. Kern *et al.*, through inhibitors of second messenger signalling molecules, was able to the detect the pathway which is responsible for this effect. Dopamine agonist coupling to dimer leads to PLC-dependent activation through $G\alpha_i$ coupling, that ultimately leads to release of Ca^{2+} via IP3 receptor in endoplasmic reticulum [145]. Additionally, Kern and colleagues demonstrated that this effect was independent of GHSR1a

high constitutive activity. Behavioural tests demonstrated that administration of a D2R-selective agonist induces a suppression of food intake in wild-type and ghrelin KO mice, but has no effect in GHSR KO mice, suggesting that anorexigenic effects of D2R agonists depend on GHSR. This provides an evidence of a central role for GHSR1a in the absence of ghrelin [145]. The same group observed a desensitization within D2R-GHSR heterodimer since pretreatment with GHSR agonists, greatly attenuated the synergic effect. This desensitization may occur by dissociation or cointernalization of the dimer [145].

4.2.3. 5-HT$_{2C}$ – GHRS1a Heterodimers

The 5-HT$_{2C}$ has been recently identified partner of GHSR1a for dimerization [285–290]. This receptor signals through the same pathway as ghrelin receptor, Gα_q, leading to Ca^{2+} accumulation [284]. However, stimulation of 5-HT$_{2C}$ leads to a decrease in food intake and adiposity [291]. Schellekens *et al.* were able to confirm the existence of this dimer as well as his behaviour. When pretreated with an inverse agonist of GHSR1a, SP-analog, cells co-expressing the two receptors show cross-sensitization to the 5-HT$_{2C}$ response [284]. These cells show a decrease of Ca^{2+} accumulation when treated with ghrelin or a synthetic agonist (MK-0677). The effect is restored when co-treated with a 5-HT$_{2C}$ antagonist. Also, exposure to ghrelin led to an increased dimer co-internalization. Authors concluded that 5-HT$_{2C}$ dimerization is able to reduce ghrelin signalling and may reduce feeding behaviour [284].

4.2.4. Targeting GHRS1a Heterodimers

Identification of GHSR heterodimers allows the development of new treatments or updates to the current treatments that would bring new hope to chronic psychiatric and metabolic conditions [148]. Ghrelin receptor action was shown to protect substantia nigra pars compact from MPTP-induced degeneration [292]. This indicates that D1R-GHSR1a can be a target to increase the remaining dopaminergic signalling and to retard the Parkinson disease's progression [148]. There is evidence that suggests that ghrelin receptor is associated with increase of reward-seeking behaviours [293–297]. So targeting this heterodimer with an antagonist of GHSR1a may aid in drug addiction, since blockade of the receptor would result in decreased reward seeking behaviour [148]. Patients suffering from schizophrenia are often treated with 5-HT2C agonists. This type of antipsychotics exhibits less side effects [298] while decreasing the ghrelin-induce food intake, suggesting cross-action within the dimer [299]. Inverse agonist of GHSR was shown to enhance 5-HT2C signalling, indicating that inverse agonists and probably antagonists may increase the effectiveness of the treatment of schizophrenia [148].

CONCLUSION

Experimental techniques which have been developed to study protein-protein interactions not only identified constituents of protein complexes but also introduced the notion that the constituents "talk" to each other, that is to say, they modulate each other's function in the complex. Therefore, dimerization/oligomerization phenomenon must be explicitly and thoroughly considered in order to develop powerful therapeutics that possess fewer/no toxicological side effects-in particular, in the field of GPCRs, which constitutes one of the most studied drug targets for treating many crucial diseases (cancer, Parkinson's disease, Alzheimer's disease, schizophrenia, obesity, etc.), and .

We presented here an overview of widely used computational and experimental methods developed for characterization of dimer/oligomer interfaces as well as their dynamics. Due to the fact that nearly all assays have their own limitations, the best approach to overcome this would be to use various relevant experimental techniques in combination and also to complement them by data obtained from *in silico* methods in order to have a holistic understanding of GPCR oligomerization and so to modulate the function of resulting complexes. In particular, fluorescence-based methods like BRET, FRET, BiFC are *e.g.* not suitable for native tissues, but especially more sophisticated methods such as TR-FRET or TIRFM are very well suited for detecting dynamic and transient interactions, while for native tissues, proximity ligation assays can be applied. Since knowledge of the interfaces is of utmost importance for targeting hot spots by modulators, it is necessary to develop more sensitive and accurate assays which can work in model systems that resemble living organisms. In this respect, microarrays have been emerged to be suitable for detecting specific interactions, but are so far limited to interactions between proteins and single domains. For transient interactions, on the other hand, MS-based methods and BioID might be used but still remains to be tested for studying GPCR dimers. When considering computational methods, CG molecular dynamics simulations can be preferred over all atomistic ones due to large system sizes and long length scales. In particular, coarser representation of the system can be used to equilibrate lipid molecules around the protein. Consequently, the CG representation can be switched back to atomistic one to study fine details of the structure and dynamics of the system. Here, it is important to emphasize that one must be careful when studying with parameters regarding system dynamics as CG force fields inherently speed up the dynamics due to usage of larger time steps.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

REFERENCES

[1]   Fuxe, K.; O. Borroto-Escuela, D.; Marcellino, D.; Romero-Fernandez, W.; Frankowska, M.; Guidolin, D.; Filip, M.; Ferraro, L.; Woods, a. S.; Tarakanov, a.; Ciruela, F.; F. Agnati, L.; Tanganelli, S. GPCR Heteromers and their Allosteric Receptor-Receptor Interactions. *Curr. Med. Chem.* **2012,** *19* (3), 356–363.

[2]   Latek, D.; Modzelewska, A.; Trzaskowski, B.; Palczewski, K.; Filipek, S. G protein-coupled receptors-recent advances. *Acta Biochim. Pol.* **2012,** *59* (4), 515–529.

[3]   Sensoy, O.; Weinstein, H. A mechanistic role of Helix 8 in GPCRs: Computational modeling of the dopamine D2 receptor interaction with the GIPC1-PDZ-domain. *Biochim. Biophys. Acta - Biomembr.* **2015**, *1848* (4), 976–983.

[4]   Han, Y.; Moreira, I. S.; Urizar, E.; Weinstein, H.; Javitch, J. A. Allosteric communication between protomers of dopamine Class A GPCR dimers modulates activation. *Nat. Chem. Biol.* **2009**, *5* (9), 688–695.

[5]   Lohse, M. J.; Hoffmann, C. Arrestins - Pharmacology and Therapeutic Potential. *Arrestins - Pharmacology and Therapeutic Potential*; 2014; Vol. 219.

[6]   Maurice, P.; Guillaume, J. L.; Benleulmi-Chaachoua, A.; Daulat, A. M.; Kamal, M.; Jockers, R. GPCR-Interacting Proteins, Major Players of GPCR Function. *GPCR-Interacting Proteins, Major Players of GPCR Function*, 1st ed.; Elsevier Inc., 2011; Vol. 62.

[7]   Daulat, A. M.; Maurice, P.; Jockers, R. Tandem Affinity Purification and Identification of GPCR-Associated Protein Complexes. In *Receptor Signal Transduction Protocols: Third Edition, Methods in Molecular Biology*; 2011; Vol. 746, pp 399–409.

[8]   Ma, Z.; Du, L.; Li, M. Toward fluorescent probes for G-protein-coupled receptors (GPCRs). *J. Med. Chem.* **2014**, *57* (20), 8187–8203.

[9]   Bornert, O.; Møller, T. C.; Boeuf, J.; Candusso, M. P.; Wagner, R.; Martinez, K. L.; Simonin, F. Identification of a Novel Protein-Protein Interaction Motif Mediating Interaction of GPCR-Associated Sorting Proteins with G Protein-Coupled Receptors. *PLoS One* **2013**, *8* (2).

[10]   Snider, J.; Kotlyar, M.; Saraon, P.; Yao, Z.; Jurisica, I.; Stagljar, I. Fundamentals of protein interaction network mapping. *Mol. Syst. Biol.* **2015**, *11* (12), 848–848.

[11]   Sokolina, K.; Kittanakom, S.; Snider, J.; Kotlyar, M.; Maurice, P.; Gandía, J.; Benleulmi-Chaachoua, A.; Tadagaki, K.; Oishi, A.; Wong, V.; Malty, R. H.; Deineko, V.; Aoki, H.; Amin, S.; Yao, Z.; Morató, X.; Otasek, D.; Kobayashi, H.; Menendez, J.; Auerbach, D.; Angers, S.; Pržulj, N.; Bouvier, M.; Babu, M.; Ciruela, F.; Jockers, R.; Jurisica, I.; Stagljar, I. Systematic protein–protein interaction mapping for clinically relevant human GPCRs. *Mol. Syst. Biol.* **2017**, *13* (3), 918.

[12]   De Filippo, E.; Schiedel, A. C.; Manga, P. Interaction between G Protein-Coupled Receptor 143 and Tyrosinase: Implications for Understanding Ocular Albinism Type 1. *J. Invest. Dermatol.* **2017**, *137* (2), 457–465.

[13]   Oeljeklaus, S.; Meyer, H. E.; Warscheid, B. New dimensions in the study of protein complexes using quantitative mass spectrometry. *FEBS Lett.* **2009**, *583* (11), 1674–1683.

[14]   Sensoy, O.; Almeida, J. G.; Shabbir, J.; Moreira, I. S.; Morra, G. Computational modeling – a way to understand GPCR structure, function and mechanism. *Methods Cell Biol.* **2017**, 205–245.

[15]   Moreira, I. S.; Shi, L.; Freyberg, Z.; Ericksen, S. S.; Weinstein, H.; Javitch, J. A. Structural Basis of Dopamine Receptor Activation. In *The Dopamine Receptors*; Neve, K. A., Ed.; Humana/Springer, 2010; pp 47–73.

[16]   Munk, C.; Isberg, V.; Mordalski, S.; Harpsøe, K.; Rataj, K.; Hauser, A. S.; Kolb, P.; Bojarski, A. J.; Vriend, G.; Gloriam, D. E. GPCRdb: the G protein-coupled receptor database – an introduction. *British Journal of Pharmacology*. July 2016, pp 2195–2207.

[17]   Pándy-Szekeres, G.; Munk, C.; Tsonkov, T. M.; Mordalski, S.; Harpsøe, K.; Hauser, A. S.; Bojarski, A. J.; Gloriam, D. E. GPCRdb in 2018: adding GPCR structure models and ligands. *Nucleic Acids Res.* **2017**, *46* (D1), D440–D446.

[18]   Torrie, G. M.; Valleau, J. P. Nonphysical sampling distributions in Monte Carlo free-energy estimation: Umbrella sampling. *J. Comput. Phys.* **1977**, *23* (2), 187–199.

[19]   Grubmüller, H.; Heymann, B.; Tavan, P. Ligand Binding: Molecular Mechanics Calculation of the Streptavidin-Biotin Rupture Force. *Science (80-.).* **1996**, *271* (5251), 997–999.

[20]   Isralewitz, B.; Gao, M.; Schulten, K. Steered molecular dynamics and mechanical functions of proteins. *Current Opinion in Structural Biology*. Elsevier Current Trends April 2001, pp 224–230.

[21]   Sensoy, O.; Moreira, I. S.; Morra, G. Understanding the Differential Selectivity of Arrestins toward the Phosphorylation State of the Receptor. *ACS Chem. Neurosci.* **2016**, *7* (9), 1212–1224.

[22]   Marrink, S. J.; Risselada, H. J.; Yefimov, S.; Tieleman, D. P.; de Vries, A. H. The MARTINI Force Field: Coarse Grained Model for Biomolecular Simulations. *J. Phys. Chem. B* **2007**, *111* (27), 7812–7824.

[23]   Periole, X.; Huber, T.; Marrink, S. J.; Sakmar, T. P. G protein-coupled receptors self-assemble in dynamics simulations of model bilayers. *J. Am. Chem. Soc.* **2007**, *129* (33), 10126–10132.

[24]   Baron, R.; Trzesniak, D.; De Vries, A. H.; Elsener, A.; Marrink, S. J.; Van Gunsteren, W. F. Comparison of thermodynamic properties of coarse-grained and atomic-level simulation models. *ChemPhysChem* **2007**, *8* (3), 452–461.

