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Drug Improvement and Design Involving G-Protein Coupled Receptors

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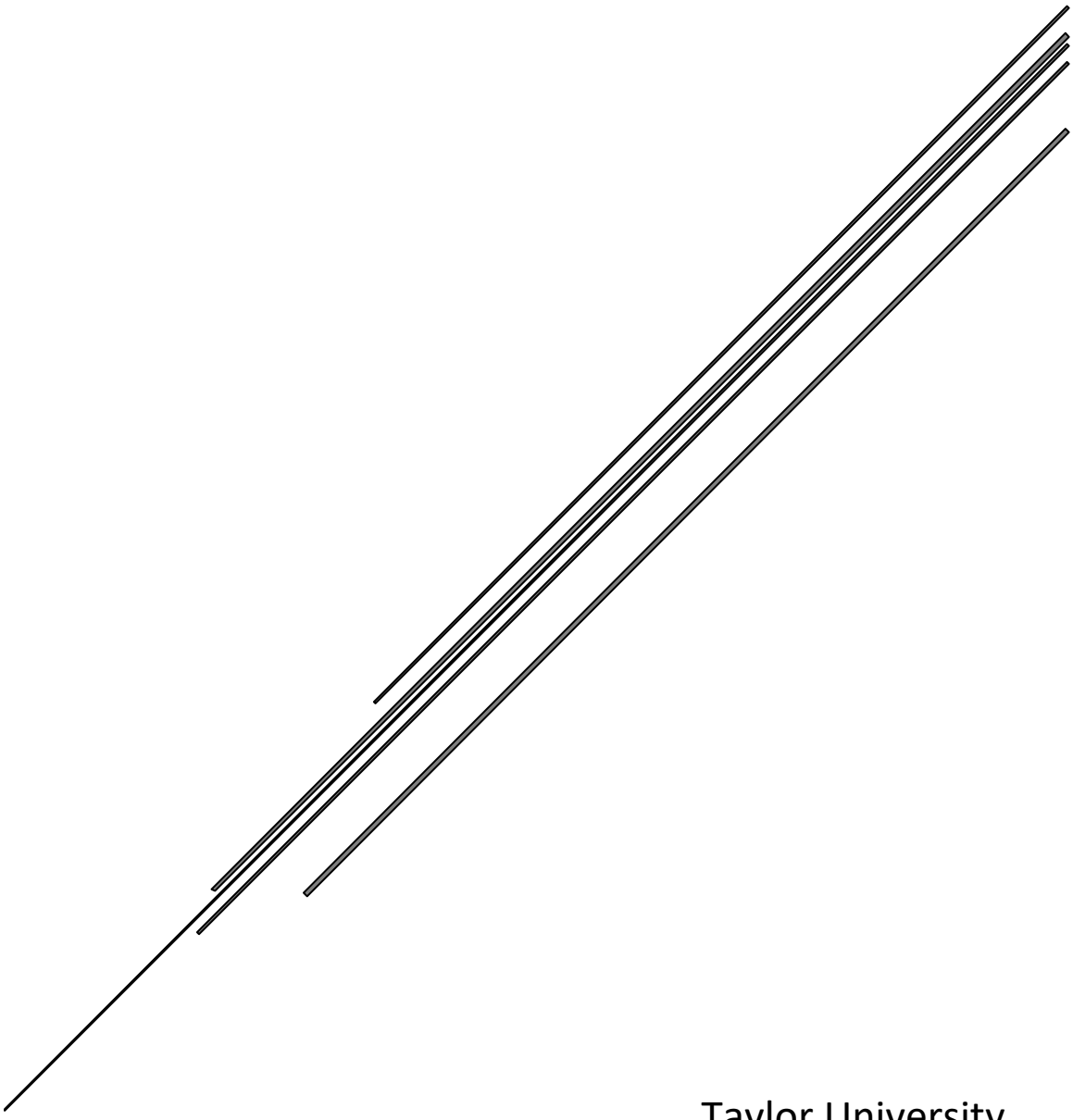

Taylor University
CHE 420

Introduction:

G protein-coupled receptors (GPCRs) are the largest family of cell surface receptors. G proteins are named after the guanine nucleotide component of the protein, they are also known as guanine-nucleotide binding proteins. They are characterized by a heptahelical structure that "snake" across a cell membrane. GPCRs have an extracellular N-terminus and an intracellular C-terminus (Figure 1). The major function of GPCRs is to transmit signals into the cell.