[25]   Marrink, S. J.; de Vries, A. H.; Mark, A. E. Coarse Grained Model for Semiquantitative Lipid Simulations. *J. Phys. Chem. B* **2004**, *108* (2), 750–760.

[26]   Provasi, D.; Boz, M. B.; Johnston, J. M.; Filizola, M. Preferred Supramolecular Organization and Dimer Interfaces of Opioid Receptors from Simulated Self-Association. *PLOS Comput. Biol.* **2015**, *11* (3), e1004148.

[27]   Wassenaar, T. A.; Pluhackova, K.; Moussatova, A.; Sengupta, D.; Marrink, S. J.; Tieleman, D. P.; Böckmann, R. A. High-Throughput Simulations of Dimer and Trimer Assembly of Membrane Proteins. The DAFT Approach. *J. Chem. Theory Comput.* **2015**, *11* (5), 2278–2291.

[28]   Pluhackova, K.; Gahbauer, S.; Kranz, F.; Wassenaar, T. A.; Böckmann, R. A. Dynamic Cholesterol-Conditioned Dimerization of the G Protein Coupled Chemokine Receptor Type 4. *PLOS Comput. Biol.* **2016**, *12* (11), e1005169.

[29]   Han, J.; Pluhackova, K.; Wassenaar, T. A.; Böckmann, R. A. Synaptobrevin Transmembrane Domain Dimerization Studied by Multiscale Molecular Dynamics Simulations. *Biophys. J.* **2015**, *109* (4), 760–771.

[30]   Periole, X.; Knepp, A. M.; Sakmar, T. P.; Marrink, S. J.; Huber, T. Structural Determinants of the Supramolecular Organization of G Protein-Coupled Receptors in Bilayers. *J. Am. Chem. Soc.* **2012**, *134* (26), 10959–10965.

[31]   Stark, A. C.; Andrews, C. T.; Elcock, A. H. Toward Optimized Potential Functions for Protein–Protein Interactions in Aqueous Solutions: Osmotic Second Virial Coefficient Calculations Using the MARTINI Coarse-Grained Force Field. *J. Chem. Theory Comput.* **2013**, *9* (9), 4176–4185.

[32]   Johnston, J. M.; Wang, H.; Provasi, D.; Filizola, M. Assessing the Relative Stability of Dimer Interfaces in G Protein-Coupled Receptors. *PLoS Comput. Biol.* **2012**, *8* (8), e1002649.

[33]   Domański, J.; Hedger, G.; Best, R. B.; Stansfeld, P. J.; Sansom, M. S. P. Convergence and Sampling in Determining Free Energy Landscapes for Membrane Protein Association. *J. Phys. Chem. B* **2017**, *121* (15), 3364–3375.

[34]   Kumar, S.; Rosenberg, J. M.; Bouzida, D.; Swendsen, R. H.; Kollman, P. A. THE weighted histogram analysis method for free-energy calculations on biomolecules. I. The method. *J. Comput. Chem.* **1992**, *13* (8), 1011–1021.

[35]   Efron, B.; Tibshirani, R. J. An introduction to the bootstrap. *Refrig. Air Cond.* **1993**, *57* (57), 436.

[36]   Cuendet, M. A.; Michielin, O. Protein-protein interaction investigated by steered molecular dynamics: The TCR-pMHC complex. *Biophys. J.* **2008**, *95* (8), 3575–3590.

[37]   Jarzynski, C. Nonequilibrium equality for free energy differences. *Phys. Rev. Lett.* **1997**, *78* (14), 2690–2693.

[38]   Gore, J.; Ritort, F.; Bustamante, C. Bias and error in estimates of equilibrium free-energy differences from nonequilibrium measurements. *Proc. Natl. Acad. Sci.* **2003**, *100* (22), 12564–12569.

[39]   Sensoy, O.; Atilgan, A. R.; Atilgan, C. FbpA iron storage and release are governed by periplasmic microenvironments. *Phys. Chem. Chem. Phys.* **2017**, *19* (8), 6064–6075.

[40]   Ostrom, R. S.; Insel, P. A. The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. *Br. J. Pharmacol.* **2004**, *143* (2), 235–245.

[41]   Ostrom, R. S.; Post, S. R.; Insel, P. A. Stoichiometry and Compartmentation in G Protein-Coupled Receptor Signaling: Implications for Therapeutic Interventions Involving Gs. *J. Pharmacol. Exp. Ther.* **2000**, *294* (2).

[42]   Goddard, A. D.; Dijkman, P. M.; Adamson, R. J.; Watts, A. Lipid-Dependent GPCR Dimerization. *Methods Cell Biol.* **2013**, *117*, 341–357.

[43]   Villar, V. A. M.; Cuevas, S.; Zheng, X.; Jose, P. A. Localization and signaling of GPCRs in lipid rafts. In *Methods in Cell Biology*; Academic Press, 2016; Vol. 132, pp 3–23.

[44]   Insel, P. A.; Head, B. P.; Patel, H. H.; Roth, D. M.; Bundey, R. A.; Swaney, J. S. Compartmentation of G-protein-coupled receptors and their signalling components in lipid rafts and caveolae. *Biochem. Soc. Trans.* **2005**, *33* (Pt 5), 1131–1134.

[45]   Jafurulla, M.; Tiwari, S.; Chattopadhyay, A. Identification of cholesterol recognition amino acid consensus (CRAC) motif in G-protein coupled receptors. *Biochem. Biophys. Res. Commun.* **2011**, *404* (1), 569–573.

[46]   Gahbauer, S.; Böckmann, R. A. Membrane-mediated oligomerization of G protein coupled receptors and its implications for GPCR function. *Front. Physiol.* **2016**, *7* (OCT), 1–17.

[47]   Gawrisch, K.; Soubias, O.; Mihailescu, M. Insights from biophysical studies on the role of polyunsaturated fatty acids for function of G-protein coupled membrane receptors. *Prostaglandins Leukot. Essent. Fat. Acids* **2008**, *79* (3–5), 131–134.

[48]   Mitchell, D. C.; Litman, B. J. Molecular Order and Dynamics in Bilayers Consisting of Highly Polyunsaturated Phospholipids. *Biophys. J.* **1998**, *74* (2), 879–891.

[49]   Arango-Lievano, M.; Sensoy, O.; Borie, A.; Corbani, M.; Guillon, G.; Sokoloff, P.; Weinstein, H.; Jeanneteau, F. A GIPC1-Palmitate Switch Modulates Dopamine Drd3 Receptor Trafficking and Signaling. *Mol. Cell. Biol.* **2016**, *36* (6), 1019–1031.

[50]   Killian, J. A. Hydrophobic mismatch between proteins and lipids in membranes. *Biochim. Biophys. Acta - Rev. Biomembr.* **1998**, *1376* (3), 401–415.

[51]   Kaczor, A. A.; Rutkowska, E.; Bartuzi, D.; Targowska-Duda, K. M.; Matosiuk, D.; Selent, J. Computational methods for studying G proteincoupled receptors (GPCRs). *Computational methods for studying G proteincoupled receptors (GPCRs)*; Elsevier Ltd, 2016; Vol. 132.

[52]   Kufareva, I.; Katritch, V.; Biggin, P.; Kim, M.; Park, K.; Jung, S. W.; Cho, A. E.; Sands, Z. A.; Ostopovici-Halip, L.; Bologa, C. G.; Norn, C.; Brylinski, M.; Skolnick, J.; Keränen, H.; Lenselink, B. E.; Van Westen, G.; Overington, J. P.; De Terán, H. G.; Isberg, V.; Fidom, K. M.; Lehto, T. M.; Gloriam, D. E.; Ghosh, A.; Sonavane, U.; Joshi, R.; Xia, J.; Hsieh, J. H.; Zhang, L.; Wang, X. S.; Vogel, H.; Yuan, S.; Feng, X.; Chen, M.; Ambia, J.; Barth, P.; Gageat, C.; Stepniewski, M.; Xhaard, H.; Kelm, S.; Pitt, W. R.; Shi, J.; Larsen, A.; Li, H.; Wagner, J.; Bhattacharya, S.; Vaidehi, N.; Kanou, K.; Cvicek, V.; Kim, S. K.; Trzaskowski, B.; Goddard, W. A.; Abrol, R.; Selvam, B.; Tikhonova, I. G.; Cuzzolin, A.; Sabbadin, D.; Ciancetta, A.; Moro, S.; Freyd, T.; Gabrielsen, M.; Kristiansen, K.; Sylte, I.; Gaffney, K. J.; Petasis, N. A.; Latek, D.; Bajda, M.; Młynarczyk, K.; Filipek, S.; López, J.; Kuiper, M.; Beuming, T.; Perez-Aguilar, J. M.; Wang, R. Y. R.; Park, H.; Greisen, P.; Song, Y.; DiMaio, F.; Baker, D.; Shin, W. H.; Heo, L.; Lee, G. R.; Seok, C.; Yang, J.; Zhang, Y.; Ponassi, M.; Rosano, C.; Cheremovskiy, G.; Grudinin, S.; Chaudhari, R.; Heim, A. J.; Li, Z.; Lv, Q.; Grigorov, M. G.; Hu, X.; Sun, H.; Shen, M.; Southall, N.; Jadhav, A.; Rodríguez, D.; Ranganathan, A.; Carlsson, J.; Najmanovich, R.; Durdagi, S.; De March, C.; Diharce, J.; Golebiowski, J.; Antonczak, S.; Fiorucci, S.; Nguyen, E.; Meiler, J.; Gutcaits, A.; Marti-Solano, M.; Pastor, M.; Selent, J.; Stevens, R. C.; Abagyan, R. Advances in GPCR modeling evaluated by the GPCR Dock 2013 assessment: Meeting new challenges. *Structure* **2014**, *22* (8), 1120–1139.

[53]   Latek, D.; Bajda, M.; Filipek, S. A Hybrid Approach to Structure and Function Modeling of G Protein-Coupled Receptors. *J. Chem. Inf. Model.* **2016**, *56* (4), 630–641.

[54]   Lin, H.; Sassano, M. F.; Roth, B. L.; Shoichet, B. K. A pharmacological organization of G protein–coupled receptors. *Nat. Methods* **2013**, *10* (2), 140–146.

[55]   Kaczor, A. A.; Selent, J.; Poso, A. Structure-Based Molecular Modeling Approaches to GPCR Oligomerization. *Methods Cell Biol.* **2013**, *117*, 91–104.

[56]   Baker, F. N.; Porollo, A. CoeViz: a web-based tool for coevolution analysis of protein residues. *BMC Bioinformatics* **2016**, *17* (1), 119.

[57]   Chen, Z.; Meyer, W.; Rappert, S.; Sun, J.; Zeng, A.-P. Coevolutionary analysis enabled rational deregulation of allosteric enzyme inhibition in Corynebacterium glutamicum for lysine production. *Appl. Environ. Microbiol.* **2011**, *77* (13), 4352–4360.

[58]   Yin, C.; Yau, S. S.-T. A coevolution analysis for identifying protein-protein interactions by Fourier transform. *PLoS One* **2017**, *12* (4), e0174862.

[59]   Nichols, S. E.; Hernández, C. X.; Wang, Y.; McCammon, J. A. Structure-based network analysis of an evolved G protein-coupled receptor homodimer interface. *Protein Sci.* **2013**, *22* (6), 745–754.

[60]   Almeida, J. G.; Preto, A. J.; Koukos, P. I.; Bonvin, A. M. J. J.; Moreira, I. S. Membrane proteins structures: A review on computational modeling tools. *Biochim. Biophys. Acta - Biomembr.* **2017**, *1859* (10), 2021–2039.

[61]   Zaki, N.; Bouktif, S.; Lazarova-Molnar, S. A Combination of Compositional Index and Genetic Algorithm for Predicting Transmembrane Helical Segments. *PLoS One* **2011**, *6* (7), e21821.

[62]   Hayat, M.; Khan, A. WRF-TMH: Predicting transmembrane helix by fusing composition index and physicochemical properties of amino acids. *Amino Acids* **2013**, *44* (5), 1317–1328.

[63]   Park, Y.; Hayat, S.; Helms, V. Prediction of the burial status of transmembrane residues of helical membrane proteins. *BMC Bioinformatics* **2007**, *8* (1), 302.