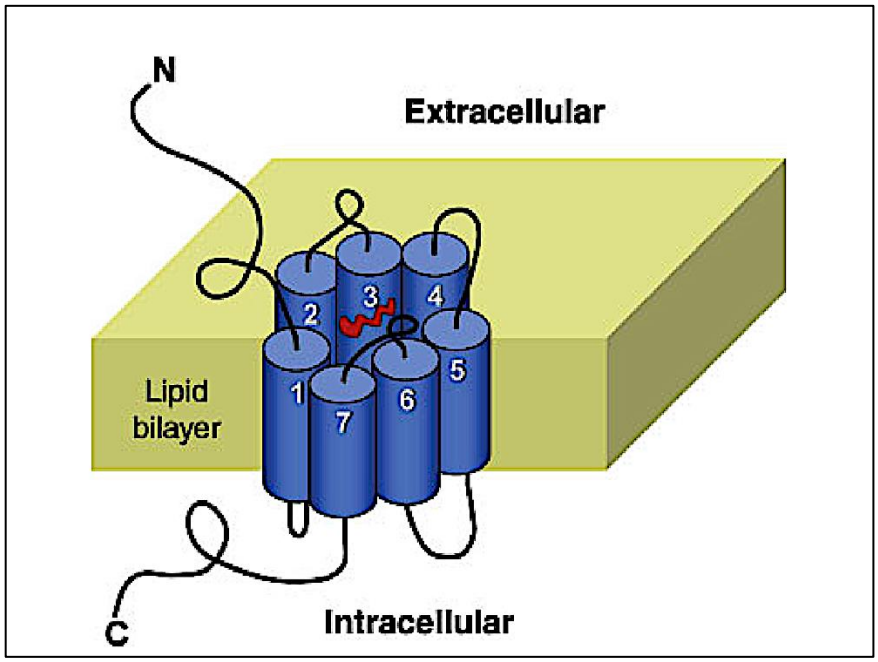

Figure 1. Simplistic representation of GPCR with characteristic seven-transmembrane helices.[1]

These receptors transduce extracellular signals into intracellular biochemical responses. GPCRs and proteins associated with GPCRs can function in a several different ways. The mediation of receptor signaling is most commonly observed when studying G proteins. A few examples of the physiological responses mediated by GPCRs include neurotransmitters, hormones, and exogenous sensory stimuli perceived by the senses (i.e. light, odor, and tastes).

Regulation of receptor signaling is another function of GPCRs and GPCR-associated proteins. This regulation happens, by controlling the localization of receptors or by trafficking the receptors in a certain way. GPCRs can also be physically linked to a receptor functioning as a scaffold to various effectors. They can act as allosteric modulator of receptor conformation, which alters receptor pharmacology and other aspects of receptor function. The mechanism of action for GPCRs is completed when, molecules called "second messengers" relay the signals received by receptors on the cell surface. An example of a signal could be, the arrival of a protein to a target molecule in the cytosol or nucleus. Along with their jobs as relay molecules, second messengers also serve to amplify the strength of the signal.

GPCRs are classified into six major families/classes, and if a family is large enough they can be further classified into subclasses.[2] Class A includes, GPCRs that share similarities with the rhodopsin protein, and are commonly known as rhodopsin-like receptors. Rhodopsin-like receptors are the largest and best understood family of GPCRs.[3] The grouping of GPCRs is observed as follows: class A-rhodopsin like, class B-secretin like receptors, class C-metabotrophic/glutamate/pheromone like receptors, class D-fungal pheromone like receptors, class E-cAMP receptor, and class F-frizzled/smoothened like receptors.[1,2] Sensing intercellular messenger molecules and sensory messages, shows why GPCRs have critical roles in intercellular communication.[4] Belonging to a family of cell-surface receptors, GPCRs are of pharmacological interest because of the numerous physiological processes mediated by GPCRs. Making it possible for them to be the targets of several different types of medication. The latest estimate of the number of licensed medicinal drugs targeting GPCRs is about 36%.[5] This includes hundreds of drug types, including: antihistamines, neuroleptics, antidepressants, and antihypertensive.[2] Opioid receptors, as the name suggests, are another target for drugs, developed for treatment of extreme pain.[6]

GPCRs can also mediate the mechanisms and actions of certain medications, helping to treat disorders like drug dependency and mental illness. Studies of GPCRs may greatly assist in the process of drug discovery because of the large amounts of drugs targeting GPCRs.[5] Understanding molecular details of ligand binding is critical for better information regarding receptor function, and development of new therapeutic compounds.[7]

The exact mechanism of G protein-coupled receptors is complex, and has traditionally been thought of as a linear process.[8] An agonist, which is a ligand/substance that initiates a physiological response when combined with a receptor,[9] first binds to the interior of the hydrophobic pockets of the extracellular surface (Figure 2).[10]