[64]   Zheng Yuan; Zhang, F.; Davis, M. J.; Bodén, M.; Teasdale, R. D. Predicting the Solvent Accessibility of Transmembrane Residues from Protein Sequence. *J. Proteome Res.* **2006**, *5* (5), 1063–1070.

[65]   Fuchs, A.; Kirschner, A.; Frishman, D. Prediction of helix-helix contacts and interacting helices in polytopic membrane proteins using neural networks. *Proteins Struct. Funct. Bioinforma.* **2009**, *74* (4), 857–871.

[66]   Ahmad, S.; Singh, Y.; Paudel, Y.; Mori, T.; Sugita, Y.; Mizuguchi, K. Integrated prediction of one-dimensional structural features and their relationships with conformational flexibility in helical membrane proteins. *BMC Bioinformatics* **2010**, *11* (1), 533.

[67]   Hollup, S.; Salensminde, G.; Reuter, N. WEBnm@: a web application for normal mode analyses of proteins. *BMC Bioinformatics* **2005**, *6* (1), 52.

[68]   Niv, M. Y.; Filizola, M. Influence of oligomerization on the dynamics of G-protein coupled receptors as assessed by normal mode analysis. *Proteins Struct. Funct. Bioinforma.* **2008**, *71* (2), 575–586.

[69]   Moreira, I. S. Structural features of the G-protein/GPCR interactions. *Biochim. Biophys. Acta - Gen. Subj.* **2014**, *1840* (1), 16–33.

[70]   Haliloglu, T.; Bahar, I. Adaptability of protein structures to enable functional interactions and evolutionary implications. *Current Opinion in Structural Biology*. Elsevier Current Trends December 2015, pp 17–23.

[71]   Tirion, M. M. Large amplitude elastic motions in proteins from a single-parameter, atomic analysis. *Phys. Rev. Lett.* **1996**, *77* (9), 1905–1908.

[72]   Micheletti, C.; Carloni, P.; Maritan, A. Accurate and Efficient Description of Protein Vibrational Dynamics: Comparing Molecular Dynamics and Gaussian Models. *Proteins Struct. Funct. Genet.* **2004**, *55* (3), 635–645.

[73]   Kolan, D.; Fonar, G.; Samson, A. O. Elastic network normal mode dynamics reveal the GPCR activation mechanism. *Proteins Struct. Funct. Bioinforma.* **2014**, *82* (4), 579–586.

[74]   Raimondi, F.; Seeber, M.; De Benedetti, P. G.; Fanelli, F. Mechanisms of inter- and intramolecular communication in GPCRs and G proteins. *J. Am. Chem. Soc.* **2008**, *130* (13), 4310–4325.

[75]   Shan, J.; Khelashvili, G.; Mondal, S.; Mehler, E. L.; Weinstein, H. Ligand-dependent conformations and dynamics of the serotonin 5-HT2A receptor determine its activation and membrane-driven oligomerization properties. *PLoS Comput. Biol.* **2012**, *8* (4), e1002473.

[76]   Perez-Aguilar, J. M.; Shan, J.; Levine, M. V.; Khelashvili, G.; Weinstein, H. A functional selectivity mechanism at the serotonin-2A GPCR involves ligand-dependent conformations of intracellular loop 2. *J. Am. Chem. Soc.* **2014**, *136* (45), 16044–16054.

[77]   Vijayabaskar, M. S.; Vishveshwara, S. Interaction energy based protein structure networks. *Biophys. J.* **2010**, *99* (11), 3704–3715.

[78]   Chennubhotla, C.; Bahar, I. Signal propagation in proteins and relation to equilibrium fluctuations. *PLoS Comput. Biol.* **2007**, *3* (9), 1716–1726.

[79]   Morra, G.; Verkhivker, G.; Colombo, G. Modeling signal propagation mechanisms and ligand-based conformational dynamics of the Hsp90 molecular chaperone full-length dimer. *PLoS Comput. Biol.* **2009**, *5* (3), e1000323.

[80]   LeVine, M. V.; Weinstein, H. NbIT - A New Information Theory-Based Analysis of Allosteric Mechanisms Reveals Residues that Underlie Function in the Leucine Transporter LeuT. *PLoS Comput. Biol.* **2014,** *10* (5), e1003603.

[81]   Borroto-Escuela, D. O.; Carlsson, J.; Ambrogini, P.; Narváez, M.; Wydra, K.; Tarakanov, A. O.; Li, X.; Millón, C.; Ferraro, L.; Cuppini, R.; Tanganelli, S.; Liu, F.; Filip, M.; Diaz-Cabiale, Z.; Fuxe, K. Understanding the Role of GPCR Heteroreceptor Complexes in Modulating the Brain Networks in Health and Disease. *Front. Cell. Neurosci.* **2017,** *11* (February), 1–20.

[82]   Fanelli, F.; Felline, A. Dimerization and ligand binding affect the structure network of A 2A adenosine receptor. *Biochim. Biophys. Acta - Biomembr.* **2011,** *1808* (5), 1256–1266.

[83]   Vishveshwara, S.; Brinda, K. V.; Kannan, N. Protein Structure: Insights From Graph Theory. *J. Theor. Comput. Chem.* **2002,** *1* (1), 187–211.

[84]   Borroto-Escuela, D. O.; Brito, I.; Romero-Fernandez, W.; Di Palma, M.; Oflijan, J.; Skieterska, K.; Duchou, J.; Van Craenenbroeck, K.; Suárez-Boomgaard, D.; Rivera, A.; Guidolin, D.; Agnati, L. F.; Fuxe, K. The G protein-coupled receptor heterodimer network (GPCR-HetNet) and its hub components. *Int. J. Mol. Sci.* **2014,** *15* (5), 8570–8590.

[85]   Casciari, D.; Seeber, M.; Fanelli, F. Quaternary structure predictions of transmembrane proteins starting from the monomer: A docking-based approach. *BMC Bioinformatics* **2006,** *7* (1), 340.

[86]   Lange, O. F.; Grubmüller, H. Generalized correlation for biomolecular dynamics. *Proteins Struct. Funct. Genet.* **2006,** *62* (4), 1053–1061.

[87]   Lockless, S. W.; Ranganathan, R. Evolutionarily conserved pathways of energetic connectivity in protein families. *Science (80-.).* **1999,** *286* (5438), 295–299.

[88]   Young, L.; Jernigan, R. L.; Covell, D. G. A role for surface hydrophobicity in protein‐protein recognition. *Protein Sci.* **1994,** *3* (5), 717–729.

[89]   Fernandez, A.; Scheraga, H. A. Insufficiently dehydrated hydrogen bonds as determinants of protein interactions. *Proc. Natl. Acad. Sci.* **2003,** *100* (1), 113–118.

[90]   Dill, K. A. Dominant Forces in Protein Folding. *Biochemistry* **1990,** *29* (31), 7133–7155.

[91]   Gurung, A. B.; Bhattacharjee, A.; Ajmal Ali, M.; Al-Hemaid, F.; Lee, J. Binding of small molecules at interface of protein–protein complex – A newer approach to rational drug design. *Saudi Journal of Biological Sciences*. Elsevier February 2017, pp 379–388.

[92]   Thorn, K. S.; Bogan, A. A. ASEdb: a database of alanine mutations and their effects on the free energy of binding in protein interactions. *Bioinformatics* **2001,** *17* (3), 284–285.

[93]   Moreira, I. S.; Fernandes, P. A.; Ramos, M. J. Hot spots-A review of the protein-protein interface determinant amino-acid residues. *Proteins Struct. Funct. Bioinforma.* **2007,** *68* (4), 803–812.

[94]   David, A.; Razali, R.; Wass, M. N.; Sternberg, M. J. E. Protein-protein interaction sites are hot spots for disease-associated nonsynonymous SNPs. *Hum. Mutat.* **2012,** *33* (2), 359–363.

[95]   Hu, G.; Xiao, F.; Li, Y.; Li, Y.; Vongsangnak, W. Protein–Protein Interface and Disease: Perspective from Biomolecular Networks. Springer, Cham, 2016; pp 57–74.

[96]   Fischer, T. B.; Arunachalam, K. V.; Bailey, D.; Mangual, V.; Bakhru, S.; Russo, R.; Huang, D.; Paczkowski, M.; Lalchandani, V.; Ramachandra, C.; Ellison, B.; Galer, S.; Shapley, J.; Fuentes, E.; Tsai, J. The binding interface database (BID): a compilation of amino acid hot spots in protein interfaces. *Bioinformatics* **2003,** *19* (11), 1453–1454.

[97]   Kumar, M. D. S.; Gromiha, M. M. PINT: Protein-protein Interactions Thermodynamic Database. *Nucleic Acids Res.* **2006,** *34* (Database issue), D195-8.

[98]   Moal, I. H.; Fernández-Recio, J. SKEMPI: a Structural Kinetic and Energetic database of Mutant Protein Interactions and its use in empirical models. *Bioinformatics* **2012,** *28* (20), 2600–2607.

[99]   Moreira, I. S.; Koukos, P. I.; Melo, R.; Almeida, J. G.; Preto, A. J.; Schaarschmidt, J.; Trellet, M.; Gümüş, Z. H.; Costa, J.; Bonvin, A. M. J. J. SpotOn: High Accuracy Identification of Protein-Protein Interface Hot-Spots. *Sci. Rep.* **2017,** *7* (1), 8007.

[100]   Moreira, I. S.; Fernandes, P. A.; Ramos, M. J. Computational alanine scanning mutagenesis—An improved methodological approach. *J. Comput. Chem.* **2007,** *28* (3), 644–654.

[101]   Melo, R.; Fieldhouse, R.; Melo, A.; Correia, J. D. G.; Cordeiro, M. N. D. S.; Gümüş, Z. H.; Costa, J.; Bonvin, A. M. J. J.; Moreira, I. S. A Machine Learning Approach for Hot-Spot Detection at Protein-Protein Interfaces. *Int. J. Mol. Sci.* **2016,** *17* (8), 1215.

[102]   Grosdidier, S.; Fernández-Recio, J. Identification of hot-spot residues in protein-protein interactions by computational docking. *BMC Bioinformatics* **2008,** *9* (1), 447.

[103]   Bromberg, Y.; Rost, B. Comprehensive in silico mutagenesis highlights functionally important residues in proteins. In *Bioinformatics*; Oxford University Press, 2008; Vol. 24, pp i207–i212.

[104]   Munteanu, C. R.; Pimenta, A. C.; Fernandez-Lozano, C.; Melo, A.; Cordeiro, M. N. D. S.; Moreira, I. S. Solvent accessible surface area-based hot-spot detection methods for protein-protein and protein-nucleic acid interfaces. *J. Chem. Inf. Model.* **2015,** *55* (5), 1077–1086.

[105]   Hu, S.-S.; Chen, P.; Wang, B.; Li, J. Protein binding hot spots prediction from sequence only by a new ensemble learning method. *Amino Acids* **2017,** *49* (10), 1773–1785.

[106]   Cukuroglu, E.; Engin, H. B.; Gursoy, A.; Keskin, O. Hot spots in protein-protein interfaces: Towards drug discovery. *Prog. Biophys. Mol. Biol.* **2014,** *116* (2–3), 165–173.

[107]   Xue, L. C.; Dobbs, D.; Bonvin, A. M. J. J.; Honavar, V. Computational prediction of protein interfaces: A review of data driven methods. *FEBS Letters*. November 2015, pp 3516–3526.

[108]   Moreira, I. S. The Role of Water Occlusion for the Definition of a Protein Binding Hot-Spot. *Curr. Top. Med. Chem.* **2015,** *15* (20), 2068–2079.

[109]   Guerois, R.; Nielsen, J. E.; Serrano, L. Predicting changes in the stability of proteins and protein complexes: A study of more than 1000 mutations. *J. Mol. Biol.* **2002,** *320* (2), 369–387.

[110]   Zhu, X.; Mitchell, J. C. KFC2: A knowledge-based hot spot prediction method based on interface solvation, atomic density, and plasticity features. *Proteins Struct. Funct. Bioinforma.* **2011,** *79* (9), 2671–2683.

[111]   Tuncbag, N.; Keskin, O.; Gursoy, A. HotPoint: hot spot prediction server for protein interfaces. *Nucleic Acids Res.* **2010,** *38* (Web Server), W402–W406.

[112]   Guney, E.; Tuncbag, N.; Keskin, O.; Gursoy, A. HotSprint: database of computational hot spots in protein interfaces. *Nucleic Acids Res.* **2007,** *36* (Database), D662–D666.

[113]   Cukuroglu, E.; Gursoy, A.; Keskin, O. HotRegion: a database of predicted hot spot clusters. *Nucleic Acids Res.* **2012,** *40* (D1), D829–D833.

[114]   Hu, C. D.; Kerppola, T. K. Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. *Nat Biotechnol* **2003,** *21* (5), 539–545.

[115]   Ofran, Y.; Rost, B. Protein–Protein Interaction Hotspots Carved into Sequences. *PLoS Comput. Biol.* **2007,** *3* (7), e119.

[116]   Shulman-Peleg, A.; Shatsky, M.; Nussinov, R.; Wolfson, H. J. Spatial chemical conservation of hot spot interactions in protein-protein complexes. *BMC Biol.* **2007,** *5* (1), 43.

[117]   Shulman-Peleg, A.; Shatsky, M.; Nussinov, R.; Wolfson, H. J. MultiBind and MAPPIS: webservers for multiple alignment of protein 3D-binding sites and their interactions. *Nucleic Acids Res.* **2008,** *36* (Web Server), W260–W264.

[118]   Assi, S. A.; Tanaka, T.; Rabbitts, T. H.; Fernandez-Fuentes, N. PCRPi: Presaging Critical Residues in Protein interfaces, a new computational tool to chart hot spots in protein interfaces. *Nucleic Acids Res.* **2010,** *38* (6), e86–e86.

[119]   Koes, D. R.; Camacho, C. J. Small-molecule inhibitor starting points learned from protein–protein interaction inhibitor structure. *Bioinformatics* **2012,** *28* (6), 784–791.

[120]   Lise, S.; Buchan, D.; Pontil, M.; Jones, D. T. Predictions of Hot Spot Residues at Protein-Protein Interfaces Using Support Vector Machines. *PLoS One* **2011,** *6* (2), e16774.

[121]   Deng, L.; Zhang, Q. C.; Chen, Z.; Meng, Y.; Guan, J.; Zhou, S. PredHS: a web server for predicting protein–protein interaction hot spots by using structural neighborhood properties. *Nucleic Acids Res.* **2014,** *42* (W1), W290–W295.

[122]   Kim, D. E.; Chivian, D.; Baker, D. Protein structure prediction and analysis using the Robetta server. *Nucleic Acids Res.* **2004,** *32* (Web Server), W526–W531.

[123]   Sable, R.; Jois, S. Surfing the protein-protein interaction surface using docking methods: Application to the design of PPI inhibitors.

Molecules. Multidisciplinary Digital Publishing Institute June 2015, pp 11569–11603.

[124] Kitchen, D. B.; Decornez, H.; Furr, J. R.; Bajorath, J. Docking and scoring in virtual screening for drug discovery: methods and applications. *Nat. Rev. Drug Discov.* **2004**, *3* (11), 935–949.

[125] Falchi, F.; Caporuscio, F.; Recanatini, M. Structure-based design of small-molecule protein–protein interaction modulators: the story so far. *Future Med. Chem.* **2014**, *6* (3), 343–357.

[126] Wolber, G.; Langer, T. LigandScout: 3-D Pharmacophores Derived from Protein-Bound Ligands and Their Use as Virtual Screening Filters. *J. Chem. Inf. Model.* **2005**, *45* (1), 160–169.

[127] Dixon, S. L.; Smondyrev, A. M.; Rao, S. N. PHASE: A Novel Approach to Pharmacophore Modeling and 3D Database Searching. *Chem. Biol. Drug Des.* **2006**, *67* (5), 370–372.

[128] Neudert, G.; Klebe, G. *DSX* : A Knowledge-Based Scoring Function for the Assessment of Protein–Ligand Complexes. *J. Chem. Inf. Model.* **2011**, *51* (10), 2731–2745.

[129] Verdonk, M. L.; Cole, J. C.; Taylor, R. SuperStar: A knowledge-based approach for identifying interaction sites in proteins. *J. Mol. Biol.* **1999**, *289* (4), 1093–1108.

[130] Zerbe, B. S.; Hall, D. R.; Vajda, S.; Whitty, A.; Kozakov, D. Relationship between hot spot residues and ligand binding hot spots in protein-protein interfaces. *J. Chem. Inf. Model.* **2012**, *52* (8), 2236–2244.

[131] Mukherjee, P.; Desai, P.; Zhou, Y.-D.; Avery, M. Targeting the BH3 Domain Mediated Protein−Protein Interaction of Bcl-xL through Virtual Screening. *J. Chem. Inf. Model.* **2010**, *50* (5), 906–923.

[132] Mysinger, M. M.; Weiss, D. R.; Ziarek, J. J.; Gravel, S.; Doak, A. K.; Karpiak, J.; Heveker, N.; Shoichet, B. K.; Volkman, B. F. Structure-based ligand discovery for the protein-protein interface of chemokine receptor CXCR4. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (14), 5517–5522.

[133] Smith, J. S.; Lefkowitz, R. J.; Rajagopal, S. Biased signalling: from simple switches to allosteric microprocessors. *Nat. Rev. Drug Discov.* **2018**.

[134] Jansen, F.; Kalbe, B.; Scholz, P.; Fränzel, B.; Osterloh, M.; Wolters, D.; Hatt, H.; Neuhaus, E. M.; Osterloh, S. Biochemical Large-Scale Interaction Analysis of Murine Olfactory Receptors and Associated Signaling Proteins with Post-Synaptic Density 95, Drosophila Discs Large, Zona-Occludens 1 (PDZ) Domains. *Mol. Cell. Proteomics* **2015**, *14* (8), 2072–2084.

[135] Söhlemann, P.; Hekman, M.; Puzicha, M.; Buchen, C.; Lohse, M. J. Binding of Purified Recombinant β‐arrestin to Guanine‐Nucleotide‐Binding‐Protein‐Coupled Receptors. *Eur. J. Biochem.* **1995**, *232* (2), 464–472.

[136] Ferre, S.; Casado, V.; Devi, L. A.; Filizola, M.; Jockers, R.; Lohse, M. J.; Milligan, G.; Pin, J.-P.; Guitart, X. G Protein-Coupled Receptor Oligomerization Revisited: Functional and Pharmacological Perspectives. *Pharmacol. Rev.* **2014**, *66* (2), 413–434.

[137] Roux, K. J.; Kim, D. I.; Raida, M.; Burke, B. A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *J. Cell Biol.* **2012**, *196* (6), 801–810.

[138] Park, S.; Jiang, H.; Zhang, H.; Smith, R. G. Modification of ghrelin receptor signaling by somatostatin receptor-5 regulates insulin release. *Proc. Natl. Acad. Sci.* **2012**, *109* (46), 19003–19008.

[139] Rediger, A.; Piechowski, C. L.; Yi, C. X.; Tarnow, P.; Strotmann, R.; Grüters, A.; Krude, H.; Schöneberg, T.; Tschöp, M. H.; Kleinau, G.; Biebermann, H. Mutually opposite signal modulation by hypothalamic heterodimerization of ghrelin and melanocortin-3 receptors. *J. Biol. Chem.* **2011**, *286* (45), 39623–39631.

[140] Villoutreix, B. O.; Labbé, C.; Lagorce, D.; Laconde, G.; Sperandio, O. A leap into the chemical space of protein - protein interaction inhibitors. *Curr Pharm Des* **2012**, *18* (30), 4648–4667.

[141] Mary, S.; Fehrentz, J. A.; Damian, M.; Gaibelet, G.; Orcel, H.; Verdié, P.; Mouillac, B.; Martinez, J.; Marie, J.; Banères, J. L. Heterodimerization with its splice variant blocks the ghrelin receptor 1a in a non-signaling conformation: A study with a purified heterodimer assembled into lipid discs. *J. Biol. Chem.* **2013**, *288* (34), 24656–24665.

[142] Müller, T. D.; Mul ler, A.; Yi, C. X.; Habegger, K. M.; Meyer, C. W.; Gaylinn, B. D.; Finan, B.; Heppner, K.; Trivedi, C.; Bielohuby, M.; Abplanalp, W.; Meyer, F.; Piechowski, C. L.; Pratzka, J.; Stemmer, K.; Holland, J.; Hembree, J.; Bhardwaj, N.; Raver, C.; Ottaway, N.; Krishna, R.; Sah, R.; Sallee, F. R.; Woods, S. C.;

Perez-Tilve, D.; Bidlingmaier, M.; Thorner, M. O.; Krude, H.; Smiley, D.; DiMarchi, R.; Hofmann, S.; Pfluger, P. T.; Kleinau, G.; Biebermann, H.; Tschop, M. H. The orphan receptor Gpr83 regulates systemic energy metabolism via ghrelin-dependent and ghrelin-independent mechanisms. *Nat. Commun.* **2013**, *4* (May), 1–8.

[143] Müller, A.; Berkmann, J. C.; Scheerer, P.; Biebermann, H.; Kleinau, G. Insights into basal signaling regulation, oligomerization, and structural organization of the human G-Protein coupled receptor 83. *PLoS One* **2016**, *11* (12), 1–23.

[144] Schellekens, H.; Dinan, T. G.; Cryan, J. F. Taking two to tango: A role for ghrelin receptor heterodimerization in stress and reward. *Front. Neurosci.* **2013**, *7* (7 AUG), 1–18.

[145] Kern, A.; Albarran-Zeckler, R.; Walsh, H. E.; Smith, R. G. Apo-Ghrelin Receptor Forms Heteromers with DRD2 in Hypothalamic Neurons and Is Essential for Anorexigenic Effects of DRD2 Agonism. *Neuron* **2012**, *73* (2), 317–332.

[146] Kern, A.; Grande, C.; Smith, R. G. Apo-ghrelin receptor (apo-GHSR1a) regulates dopamine signaling in the brain. *Front. Endocrinol. (Lausanne).* **2014**, *5* (AUG), 1–8.

[147] Kern, A.; Mavrikaki, M.; Ullrich, C.; Albarran-Zeckler, R.; Brantley, A. F.; Smith, R. G. Hippocampal Dopamine/DRD1 Signaling Dependent on the Ghrelin Receptor. *Cell* **2015**, *163* (5), 1176–1190.

[148] Wellman, M.; Abizaid, A. Growth Hormone Secretagogue Receptor Dimers: A New Pharmacological Target(1,2,3). *eNeuro* **2015**, *2* (2), 0053–14.

[149] Srisai, D.; Yin, T. C.; Lee, A. A.; Rouault, A. A. J.; Pearson, N. A.; Grobe, J. L.; Sebag, J. A. MRAP2 regulates ghrelin receptor signaling and hunger sensing. *Nat. Commun.* **2017**, *8* (1).

[150] George, S. R.; Fan, T.; Xie, Z.; Tse, R.; Tam, V.; Varghese, G.; O'Dowd, B. F. Oligomerization of μ- and delta-opioid receptors: Generation of novel functional properties. *J. Biol. Chem.* **2000**, *275* (34), 26128–26135.

[151] Gomes, I.; Wakako, F.; Chandrakala, M. V.; Devi, L. A. Disease-Specific Heteromerization of G-Protein-Coupled Receptors That Target Drugs of Abuse. *Prog Mol Biol Transl Sci* **2013**, *117*, 207–265.

[152] Fujita, W.; Gomes, I.; Devi, L. A. Revolution in GPCR signalling: Opioid receptor heteromers as novel therapeutic targets: IUPHAR Review 10. *Br. J. Pharmacol.* **2014**, *171* (18), 4155–4176.

[153] Stockton, S. D.; Devi, L. A. Functional relevance of μ-δ opioid receptor heteromerization: A Role in novel signaling and implications for the treatment of addiction disorders: From a symposium on new concepts in mu-opioid pharmacology. *Drug Alcohol Depend.* **2012**, *121* (3), 167–172.