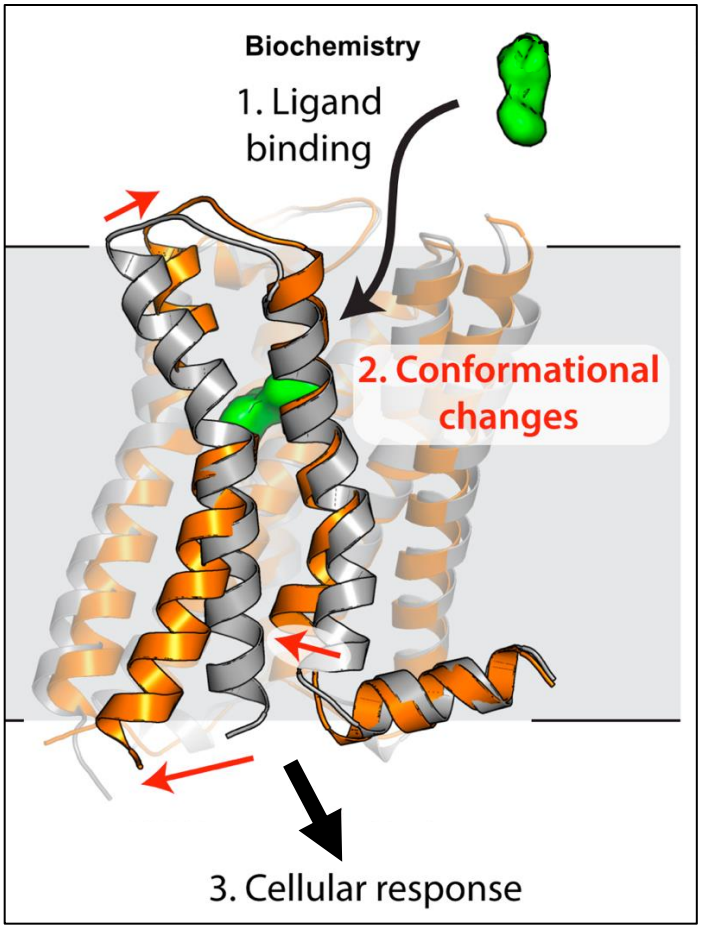

Figure 2. Illustration of ligand binding in the binding pocket of the GPCR[3]

This binding stimulates a conformational change of the GPCR from an inactive to active state.[8] This active state, catalyzes the activation of a guanine binding protein (G protein). It is important to note, that an agonist is more likely to choose the receptor with which it has the greatest affinity. After activation of the binding protein, comes the activation of the heterotrimeric G protein ($G_{\alpha\beta\gamma}$) by the phosphorylation of a guanosine diphosphate to guanosine triphosphate. The addition of this phosphate, happens within the G_{α} subunit and promotes dissociation of G_{α} from the $G_{\alpha\beta\gamma}$.[8] The respective G_{α} and $G_{\beta\gamma}$ subunits, can now lead to activation or inhibition of effector enzymes and ion channels that are able to trigger multiple signaling pathways. These effector enzymes or regulating proteins, can either stimulate or inhibit the recruitment/production of secondary messengers inside the cell.[11] Some examples of second messengers include: cyclic adenosine monophosphate (cAMP) and Calcium (Ca^{2+}) ions.

Ch. 1: GPCR Classes and Activation

GPCRs make up the biggest family/class of transmembrane receptors in humans.[3] Some of the larger classes, such as class A (rhodopsin-like), also include subclasses. Each of these classes, differ from each other in the way their extracellular binding pocket binds to an agonist or similar types of ligands (Figure 3).

There is a great demand to develop a programable way of determining classes of GPCRs to aid in the classification of drugs, but more importantly to improve the process of drug discovery.[2] Chou succeeded in using a computational method that can determine GPCR family types.[2] Using a statistical predictor, Chou tried to determine classes and subclasses of GPCRs based on their primary amino acid sequence. The amino acid arrangement represents the GPCR sample, and a predictor (Covariant Discriminant) is then used to perform the prediction.[2] A statistical predictor cannot work without a benchmark data set.[1] The G-protein coupled receptor database, was used to

create this benchmark set.[2] To determine the power of the operation engine on the predictor, a cross-validation test must be used.