[154] Suzuki, S.; Chuang, L. F.; Yau, P.; Doi, R. H.; Chuang, R. Y. Interactions of opioid and chemokine receptors: Oligomerization of mu, kappa, and delta with CCR5 on immune cells. *Exp. Cell Res.* **2002**, *280* (2), 192–200.

[155] Chen, C.; Li, J.; Bot, G.; Szabo, I.; Rogers, T. J.; Liu-Chen, L. Y. Heterodimerization and cross-desensitization between the μ-opioid receptor and the chemokine CCR5 receptor. *Eur. J. Pharmacol.* **2004**, *483* (2–3), 175–186.

[156] Pfeiffer, M.; Koch, T.; Schröder, H.; Laugsch, M.; Höllt, V.; Schulz, S. Heterodimerization of somatostatin and opioid receptors cross-modulates phosphorylation, internalization, and desensitization. *J. Biol. Chem.* **2002**, *277* (22), 19762–19772.

[157] Jorand, R.; Biswas, S.; Wakefield, D. L.; Tobin, S. J.; Golfetto, O.; Hilton, K.; Ko, M.; Ramos, J. W.; Small, A. R.; Chu, P.; Singh, G.; Jovanovic-Talisman, T. Molecular signatures of mu opioid receptor and somatostatin receptor 2 in pancreatic cancer. *Mol. Biol. Cell* **2016**, *27*, 3659–3672.

[158] Pfeiffer, M.; Kirscht, S.; Stumm, R.; Koch, T.; Wu, D.; Laugsch, M.; Schröder, H.; Höllt, V.; Schulz, S. Heterodimerization of Substance P and μ-Opioid Receptors Regulates Receptor Trafficking and Resensitization. *J. Biol. Chem.* **2003**, *278* (51), 51630–51637.

[159] Xiao, J.; Zeng, S.; Wang, X.; Babazada, H.; Li, Z.; Liu, R.; Yu, W. Neurokinin 1 and opioid receptors: relationships and interactions in nervous system. *Transl Perioper Pain Med* **2016**, *1* (3), 11–21.

[160] Wang, H. L.; Hsu, C. Y.; Huang, P. C.; Kuo, Y. L.; Li, A. H.; Yeh, T. H.; Tso, A. S.; Chen, Y. L. Heterodimerization of opioid receptor-like 1 and μ-opioid receptors impairs the potency of μ receptor agonist. *J. Neurochem.* **2005**, *92* (6), 1285–1294.

[161] Wang, D.; Sun, X.; Bohn, L.; Sadee, W. Opioid receptor homo-and heterodimerization in living cells by quantitative bioluminescence resonance energy transfer. *Mol. Pharmacol.* **2005**, *67* (6), 2173–2184.

[162] Liu, N.-J.; Chakrabarti, S.; Schnell, S.; Wessendorf, M.; Gintzler, A. R. Spinal Synthesis of Estrogen and Concomitant Signaling by Membrane Estrogen Receptors Regulate Spinnal kappa- and mu-Opioid Receptor Heterodimerization and Female-Specific Spinal Morphine Antinociception. *J. Neurosci.* **2011**, *31* (33), 11836–11845.

[163] Rios, C.; Gomes, I.; Devi, L. A. ?? opioid and CB1 cannabinoid receptor interactions: Reciprocal inhibition of receptor signaling and neuritogenesis. *Br. J. Pharmacol.* **2006**, *148* (4), 387–395.

[164] Hojo, M.; Sudo, Y.; Ando, Y.; Minami, K.; Takada, M.; Matsubara, T.; Kanaide, M.; Taniyama, K.; Sumikawa, K.; Uezono, Y. Mu-Opioid Receptor Forms a Functional Heterodimer With Cannabinoid CB Receptor: Electrophysiological and FRET Assay Analysis. *J. Pharmacol. Sci.* **2008**, *108* (3), 308–319.

[165] Costantino, C. M.; Gomes, I.; Stockton, S. D.; Lim, M. P.; Devi, L. A. Opioid receptor heteromers in analgesia. *Expert Rev. Mol. Med.* **2012**, *14*.

[166] Jordan, B. A.; Gomes, I.; Rios, C.; Filipovska, J.; Devi, L. A. Functional Interactions between μ Opioid and α2A-Adrenergic Receptors. *Mol. Pharmacol.* **2003**, *64* (6), 1317–1324.

[167] Vilardaga, J. P.; Nikolaev, V. O.; Lorenz, K.; Ferrandon, S.; Zhuang, Z.; Lohse, M. J. Conformational cross-talk between α2A-adrenergic and μ-opioid receptors controls cell signaling. *Nat. Chem. Biol.* **2008**, *4* (2), 126–131.

[168] Tan, M.; Walwyn, W. M.; Evans, C. J.; Xie, C. W. p38 MAPK and β-arrestin 2 mediate functional interactions between endogenous mu-opioid and α2A-adrenergic receptors in neurons. *J. Biol. Chem.* **2009**, *284* (10), 6270–6281.

[169] Schröder, H.; Wu, D. F.; Seifert, A.; Rankovic, M.; Schulz, S.; Höllt, V.; Koch, T. Allosteric modulation of metabotropic glutamate receptor 5 affects phosphorylation, internalization, and desensitization of the ??-opioid receptor. *Neuropharmacology* **2009**, *56* (4), 768–778.

[170] Liu, X.; Liu, Z.; Sun, Y.; Ross, M.; Kim, S.; Tsai, F.; Li, Q.; Jeffry, J.; Kim, J.; Loh, H. H.; Chen, Z. Unidirectional Cross-activation of GRPR by MOR1D Uncouples Itch and Analgesia Induced by Opioids. *Cell* **2011**, *14* (2), 447–458.

[171] Cussac, D.; Rauly-Lestienne, I.; Heusler, P.; Finana, F.; Cathala, C.; Bernois, S.; De Vries, L. μ-opioid and 5-HT1A receptors heterodimerize and show signalling crosstalk via G protein and MAP-kinase pathways. *Cell. Signal.* **2012**, *24* (8), 1648–1657.

[172] Moreno, E.; Quiroz, C.; Rea, W.; Cai, N.-S.; Mallol, J.; Cortés, A.; Lluís, C.; Canela, E. I.; Casadó, V.; Ferré, S. Functional μ-Opioid-Galanin Receptor Heteromers in the Ventral Tegmental Area. *J. Neurosci.* **2017**, *37* (5), 1176–1186.

[173] Rios, C.; Gomes, I.; Devi, L. A. Interactions Between Delta Opioid Receptors and α2A-adrenoceptors. *Clin. Exp. Pharmacol. Physiol.* **2004**, 833–836.

[174] Riedl, M. S.; Schnell, S. A.; Overland, A. C.; Chabot-Doré, A.-J.; Taylor, A. M.; Ribeiro-Da-Silva, A.; Elde, R. P.; Wilcox, G. L.; Stone4, L. S. Co-expresion of alpha-2A-adrenergic and delta opioid receptors in substance P terminales in rat dorsal horn. *J Comp Neurol* **2009**, *513* (4), 385–398.

[175] Jordan, B. A.; Trapaidze, N.; Gomes, I.; Nivarthi, R.; Devi, L. A. Oligomerization of opioid receptors with β2-adrenergic receptors: A role in trafficking and mitogen-activated protein kinase activation. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98* (1), 343–348.

[176] Ramsay, D.; Kellett, E.; Mcvey, M.; Rees, S.; Milligan, G. Subtypes Form More Efficiently Than Between Less Closely Related Sequences. *Media* **2002**, *440*, 429–440.

[177] Breit, A.; Gagnidze, K.; Devi, L. a; Lagacé, M.; Bouvier, M. Simultaneous activation of the delta opioid receptor (deltaOR)/sensory neuron-specific receptor-4 (SNSR-4) hetero-oligomer by the mixed bivalent agonist bovine adrenal medulla peptide 22 activates SNSR-4 but inhibits deltaOR signaling. *Mol. Pharmacol.* **2006**, *70* (2), 686–696.

[178] Rozenfeld, R.; Bushlin, I.; Gomes, I.; Tzavaras, N.; Gupta, A.; Neves, S.; Battini, L.; Gusella, G. L.; Lachmann, A.; Ma'ayan, A.; Blitzer, R. D.; Devi, L. A. Receptor heteromerization expands the repertoire of cannabinoid signaling in rodent neurons. *PLoS One* **2012**, *7* (1).

[179] Pello, O. M.; Martínez-Muñoz, L.; Parrillas, V.; Serrano, A.; Rodríguez-Frade, J. M.; Toro, M. J.; Lucas, P.; Monterrubio, M.; Martínez-A., C.; Mellado, M. Ligand stabilization of CXCR4/δ-opioid receptor heterodimers reveals a mechanism for immune response regulation. *Eur. J. Immunol.* **2008**, *38* (2), 537–549.

[180] Berg, K. A.; Rowan, M. P.; Gupta, A.; Sanchez, T. A.; Silva, M.; Gomes, I.; McGuire, B. A.; Portoghese, P. S.; Hargreaves, K. M.; Devi, L. A.; Clarke, W. P. Allosteric Interactions between and Opioid Receptors in Peripheral Sensory Neurons. *Mol. Pharmacol.* **2012**, *81* (2), 264–272.

[181] Li, Y.; Chen, J.; Bai, B.; Du, H.; Liu, Y.; Liu, H. Heterodimerization of human apelin and kappa opioid receptors: Roles in signal transduction. *Cell. Signal.* **2012**, *24* (5), 991–1001.

[182] Ji, B.; Liu, H.; Zhang, R.; Jiang, Y.; Wang, C.; Li, S.; Chen, J.; Bai, B. Novel signaling of dynorphin at κ-opioid receptor/bradykinin B2 receptor heterodimers. *Cell. Signal.* **2017**, *31*, 66–78.

[183] Lee, J.; Ghil, S. Regulator of G protein signaling 8 inhibits protease-activated receptor 1/Gi/osignaling by forming a distinct G protein-dependent complex in live cells. *Cell. Signal.* **2016**, *28* (5), 391–400.

[184] AbdAlla, S.; Lother, H.; Quitterer, U. AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. *Nature* **2000**, *407* (6800), 94–98.

[185] AbdAlla, S.; Abdel-Base, A.; Lother, H.; Massiery, A. el; Ursula, Q. Mesangial AT1/B2 Receptor Heterodimers Contribute to Angiotensin II Hyperresponsiveness in Experimental Hypertension. *J. Mol. Neurosci.* **2005**, *26* (2–3), 185–192.

[186] Rozenfeld, R.; Gupta, A.; Gagnidze, K.; Lim, M. P.; Gomes, I.; Lee-Ramos, D.; Nieto, N.; Devi, L. A. AT1R-CB1R heteromerization reveals a new mechanism for the pathogenic properties of angiotensin II. *EMBO J.* **2011**, *30* (12), 2350–2363.

[187] Bellot, M.; Galandrin, S.; Boularan, C.; Matthies, H. J.; Despas, F.; Colette, D.; Javitch, J.; Mazeres, S.; Sanni, S. J.; Pons, V.; Seguelas, M.-H.; Hansen, J. L.; Atul, P.; Galli, A.; Senard, J.-M.; Gales, C. Dual agonist occupancy of AT1-alpha2C-AR heterodimers results in atypical Gs-PKA signaling. *Nat Chem Biol* **2015**, *11* (4), 271–279.

[188] Devost, D.; Zingg, H. H. Identification of dimeric and oligomeric complexes of the human oxytocin receptor by co-immunoprecipitation and bioluminescence resonance energy transfer. *J. Mol. Endocrinol.* **2003**, *31* (3), 461–471.

[189] Albizu, L.; Cottet, M.; Kralikova, M.; Stoev, S.; Seyer, R.; Brabet, I.; Roux, T.; Bazin, H.; Bourrier, E.; Lamarque, L.; Breton, C.; Rives, M.-L.; Newman, A.; Trinquet, E.; Manning, M.; Pin, J.-P.; Mouillac, B.; Durroux, T. Time-resolved FRET between GPCR ligands reveals oligomers in native tissues. *Nat Chem Biol* **2012**, *6* (8), 587–594.

[190] Terrillon, S.; Durroux, T.; Mouillac, B.; Breit, A.; Ayoub, M. A.; Taulan, M.; Jockers, R.; Barberis, C.; Bouvier, M. Oxytocin and Vasopressin V1a and V2 Receptors Form Constitutive Homo- and Heterodimers during Biosynthesis. *Mol. Endocrinol.* **2003**, *17* (4), 677–691.

[191] Kroeger, K. M.; Hanyaloglu, A. C.; Seeber, R. M.; Miles, L. E. C.; Eidne, K. A. Constitutive and agonist-dependent homo-oligomerization of the thyrotropin-releasing hormone receptor. Detection in living cells using bioluminescence resonance energy transfer. *J. Biol. Chem.* **2001**, *276* (16), 12736–12743.

[192] Urizar, E.; Montanelli, L.; Loy, T.; Bonomi, M.; Swillens, S.; Gales, C.; Bouvier, M.; Smits, G.; Vassart, G.; Costagliola, S. Glycoprotein hormone receptors: Link between receptor homodimerization and negative cooperativity. *EMBO J.* **2005**, *24* (11), 1954–1964.

[193] Pei, L.; Li, S.; Wang, M.; Diwan, M.; Anisman, H.; Fletcher, P. J.; Nobrega, J. N.; Liu, F. Uncoupling the dopamine D1-D2 receptor complex exerts antidepressant-like effects. *Nat. Med.* **2010**, *16* (12), 1393–1395.

[194] Hasbi, A.; Perreault, M. L.; Shen, M. Y. F.; Zhang, L.; To, R.; Fan, T.; Nguyen, T.; Ji, X.; O'Dowd, B. F.; George, S. R. A peptide targeting an interaction interface disrupts the dopamine D1-D2 receptor heteromer to block signaling and function in vitro and in vivo: Effective selective antagonism. *FASEB J.* **2014**, *28* (11), 4806–4820.

[195] Perreault, M. L.; Hasbi, A.; O'dowd, B. F.; George, S. R. Heteromeric dopamine receptor signaling complexes: Emerging neurobiology and disease relevance. *Neuropsychopharmacology* **2014**, *39* (1), 156–168.

[196] Hounsou, C.; Margathe, J. F.; Oueslati, N.; Belhocine, A.; Dupuis, E.; Thomas, C.; Mann, A.; Ilien, B.; Rognan, D.; Trinquet, E.; Hibert, M.; Pin, J. P.; Bonnet, D.; Durroux, T. Time-resolved FRET binding assay to investigate hetero-oligomer binding properties: Proof of concept with dopamine D1/D3 heterodimer. *ACS Chem. Biol.* **2015**, *10* (2), 466–474.

[197] Marcellino, D.; Ferré, S.; Casadó, V.; Cortés, A.; Le Foll, B.; Mazzola, C.; Drago, F.; Saur, O.; Stark, H.; Soriano, A.; Barnes, C.; Goldberg, S. R.; Lluis, C.; Fuxe, K.; Franco, R. Identification of dopamine D1-D3receptor heteromers: Indications for a role of synergistic D1-D3receptor interactions in the striatum. *J. Biol. Chem.* **2008**, *283* (38), 26016–26025.

[198] Fuenzalida, J.; Galaz, P.; Araya, K. A.; Slater, P. G.; Blanco, E. H.; Campusano, J. M.; Ciruela, F.; Gysling, K. Dopamine D1and corticotrophin-releasing hormone type-2α receptors assemble into functionally interacting complexes in living cells. *Br. J. Pharmacol.* **2014**, *171* (24), 5650–5664.

[199] Rodríguez-Ruiz, M.; Moreno, E.; Moreno-Delgado, D.; Navarro, G.; Mallol, J.; Cortés, A.; Lluís, C.; Canela, E. I.; Casadó, V.; McCormick, P. J.; Franco, R. Heteroreceptor Complexes Formed by Dopamine D1, Histamine H3, and N-Methyl-D-Aspartate Glutamate Receptors as Targets to Prevent Neuronal Death in Alzheimer's Disease. *Mol. Neurobiol.* **2017**, *54* (6), 4537–4550.

[200] Tao, Y.-M.; Yu, C.; Wang, W.-S.; Hou, Y.-Y.; Xu, X.-J.; Chi, Z.-Q.; Ding, Y.-Q.; Wang, Y.-J.; Liu, J.-G. Heteromers of μ opioid and dopamine D₁ receptors modulate opioid-induced locomotor sensitization in a dopamine-independent manner. *Br. J. Pharmacol.* **2017**, *174* (17), 2842–2861.

[201] Guo, W.; Urizar, E.; Kralikova, M.; Mobarec, J. C.; Shi, L.; Filizola, M.; Javitch, J. A. Dopamine D2 receptors form higher order oligomers at physiological expression levels. *EMBO J.* **2008**, *27* (17), 2293–2304.

[202] Kaczor, A. A.; Jörg, M.; Capuano, B. The dopamine D2 receptor dimer and its interaction with homobivalent antagonists: homology modeling, docking and molecular dynamics. *J. Mol. Model.* **2016**, *22* (9).

[203] Scarselli, M.; Novi, F.; Schallmach, E.; Lin, R.; Baragli, A.; Colzi, A.; Griffon, N.; Corsini, G. U.; Sokoloff, P.; Levenson, R.; Vogel, Z.; Maggio, R. D2/D3 Dopamine Receptor Heterodimers Exhibit Unique Functional Properties. *J. Biol. Chem.* **2001**, *276* (32), 30308–30314.

[204] González, S.; Rangel-Barajas, C.; Peper, M.; Lorenzo, R.; Ciruela, F.; Borycz, J.; Ortiz, J.; Lluís, C.; Franco, R.; Mccormick, P. J.; Volkow, N. D.; Rubinstein, M.; Floran, B. Dopamine D4 receptor, but not the ADHD-associated D4.7 variant, forms functional heteromers with the dopamine D2S receptor in the brain. *Mol Psychiatry* **2012**, *17* (6), 650–662.

[205] Kearn, C. S.; Blake-Palmer, K.; Daniel, E.; Mackie, K.; Glass, M. Concurrent Stimulation of Cannabinoid CB1 and Dopamine D2 Receptors Enhances Heterodimer Formation: A Mechanism for Receptor Cross-Talk? *Mol. Pharmacol.* **2005**, *67* (5), 1697–1704.

[206] Rocheville, M. Receptors for Dopamine and Somatostatin: Formation of Hetero-Oligomers with Enhanced Functional Activity. *Science (80-.).* **2000**, *288* (5463), 154–157.

[207] Somvanshi, R. K.; Kumar, U. Pathophysiology of GPCR homo- and heterodimerization: Special emphasis on Somatostatin receptors. *Pharmaceuticals* **2012**, *5* (5), 417–446.

[208] Borroto-Escuela, D. O.; Romero-Fernandez, W.; Tarakanov, A. O.; Marcellino, D.; Ciruela, F.; Agnati, L. F.; Fuxe, K. Dopamine D2 and 5-hydroxytryptamine 5-HT2A receptors assemble into functionally interacting heteromers. *Biochem. Biophys. Res. Commun.* **2010**, *401* (4), 605–610.

[209] Koschatzky, S.; Tschammer, N.; Gmeiner, P. Cross-Receptor Interactions between Dopamine D2L and Neurotensin NTS1 Modulate Binding Affinites of Dopaminergics. **2011**, 308–316.

[210] Borroto-Escuela, D. O.; Ravani, A.; Tarakanov, A. O.; Brito, I.; Narvaez, M.; Romero-Fernandez, W.; Corrales, F.; Agnati, L. F.; Tanganelli, S.; Ferraro, L.; Fuxe, K. Dopamine D2 receptor signaling dynamics of dopamine D2-neurotensin 1 receptor heteromers. *Biochem. Biophys. Res. Commun.* **2013**, *435* (1), 140–146.

[211] Martínez-Pinilla, E.; Rodríguez-Pérez, A. I.; Navarro, G.; Aguinaga, D.; Moreno, E.; Lanciego, J. L.; Labandeira-García, J. L.; Franco, R. Dopamine D2 and angiotensin II type 1 receptors form functional heteromers in rat striatum. *Biochem. Pharmacol.* **2015**, *96* (2), 131–142.

[212] Bontempi, L.; Savoia, P.; Bono, F.; Fiorentini, C.; Missale, C. Dopamine D3 and acetylcholine nicotinic receptor heteromerization in midbrain dopamine neurons: Relevance for neuroplasticity. *Eur. Neuropsychopharmacol.* **2017**, *27* (4), 313–324.

[213] Borroto-Escuela, D. O.; Narvaez, M.; Marcellino, D.; Parrado, C.; Narvaez, J. A.; Tarakanov, A. O.; Agnati, L. F.; Díaz-Cabiale, Z.; Fuxe, K. Galanin receptor-1 modulates 5-hydroxtryamine-1A signaling via heterodimerization. *Biochem. Biophys. Res. Commun.* **2010**, *393* (4), 767–772.

[214] Fuxe, K.; Marcellino, D.; Rivera, A.; Diaz-Cabiale, Z.; Filip, M.; Gago, B.; Roberts, D. C. S.; Langel, U.; Genedani, S.; Ferraro, L.; de la Calle, A.; Narvaez, J.; Tanganelli, S.; Woods, A.; Agnati, L. F. Receptor-receptor interactions within receptor mosaics. Impact on neuropsychopharmacology. *Brain Res. Rev.* **2008**, *58* (2), 415–452.

[215] Renner, U.; Zeug, A.; Woehler, A.; Niebert, M.; Dityatev, A.; Dityateva, G.; Gorinski, N.; Guseva, D.; Abdel-Galil, D.; Fröhlich, M.; Döring, F.; Wischmeyer, E.; Richter, Diethelm, W.; Neher, E.; Ponimaskin, E. G. Heterodimerization of serotonin receptors 5-HT1A and 5-HT7 differentially regulates receptor signalling and trafficking Journal. *J. Cell Sci.* **2012**, No. February.

[216] Lukasiewicz, S.; Błasiak, E.; Szafran-Pilch, K.; Dziedzicka-Wasylewska, M. Dopamine D 2 and serotonin 5-HT 1A receptor interaction in the context of the effects of antipsychotics - in vitro studies. *J. Neurochem.* **2016**, *137* (4), 549–560.

[217] Ciruela, F.; Casadó, V.; Rodrigues, R. J.; Lujan, R.; Burgueño, J.; Canals, M.; Borycz, J.; Rebola, N.; Goldberg, S. R.; Mallol, J.; Cortes, A.; Canela, E. I.; Lopez-Gimenez, J. F.; Milligan, G.; Lluís, C.; Cunha, R. A.; Ferre, S.; Franco, R. Presynaptic Control of Striatal Glutamatergic Neurotransmission by Adenosine A1-A2A Receptor Heteromers. *J. Neurosci.* **2006**, *26* (7), 2080–2087.

[218] Gines, S.; Hillion, J.; Torvinen, M.; Le Crom, S.; Casado, V.; Canela, E. I.; Rondin, S.; Lew, J. Y.; Watson, S.; Zoli, M.; Agnati, L. F.; Verniera, P.; Lluis, C.; Ferre, S.; Fuxe, K.; Franco, R. Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. *Proc. Natl. Acad. Sci.* **2000**, *97* (15), 8606–8611.

[219] Yoshioka, K.; Saitoh, O.; Nakata, H. Heteromeric association creates a P2Y-like adenosine receptor. *Proc. Natl. Acad. Sci.* **2001**, *98* (13), 7617–7622.

[220] Yoshioka, K.; Hosoda, R.; Kuroda, Y.; Nakata, H. Hetero-oligomerization of adenosine A 1 receptors with P2Y 1 receptors in rat brains. *FEBS Lett.* **2002**, *531*, 299–303.

[221] Ciruela, F.; Escriche, M.; Burgueño, J.; Angulo, E.; Casado, V.; Soloviev, M. M.; Canela, E. I.; Mallol, J.; Chan, W.-Y.; Lluís, C.; McIlhinney, R. A. J.; Franco, R. Metabotropic Glutamate 1 ? and Adenosine A1 Receptors Assemble into Functionally Interacting Complexes. *J. Biol. Chem.* **2001**, *276, No. 2* (25), 18345–18351.