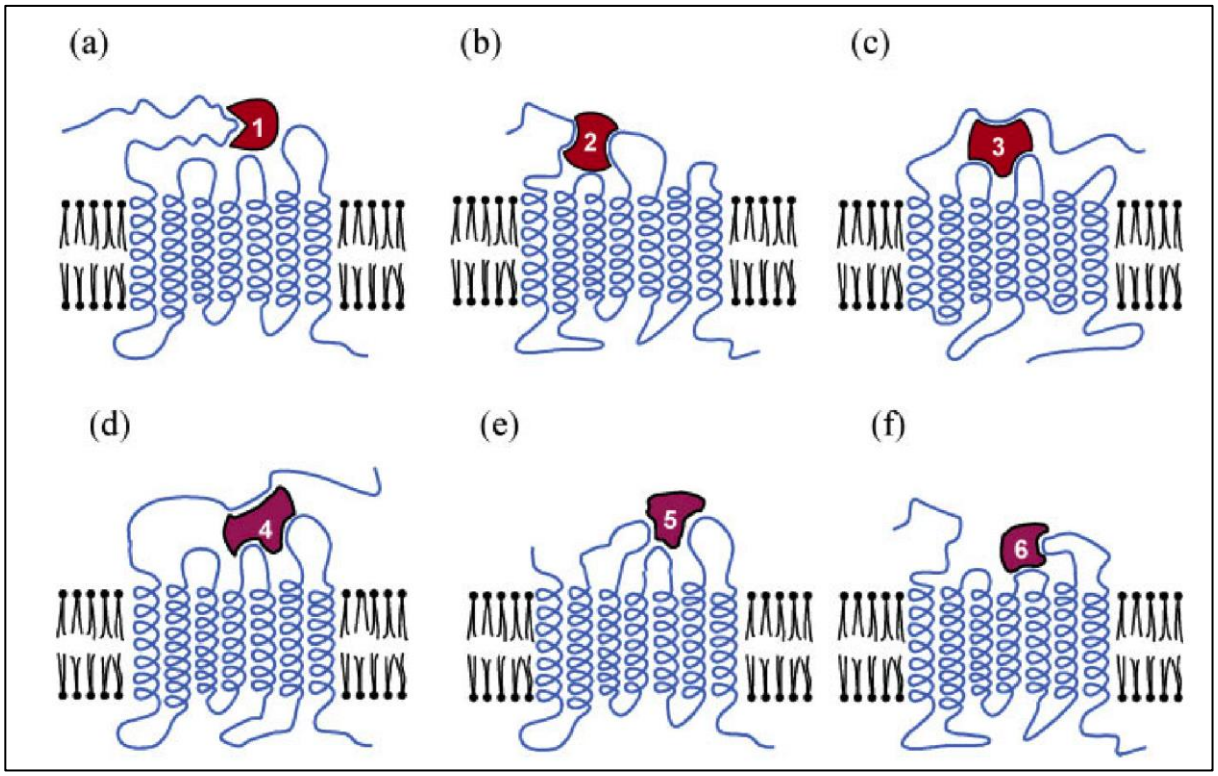

Figure 3. Schematic showing the difference in six main family classes of GPCRs: a) rhodopsin-like, b) secretin- like, c) metabotrophic/glutamate, d) fungal pheromone, e) cAMP receptor, and f) frizzled/smoothened family[1]

Two different types of cross-validation tests are; the re-substitution test and the jack-knife test. Of these two statistical methods, the jack-knife, is thought of as the most exact and unbiased of cross-validation tests.[2] The overall success rate, for identification by the jack-knife test within the data set of 1,238 GPCRs was 97.42%. The researcher believes that this accuracy, implies that the families of GPCRs are correlated by their amino acid sequence.[2] However, the covariant-discriminant algorithm used in these predictions is still in need of improvement.[1]

A different type of predictor was developed by Brooks *et al.*, in an attempt to improve upon the covariant discriminant. It is called the GPCR-Cellular Automaton. This predictor uses cellular

automaton (CA) images, to find patterns and hidden features in the complex protein composition.[1] There are two levels to this predictor. The first is, to determine if the protein sequence comes from a GPCR. The algorithm will then automatically move to the next prediction engine and classify which of the six GPCR families the sequence belongs.[1] Once again, the G protein coupled receptor database was used to create the benchmark data set. However, the pseudo amino acid composition is used to represent the protein sample. A set of data from a pseudo amino acid sequence will have the 20 known amino acids but will also include a λ term. This term, will account for the sequence-order information, usually lost by using the conventional 20 amino acids.[1] The cellular automaton images (Figure 4) were analyzed by a new approach called the gray level co-occurrence matrix (GLCM). This method, essentially, is able to characterize the texture of an image statistically, at different pixels and at different locations.[1] Each class of GPCR produces a CA image, with similar textures not found in the other families. Thus, a predictor, can determine which textures belong to which class of GPCR. The jack-knife test was used to cross-validate the method, and examine the usefulness of the predictor.[1] The results of the predictor after testing 730 proteins was 91.64% (Table 1).

There are many challenges involved when trying to determine classification or function of a GPCR. Due to the costly and time-consuming nature of new NMR and X-ray crystallography techniques, it is desirable for a computational method that can efficiently and effectively identify functional families of GPCRs. Classifying an unknown sample of GPCR without having to determine protein sequence is the main advantage of using cellular automaton images and GLCM approach. Brooks *et al*., have made a web-server that is accessible to the public for free online[22], it includes the GPCR-CA predictor.[1] This predictor could have a major influence on the field of pharmacology, by computationally classifying the many GPCRs targeted by therapeutic drugs.