[222] Chandrasekera, P. C.; Wan, T. C.; Gizewski, E. T.; Auchampach, J. A.; D, L. R. Adenosine A1 receptors heterodimerize with beta1- and beta2-adrenergic receptors creating novel receptor complexes with altered G protein coupling and signaling. *Cell Signal* **2013**, *25* (4), 736–742.

[223] Canals, M.; Burgueño, J.; Marcellino, D.; Cabello, N.; Canela, E. I.; Mallol, J.; Agnati, L.; Ferré, S.; Bouvier, M.; Fuxe, K.; Ciruela, F.; Lluis, C.; Franco, R. Homodimerization of adenosine A2A receptors: Qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J. Neurochem.* **2004**, *88* (3), 726–734.

[224] Hinz, S.; Navarro, G.; Borroto-Escuela, D.; Seibt, B. F.; Ammon, Y.-C.; De Filippo, E.; Danish, A.; Lacher, S. K.; Červinková, B.; Rafehi, M.; Fuxe, K.; Schiedel, A. C.; Franco, R.; Müller, C. E. Adenosine A2A receptor ligand recognition and signaling is blocked by A2B receptors. *Oncotarget* **2018**, *9* (17), 13593–13611.

[225] Canals, M.; Marcellino, D.; Fanelli, F.; Ciruela, F.; De Benedetti, P.; Goldberg, S. R.; Neve, K.; Fuxe, K.; Agnati, L. F.; Woods, A. S.; Ferré, S.; Lluis, C.; Bouvier, M.; Franco, R. Adenosine A2A-dopamine D2receptor-receptor heteromerization: Qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J. Biol. Chem.* **2003**, *278* (47), 46741–46749.

[226] Borroto-Escuela, D. O.; Romero-Fernandez, W.; Tarakanov, A. O.; Gómez-Soler, M.; Corrales, F.; Marcellino, D.; Narvaez, M.; Frankowska, M.; Flajolet, M.; Heintz, N.; Agnati, L. F.; Ciruela, F.; Fuxe, K. Characterization of the A2AR-D2R interface: Focus on

the role of the C-terminal tail and the transmembrane helices. *Biochem. Biophys. Res. Commun.* **2010**, *402* (4), 801–807.

[227] Trifilieff, P.; Rives, M.; Urizar, E.; Piskorowski, R. A.; Vishwasrao, H. D.; Castrillon, J.; Schmauss, C.; Slättman, M.; Javitch, J. A. Detection of antigen interactions ex vivo by proximity ligation assay: endogenous dopamine D2-adenosine A2A receptor complexes in the striatum. *Biotechniques* **2011**, *51* (2), 111–118.

[228] Bonaventura, J.; Navarro, G.; Casadó-Anguera, V.; Azdad, K.; Rea, W.; Moreno, E.; Brugarolas, M.; Mallol, J.; Canela, E. I.; Lluís, C.; Cortés, A.; Volkow, N. D.; Schiffmann, S. N.; Ferré, S.; Casadó, V. Allosteric interactions between agonists and antagonists within the adenosine A $_{2A}$ receptor-dopamine D $_2$ receptor heterotetramer. *Proc. Natl. Acad. Sci.* **2015**, *112* (27), E3609–E3618.

[229] Torvinen, M.; Marcellino, D.; Canals, M.; Agnati, L.; Lluis, C.; Franco, R.; Fuxe, K. Adenosine A2A receptor and dopamine D3 receptor interactions: evidence of functional A2A/D3 heteromeric complexes. *Mol. Pharmacol.* **2005**, *67* (2), 400–407.

[230] Ferre, S.; Karcz-Kubicha, M.; Hope, B. T.; Popoli, P.; Burgueno, J.; Gutierrez, M. A.; Casado, V.; Fuxe, K.; Goldberg, S. R.; Lluis, C.; Franco, R.; Ciruela, F. Synergistic interaction between adenosine A2A and glutamate mGlu5 receptors: Implications for striatal neuronal function. *Proc. Natl. Acad. Sci.* **2002**, *99* (18), 11940–11945.

[231] Milligan, G. G protein-coupled receptor hetero-dimerization: Contribution to pharmacology and function. *Br. J. Pharmacol.* **2009**, *158* (1), 5–14.

[232] Márquez-Gómez, R.; Robins, M. T.; Gutiérrez-Rodelo, C.; Arias, J.-M.; Olivares-Reyes, J.-A.; van Rijn, R. M.; Arias-Montaño, J.-A. Functional histamine H3 and adenosine A2A receptor heteromers in recombinant cells and rat striatum. *Pharmacol. Res.* **2017**.

[233] Moreno, E.; Chiarlone, A.; Medrano, M.; Puigdellívol, M.; Bibic, L.; Howell, L. A.; Resel, E.; Puente, N.; Casarejos, M. J.; Perucho, J.; Botta, J.; Suelves, N.; Ciruela, F.; Ginés, S.; Galve-Roperh, I.; Casadó, V.; Grandes, P.; Lutz, B.; Monory, K.; Canela, E. I.; Lluís, C.; McCormick, P. J.; Guzmán, M. Singular Location and Signaling Profile of Adenosine A2A-Cannabinoid CB1Receptor Heteromers in the Dorsal Striatum. *Neuropsychopharmacology* **2017**, 1–14.

[234] May, L. T.; Bridge, L. J.; Stoddart, L. A.; Briddon, S. J.; Hill, S. J. Allosteric interactions across native adenosine-A3 receptor homodimers: quantification using single-cell ligand-binding kinetics. *FASEB J.* **2011**, *25* (10), 3465–3476.

[235] Jacobson, K. a.; Gao, Z.-G. Adenosine receptors as therapeutic targets. *Nat. Rev. Drug Discov.* **2006**, *5* (3), 247–264.

[236] Ecke, D.; Hanck, T.; Tulapurkar, M. E.; Schäfer, R.; Kassack, M.; Stricker, R.; Reiser, G. Hetero-oligomerization of the P2Y $_{11}$ receptor with the P2Y $_1$ receptor controls the internalization and ligand selectivity of the P2Y $_{11}$ receptor. *Biochem. J.* **2008**, *409* (1), 107–116.

[237] Nishimura, A.; Sunggip, C.; Oda, S.; Numaga-Tomita, T.; Tsuda, M.; Nishida, M. Purinergic P2Y receptors: Molecular diversity and implications for treatment of cardiovascular diseases. *Pharmacol. Ther.* **2017**, *180*, 113–128.

[238] Levoye, A.; Dam, J.; Ayoub, M. A.; Guillaume, J. L.; Couturier, C.; Delagrange, P.; Jockers, R. The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. *EMBO J.* **2006**, *25* (13), 3012–3023.

[239] Hirsch-Rodriguez, E.; Imbesi, M.; Manev, R.; Uz, T.; Manev, H. The pattern of melatonin receptor expression in the brain may influence antidepressant treatment. *Med Hypotheses* **2007**, *61* (1), 120–124.

[240] Gomes, I.; Bobeck, E. N.; Margolis, E. B.; Gupta, A.; Sierra, S.; Fakira, A. K.; Fujita, W.; Müller, T. D.; Müller, A.; Tschöp, M. H.; Kleinau, G.; Fricker, L. D.; Devi, L. A. Identification of GPR83 as the receptor for the neuroendocrine peptide PEN. *Sci. Signal.* **2016**, *9* (425), 1–15.

[241] Orlandi, C.; Cao, Y.; Martemyanov, K. A. Orphan receptor GPR179 forms macromolecular complexes with components of metabotropic signaling cascade in retina ON-bipolar neurons. *Invest. Ophthalmol. Vis. Sci.* **2013**, *54* (10), 7153–7161.

[242] Milasta, S.; Pediani, J.; Appelbe, S.; Trim, S.; Wyatt, M.; Cox, P.; Fidock, M.; Milligan, G. Interactions between the Mas-Related Receptors MrgD and MrgE Alter Signalling and Trafficking of MrgD. *Mol. Pharmacol.* **2006**, *69* (2), 479–491.

[243] Harikumar, K. G. Constitutive Formation of Oligomeric Complexes Between Family B G Protein-Coupled Vpac and Secretin Receptors. *Mol. Pharmacol.* **2006**, *69* (1), 363–373.

[244] Harikumar, K. G.; Morfis, M. M.; Sexton, P. M.; Miller, L. J. Pattern of Intra-Family Hetero-Oligomerization Involving the G-Protein-Coupled Secretin Receptor. *J. Mol. Neurosci.* **2008**, *36* (10), 1883–1889.

[245] Lee, L. T. O.; Ng, S. Y. L.; Chu, J. Y. S.; Sekar, R.; Harikumar, K. G.; Miller, L. J.; Chow, B. K. C. Transmembrane peptides as unique tools to demonstrate the in vivo action of a cross-class GPCR heterocomplex. *FASEB J.* **2014**, *28* (6), 2632–2644.

[246] Vrecl, M.; Drinovec, L.; Elling, C.; Heding, A. Opsin oligomerization in a heterologous cell system. *J. Recept. Signal Transduct.* **2006**, *26* (5–6), 505–526.

[247] Young, S. F.; Griffante, C.; Aguilera, G. Dimerization between vasopressin V1b and corticotropin releasing hormone type 1 receptors. *Cell. Mol. Neurobiol.* **2007**, *27* (4), 439–461.

[248] Bai, M.; Trivedi, S.; Brown, E. M. Dimerization of the extracellular calcium-sensing receptor (CaR) on the cell surface of CaR-transfected HEK293 cells. *J. Biol. Chem.* **1998**, *273* (36), 23605–23610.

[249] Margeta-Mitrovic, M.; Jan, Y. N.; Jan, L. Y. A Trafficking Checkpoint Controls GABAB Receptor Heterodimerization. *Neuron* **2000**, *27*, 97–106.

[250] Gavin, A.-C.; Bösche, M.; Krause, R.; Grandi, P.; Marzioch, M.; Bauer, A.; Schultz, J.; Rick, J. M.; Michon, A.-M.; Cruciat, C.-M.; Remor, M.; Höfert, C.; Schelder, M.; Brajenovic, M.; Ruffner, H.; Merino, A.; Klein, K.; Hudak, M.; Dickson, D.; Rudi, T.; Gnau, V.; Bauch, A.; Bastuck, S.; Huhse, B.; Leutwein, C.; Heurtier, M.-A.; Copley, R. R.; Edelmann, A.; Querfurth, E.; Rybin, V.; Drewes, G.; Raida, M.; Bouwmeester, T.; Bork, P.; Seraphin, B.; Kuster, B.; Neubauer, G.; Superti-Furga, G. Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* **2002**, *415* (6868), 141–147.

[251] Ong, S.-E.; Blagoev, B.; Kratchmarova, I.; Kristensen, D. B.; Steen, H.; Pandey, A.; Mann, M. Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC, as a Simple and Accurate Approach to Expression Proteomics. *Mol. Cell. Proteomics* **2002**, *1* (5), 376–386.

[252] Xiao, K.; Sun, J. Elucidating structural and molecular mechanisms of β-arrestin-biased agonism at GPCRs via MS-based proteomics. *Cell. Signal.* **2018**, *41*, 56—64.

[253] Kim, D. I.; KC, B.; Zhu, W.; Motamedchaboki, K.; Doye, V.; Roux, K. J. Probing nuclear pore complex architecture with proximity-dependent biotinylation. *Proc. Natl. Acad. Sci.* **2014**, *111* (24), E2453–E2461.

[254] De Munter, S.; Görnemann, J.; Derua, R.; Lesage, B.; Qian, J.; Heroes, E.; Waelkens, E.; Van Eynde, A.; Beullens, M.; Bollen, M. Split-BioID: a proximity biotinylation assay for dimerization-dependent protein interactions. *FEBS Lett.* **2017**, *591* (2), 415–424.

[255] Rylova, G.; Ozdian, T.; Varanasi, L.; Soural, M.; Hlavac, J.; Holub, D.; Dzubak, P.; Hajduch, M. Affinity-Based Methods in Drug-Target Discovery. **2015**, 60–76.

[256] Poetz, O.; Schwenk, J. M.; Kramer, S.; Stoll, D.; Templin, M. F.; Joos, T. O. Protein microarrays: Catching the proteome. *Mech. Ageing Dev.* **2005**, *126* (1), 161–170.

[257] Chen, C.-S.; Zhu, H. Protein Microarrays. *Biotechniques* **2006**, *40* (4), 423–429.

[258] Wisler, J. W.; Xiao, K.; Thomsen, A. R. B.; Lefkowitz, R. J. Recent developments in biased agonism. *Curr. Opin. Cell Biol.* **2014**, *27* (1), 18–24.

[259] Yasgar, A.; Jadhav, A.; Simeonov, A.; Coussens, N. P. AlphaScreen-Based Assays: Ultra-High-Throughput Screening for Small-Molecule Inhibitiors of Challenging Enzymes and Protein-Protein Interactions. *Methods Mol. Biol.* **2016**, *1439*, 77–98.

[260] Arkin, M. R.; Glicksman, M. A.; Fu, H.; Havel, J. J.; Du, Y. Inhibition of Protein-Protein Interactions : Non- Cellular Assay Formats. *Assay Guid. Man.* **2012**, 1–30.

[261] Eglen, R. M.; Reisine, T.; Roby, P.; Rouleau, N.; Illy, C.; Bossé, R.; Bielefeld, M. The Use of AlphaScreen Technology in HTS: Current Status. *Curr. Chem. Genomics* **2008**, *1*, 2–10.

[262] Yasgar, A.; Jadhav, A.; Simeonov, A.; Coussens, N. P. Alphascreen-based assays: Ultra-high-throughput screening for small-molecule inhibitors of challenging enzymes and protein-protein interactions. In *Methods in Molecular Biology*; 2016.

[263] Broussard, J. A.; Green, K. J. Research Techniques Made Simple: Methodology and Applications of Förster Resonance Energy Transfer (FRET) Microscopy. *J. Invest. Dermatol.* **2017**, *137* (11), 185–191.

[264] Leyris, J. P.; Roux, T.; Trinquet, E.; Verdié, P.; Fehrentz, J. A.; Oueslati, N.; Douzon, S.; Bourrier, E.; Lamarque, L.; Gagne, D.; Galleyrand, J. C.; M'kadmi, C.; Martinez, J.; Mary, S.; Banères, J. L.; Marie, J. Homogeneous time-resolved fluorescence-based assay to screen for ligands targeting the growth hormone secretagogue receptor type 1a. *Anal. Biochem.* **2011**, *408* (2), 253–262.

[265] Xu, Y.; Kanauchi, A.; Arnim, A. G. Von; Piston, D. W. Bioluminescence resonance energy transfer (BRET): a new technique for monitoring protein-protein interactions in living cells. *Methods Enzymol.* **2003**, *360*, 289–301.

[266] Rekas, A.; Alattia, J. R.; Nagai, T.; Miyawaki, A.; Ikura, M. Crystal structure of venus, a yellow fluorescent protein with improved maturation and reduced environmental sensitivity. *J. Biol. Chem.* **2002**, *277* (52), 50573–50578.

[267] Markwardt, M. L.; Kremers, G. J.; Kraft, C. A.; Ray, K.; Cranfill, P. J. C.; Wilson, K. A.; Day, R. N.; Wachter, R. M.; Davidson, M. W.; Rizzo, M. A. An improved cerulean fluorescent protein with enhanced brightness and reduced reversible photoswitching. *PLoS One* **2011**, *6* (3).

[268] Shyu, J.; Liu, H.; Deng, X.; Hu, C.-D. Identification of new fluorescent protein fragments for bimolecular fluorescence complementation analysis under physiological conditions. *Biotechniques* **2006**, *40* (1), 61–66.

[269] Ghosh, I.; Hamilton, A. D.; Regan, L. Antiparallel leucine zipper-directed protein reassembly: Application to the green fluorescent protein [12]. *J. Am. Chem. Soc.* **2000**, *122* (23), 5658–5659.

[270] Vidi, P.-A.; Watts, V. J. Fluorescent and bioluminescent protein-fragment complementation assays in the study of G protein-coupled receptor oligomerization and signaling. *Mol. Pharmacol.* **2009**, *75* (4), 733–739.

[271] Milligan, G. G protein-coupled receptor dimerisation: Molecular basis and relevance to function. *Biochim. Biophys. Acta - Biomembr.* **2007**, *1768* (4), 825–835.

[272] Fuxe, K.; Canals, M.; Torvinen, M.; Marcellino, D.; Terasmaa, A.; Genedani, S.; Leo, G.; Guidolin, D.; Diaz-Cabiale, Z.; Rivera, A.; Lundstrom, L.; Langel, U.; Narvaez, J.; Tanganelli, S.; Lluis, C.; Ferré, S.; Woods, A.; Franco, R.; Agnati, L. F. Intramembrane receptor-receptor interactions: A novel principle in molecular medicine. *J. Neural Transm.* **2007**, *114* (1), 49–75.

[273] Delgado-Peraza, F.; Nogueras-Ortiz, C.; Canabal Acevedo, A. M.; Roman-Vendrell, C.; Yudowski, G. A. Imaging GPCRs trafficking and signaling with total internal reflection fluorescence microscopy in cultured neurons. *Methods Cell Biol.* **2016**, *132*, 25–33.

[274] Lee, J.; Kwon, Y. J.; Choi, Y.; Kim, H. C.; Kim, K.; Kim, J.; Park, S.; Song, R. Quantum Dot-Based Screening System for Discovery of G Protein-Coupled Receptor Agonists. *ChemBioChem* **2012**, *13* (10), 1503–1508.

[275] Barroso, M. M. Quantum Dots in Cell Biology. *J. Histochem. Cytochem.* **2011**, *59* (3), 237–251.

[276] Scarselli, M.; Annibale, P.; McCormick, P. J.; Kolachalam, S.; Aringhieri, S.; Radenovic, A.; Corsini, G. U.; Maggio, R. Revealing G-protein-coupled receptor oligomerization at the single-molecule level through a nanoscopic lens: Methods, dynamics and biological function. *FEBS J.* **2016**, *283* (7), 1197–1217.

[277] Churm, R.; Davies, J. S.; Stephens, J. W.; Prior, S. L. Ghrelin function in human obesity and type 2 diabetes: a concise review. *Obes. Rev.* **2017**, *18* (2), 140–148.

[278] Jiao, Q.; Du, X.; Li, Y.; Gong, B.; Shi, L.; Tang, T.; Jiang, H. The neurological effects of ghrelin in brain diseases: Beyond metabolic functions. *Neuroscience and Biobehavioral Reviews*. 2017, pp 98–111.

[279] Laviano, A.; Molfino, A.; Rianda, S.; Fanelli, F. R. The growth hormone secretagogue receptor (Ghs-R). *Curr. Pharm. Des.* **2012**, *18*, 4749–4754.

[280] Al Massadi, O.; López, M.; Tschöp, M.; Diéguez, C.; Nogueiras, R. Current Understanding of the Hypothalamic Ghrelin Pathways Inducing Appetite and Adiposity. *Trends Neurosci.* **2017**, *40* (3), 167–180.

[281] Schrage, R.; Kostenis, E. Functional selectivity and dualsteric/bitopic GPCR targeting. *Curr. Opin. Pharmacol.* **2016**, *32*, 85–90.

[282] Seifert, R.; Wenzel-Seifert, K. Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **2002**, *366* (5), 381–416.

[283] Jiang, H.; Betancourt, L.; Smith, R. G. Ghrelin amplifies dopamine signaling by cross talk involving formation of growth hormone secretagogue receptor/dopamine receptor subtype 1 heterodimers. *Mol. Endocrinol.* **2006**, *20* (8), 1772–1785.

[284] Schellekens, H.; Van Oeffelen, W. E. P. A.; Dinan, T. G.; Cryan, J. F. Promiscuous dimerization of the growth hormone secretagogue receptor (GHS-R1a) attenuates ghrelin-mediated signaling. *J. Biol. Chem.* **2013**, *288* (1), 181–191.

[285] Nonogaki, K.; Ohashi-Nozue, K.; Oka, Y. A negative feedback system between brain serotonin systems and plasma active ghrelin levels in mice. *Biochem. Biophys. Res. Commun.* **2006**, *341* (3), 703–707.

[286] Brunetti, L.; Recinella, L.; Orlando, G.; Michelotto, B.; Di Nisio, C.; Vacca, M. Effects of ghrelin and amylin on dopamine, norepinephrine and serotonin release in the hypothalamus. *Eur. J. Pharmacol.* **2002**, *454* (2–3), 189–192.

[287] Higgins, G. A.; Fletcher, P. J. Serotonin and drug reward: Focus on 5-HT2Creceptors. *Eur. J. Pharmacol.* **2003**, *480* (1–3), 151–162.

[288] Alex, K. D.; Pehek, E. A. Pharmacologic mechanisms of serotonergic regulation of dopamine neurotransmission. *Pharmacol. Ther.* **2007**, *113* (2), 296–320.

[289] Currie, P. J.; John, C. S.; Nicholson, M. L.; Chapman, C. D.; Loera, K. E. Hypothalamic paraventricular 5-hydroxytryptamine inhibits the effects of ghrelin on eating and energy substrate utilization. *Pharmacol. Biochem. Behav.* **2010**, *97* (1), 152–155.

[290] Zigman, J. M.; Jones, J. E.; Lee, C. E.; Saper, C. B.; Elmquist, J. K. Expression of ghrelin receptor mRNA in the rat and the mouse brain. *J. Comp. Neurol.* **2006**, *494* (3), 528–548.

[291] Sargent, P. A.; Sharpley, A. L.; Williams, C.; Goodall, E. M.; Cowen, P. J. 5-HT2C receptor activation decreases appetite and body weight in obese subjects. *Psychopharmacology (Berl).* **1997**, *133* (3), 309–312.

[292] Andrews, Z. B.; Erion, D.; Beiler, R.; Liu, Z.-W.; Abizaid, A.; Zigman, J.; Elsworth, J. D.; Savitt, J. M.; DiMarchi, R.; Tschoep, M.; Roth, R. H.; Gao, X.-B.; Horvath, T. L. Ghrelin promotes and protects nigrostriatal dopamine function via a UCP2-dependent mitochondrial mechanism. *J. Neurosci.* **2009**, *29* (45), 14057–14065.

[293] Abizaid, A. Ghrelin and dopamine: New insights on the peripheral regulation of appetite. *Journal of Neuroendocrinology*. Blackwell Publishing Ltd September 2009, pp 787–793.

[294] Dickson, S. L.; Egecioglu, E.; Landgren, S.; Skibicka, K. P.; Engel, J. A.; Jerlhag, E. The role of the central ghrelin system in reward from food and chemical drugs. *Molecular and Cellular Endocrinology*. Elsevier June 2011, pp 80–87.

[295] Suchankova, P.; Steensland, P.; Fredriksson, I.; Engel, J. A.; Jerlhag, E. Ghrelin Receptor (GHS-R1A) Antagonism Suppresses Both Alcohol Consumption and the Alcohol Deprivation Effect in Rats following Long-Term Voluntary Alcohol Consumption. *PLoS One* **2013**, *8* (8), e71284.

[296] Wellman, P. J.; Shane Clifford, P.; Rodriguez, J. A. Ghrelin and ghrelin receptor modulation of psychostimulant action. *Frontiers in Neuroscience*. Frontiers September 2013, p 171.

[297] Leggio, L.; Zywiak, W. H.; Fricchione, S. R.; Edwards, S. M.; De La Monte, S. M.; Swift, R. M.; Kenna, G. A. Intravenous ghrelin administration increases alcohol craving in alcohol-dependent heavy drinkers: A preliminary investigation. *Biol. Psychiatry* **2014**, *76* (9), 734–741.

[298] Rosenzweig-Lipson, S.; Comery, T. A.; Marquis, K. L.; Gross, J.; Dunlop, J. 5-HT2C Agonists as Therapeutics for the Treatment of Schizophrenia. Springer, Berlin, Heidelberg, 2012; pp 147–165.

[299] Schellekens, H.; De Francesco, P. N.; Kandil, D.; Theeuwes, W. F.; McCarthy, T.; van Oeffelen, W. E. P. A.; Perelló, M.; Giblin, L.; Dinan, T. G.; Cryan, J. F. Ghrelin's Orexigenic Effect Is Modulated via a Serotonin 2C Receptor Interaction. *ACS Chem. Neurosci.* **2015**, *6* (7), 1186–1197.