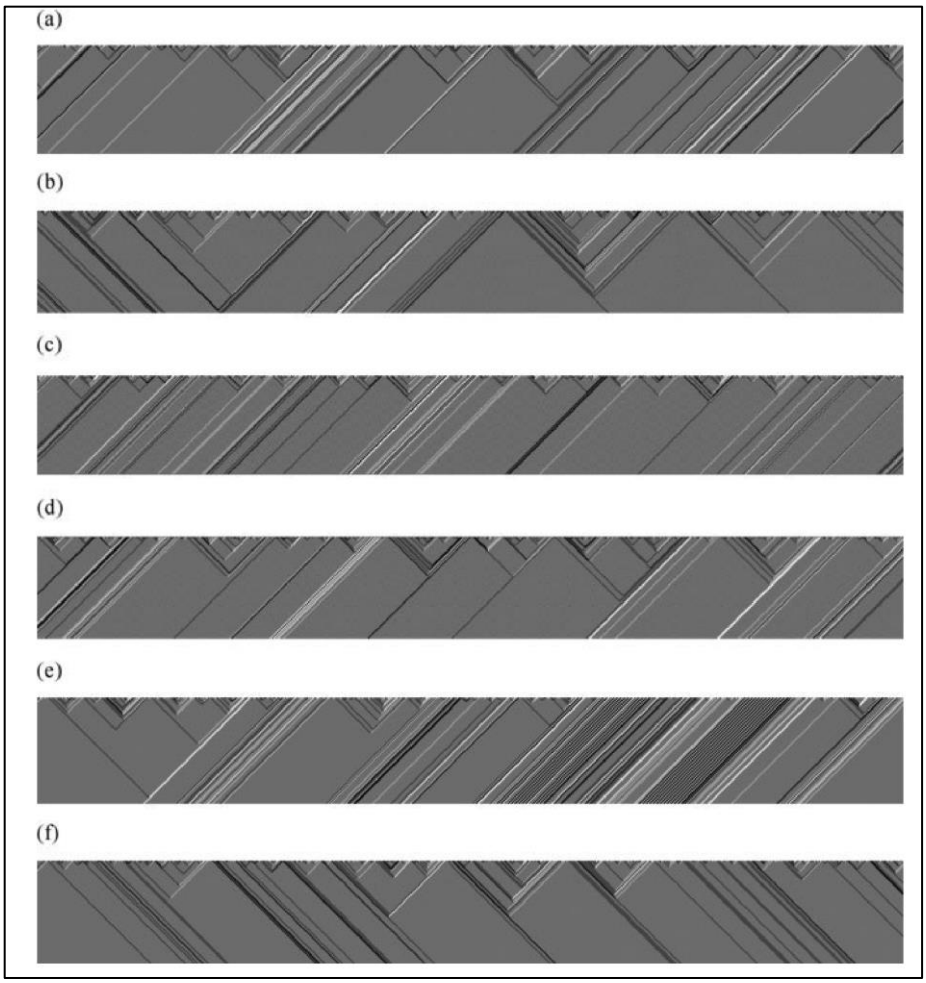

Figure 4. Cellular automaton images according to GPCR classes. a) rhodopsin-like, b) secretin-like, c) metabotrophic/glutamate, d) fungal pheromone, e) cAMP receptor, and f) frizzled/smoothened family[1]

Table 1. Results found using GPCR-CA predictor and jack-knife test for both GPCR and non-GPCR proteins[1]

Protein type	Number of proteins	Number of correct predictions	Success rate (%)
GPCR	365[b]	337	92.33
Non-GPCR	365[c]	332	90.96
Overall	730	669	91.64

The classification of GPCRs is only the beginning when developing new therapeutic drug targets. Data about the structure of GPCRs and the details about the states in which they exist, is essential to understanding more about how the activation mechanism works. In the past, the mystery of why GPCR structure determination was so difficult, is caused by the significant conformational plasticity of GPCRs.[12] The ability to adopt many different conformations, or conformational plasticity, is what makes GPCRs crystallization so complex when trying to determine their structure.[3] However, improved crystallographic techniques have led to better structural studies of GPCRs.[3] NMR and electron paramagnetic resonance, along with holo-form X-ray crystal structures are also some of the techniques that have made great lengths in contributing to the understanding of GPCR signaling.[3,4] It is well understood that for activation of a GPCR, a conformational change must take place.[13] Crystallographic data and other structure determining techniques, have led to the conclusion of unique conformation states of GPCRs. As the techniques continue to improve, researches will continue to get more accurate structures. This is verified by the 42 distinct receptors have been determined.[3] The receptors seem to exist in three states: active states, active-like states, or inactive states. Active states, are defined as the conformation that the receptor takes when it is able to interact with the heterotrimeric G proteins. There are many possible types of "active intermediate" states, but they are all steps that are leading to active state conformations and allow G-protein interaction. Inactive states, can be identified as conformations that have some type of blockage involving their G protein binding surface making them unable to interact with $G_{\alpha\beta\gamma}$.[3] From studying the structures of different receptor states, researchers are able to gather a better understanding of the aspects of GPCR activation.

Based on the activation mechanism, it is assumed that the lowest energy state of GPCRs is in the inactive conformation of the receptor. Support for this generality, are the numerous GPCRs

that have been crystallized in the inactive state.[3] Active-state structures are difficult to determine with agonist binding alone. They have primarily been established with the help of proteins that stabilize the active conformation. These proteins are known as GPCR-interacting proteins (GIPs) and contain receptor interaction domains that maintain the signaling mechanism.[8] G proteins and GIPs, are required to stabilize the active state. Proven by agonist bound receptors with the absence of G proteins the GPCRs crystallized in the inactive state.[10] A strategy, worthy of mention, in the complicated process of active-state structure determination is the use of nanobodies.[3] Generally, it has been determined that agonist binding does increase the tendency for GPCRs to adopt the active conformation; however, a bound agonist on its own is not enough to stabilize the active state of the receptor.[3] Looking carefully at GPCR activation indicates that, for the receptor to accommodate binding of the alpha subunit in the heterotrimeric G protein, transmembrane helix 6 (TM6) must rotate and be displaced (Figure 5). Receptors from other families share similar structures in the activated state, including the transmembrane helix 6 rotation. Comparing inactive and active state crystal structures for class A receptors, shows the presence of a salt bridge, the binding of a sodium ion to an aspartic acid residue stabilizes the inactive state of the receptor.[12] The stabilization of the inactive state is due to this conserved salt bridge or "ionic lock", and is found at the intracellular end of transmembrane 3 (Figure 6).[3] It is highly likely that the ionic interactions from the salts are what leads to the stability of this state. The ionic lock, is able to establish an energy minimum for the inactive conformation. In active receptor conformations, a comparable feature to the ionic lock seems to exist, called the "water lock" (Figure 5).

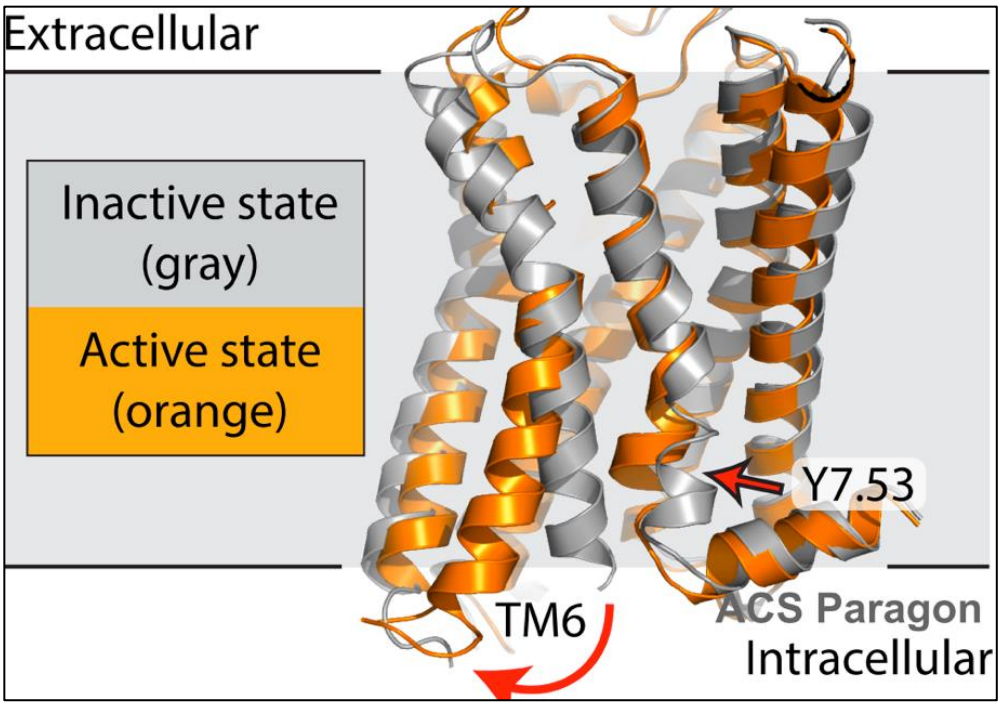

Figure 5. Inactive/Active state comparison of acetylcholine receptor. Red arrow indicates conformational change upon activation[3]

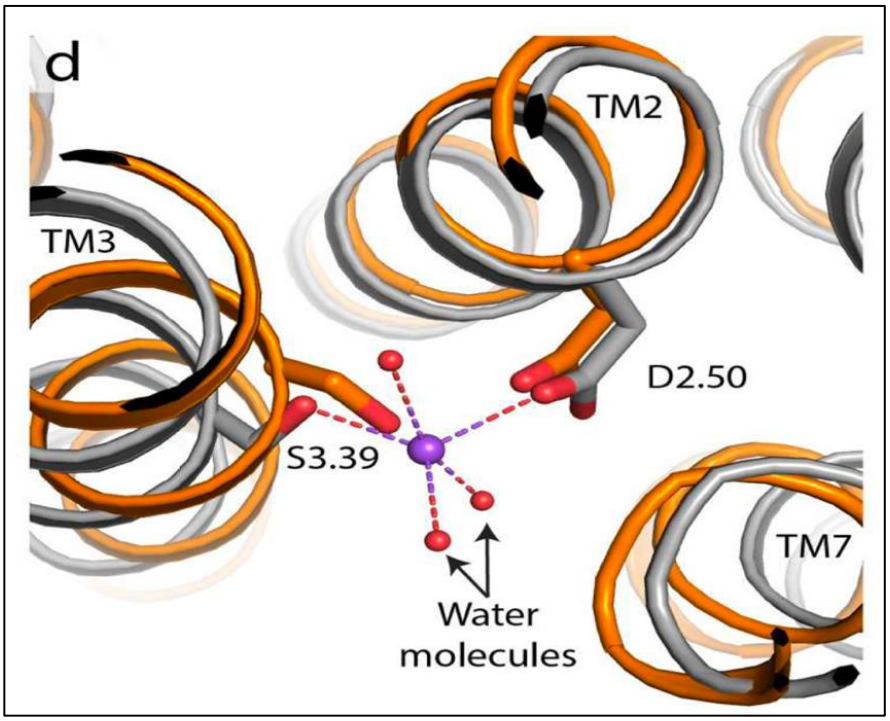

Figure 6. Sodium ion stabilization of inactive state receptor (gray). Water lock for active state receptor (orange).[3]

Hydrogen bonds, connecting a transmembrane 7 tyrosine to a transmembrane 5 tyrosine through a bridging water molecule.[3] The intermolecular forces of the water lock have similar stabilization roles to the ionic lock for the active state. Structural sodium ions (S3.39 in Figure 6) can be found in most GPCRs connected by amino acid residues of transmembrane 2 and 3 sequences. Conformational rearrangement by the receptor when activated breaks the bonds connecting the sodium ions. Sodium ions serve as negative allosteric modulators of GPCR activation. They decrease agonist affinity and stabilize the inactive state of the receptor.[3]

GPCRs facilitate the physiological responses to a plethora of cellular signals including: neurotransmitters, hormones, and sensory stimuli (light, odor, and taste). The transmission of signal into the cell completed by GPCRs is crucial to cellular communication. The mechanism that mediates this response is complex and involves many parts to ensure completion. The foundation of the activation of GPCRs lies in the change in conformation from an inactive to an active state upon ligand binding (agonist binding).[13] Thanks to the increase of crystal structures identified by improved crystallographic and similar techniques, the elements involved in GPCR signaling have been made clear. However, questions about GPCR interactions at the cellular level, and the role of protein dynamics involving receptors are still controversially debated.[4] To answer these questions the dynamics of GPCRs must be investigated.

Ch. 2: GPCR Dynamics and Labeling:

While crystallography is the primary method for spatial information of GPCRs the receptor must be crystallized and cannot exist in solution.[5] Owing to their static nature, crystal structures offer very little information about the dynamics of conformations in the living cell. Comprehension of molecular details involving ligand binding is essential for better discernment of receptor

function. More information about the dynamics in vitro will ideally lead to the design and development of innovative therapeutic medications.[7] In order to monitor ligand-receptor binding in living cells some type of labeling of either the receptor or the ligand is needed. Radioactive and fluorescent compounds are the best approach to use in this type of situation. There are many approaches to using fluorescent/radioactive probes to study ligand-receptor or receptor-receptor dynamics. GPCR dynamics studies have required the development of fluorescence energy resonance transfer (FRET), radioactive ligand, and time-resolved FRET approaches.[8] There are advantages and disadvantages of using either radioactive or fluorescent ligands to study ligand-receptor interactions.

FRET, one of the more successful approaches using luminescent probes to study ligand or receptor-receptor interactions, takes advantage of the energy transfer process of chromophores.[8] As the name suggests, after labeling the GPCR and ligand with specific chromophores (light sensitive molecules) a resonance energy transfer process can be engaged, if the chromophores are close enough (Figure 7).[8] The resonance energy transfer process, diverges from the typical ideas of how fluorescence is portrayed. Rather than one molecule absorbing and emitting the energy from the radiation, there is an energy transfer between two molecules. The fluorescently tagged chromophore on the receptor absorbs the energy from the radiation, and when a fluorescently tagged ligand chromophore is close enough, the energy is transferred and emitted by the ligand chromophore. Designing a fluorescent GPCR ligand is not a simple process. The binding sites of GPCRs are different for each class (Figure 3), and the fluorescently tagged ligands used must account for structure activity relationships in order to have a high binding affinity.[1,8] The size of the fluorophore is an important parameter when choosing ligands.[14] This is confirmed by the majority of ligands being smaller molecules. When done correctly the fluorescent derivatives are

able to maintain high affinities. GPCR fluorescent ligand chemistry, has been advanced by the synthesis of low molecular weight organic dyes, with the ability to adjust the fluorescent signaling that fits the instrument readout requirements.[8]

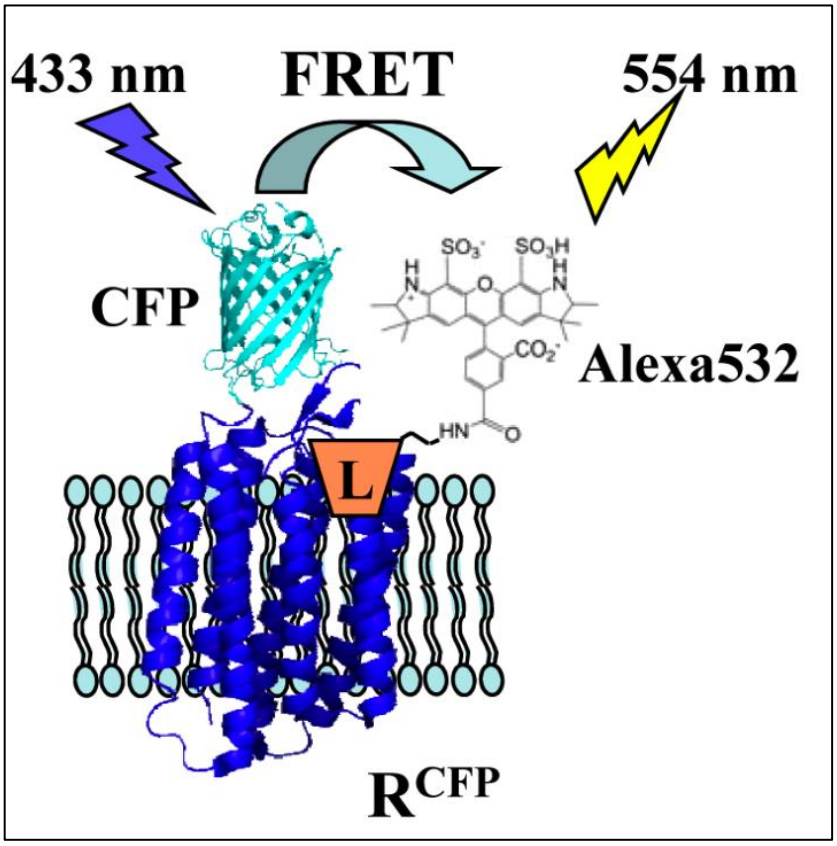

Figure 7. Schematic representation of FRET between a fluorescent ligand and a corresponding fluorescently tagged receptor[8]

Researchers are looking for linkers and dyes that have higher chemical and photostability with donors which can lead to multi-wavelength detection. Possible fluorophores for small GPCR ligands and peptide ligands are quantum dots, which are an alternative to the classic dyes being used. These dots, are fluorescent nanotube semiconductors with possible emission over the visible and near infrared range.[8] Quantum dots are larger in size, which could be a problem when studying membranes in vitro, because they can impede diffusion across the membrane. The synthesis of small fluorescent ligands of GPCRs is significant in the FRET approach with many challenges

dealing with the affinity and efficacy of the ligand. However, when done properly it helps to further our interpretation of ligand receptor interactions of GPCRs in the cell.

Another type of compound used for the tagging of GPCR ligands, are radioactive or isotope labeled ligands.[8] The very existence of adrenergic (class C) receptors on the cell membrane, was revealed by using radioactive high-affinity ligands.[5] When using radioactive ligands (radioligands), a separation of the free ligand and bound ligand is required. The sensitivity of studying ligand-receptor interactions, is better in radioactive ligands than in FRET based approaches.[8] However, while the sensitivity for radioligands is greater than FRET the hazards involved in the use and disposal of radioligands makes them less appealing than fluorescent approaches.[5] The use of fluorescent tracers to visualize GPCRs has advantages over radioactively labeled ligands. Fluorescent ligands have easier delivery and disposal of materials, a longer shelf life, and shorter signal acquisition times needed to reach an acceptable sensitivity. They can also provide instantaneous real time readouts of the ligand-receptor interactions. Overall, radioactive ligands are more expensive, time consuming, and difficult to miniaturize or automate.[8] Clearly, using fluorescent tracers to visualize GPCRs enhances safety and reduces costs.

Due to the decreased sensitivity of FRET approaches and the hazards of radioactive approaches in ligand-receptor interface studies, researchers investigated ways of improving the sensitivity.[8] Time-resolved FRET (TR-FRET) has provided a potential solution to the sensitivity problem of FRET.[5] TR-FRET uses lanthanides, like europium (Eu^{3+}) or terbium (Tb^{3+}) complexed with chelates as donor molecules in binding to the ligand. The long-lived emission found in these lanthanides allows for separation of the excitation and detection events in the classic FRET approach.[8] The excellent fluorescent properties of lanthanide complexes make it possible for the development of a high throughput screening (HTS), which uses robotics and data processors to

conduct millions of chemical, genetic, or pharmacological tests.[14] High throughput time-resolved FRET assays have been used to scan for drug candidates.[5] Generally, time-resolved FRET assays has overcome classical FRET approaches largely because of its temporal selectivity and spectral affinity.[8]

One of the biggest challenges for modern pharmacology is the study of receptor-receptor interactions (i.e. GPCR dimerization/oligomerization).[8] It is well understood that GPCRs will have receptor-receptor interactions in certain environments. The detection of receptor oligomer complexes on the plasma membrane of living cells and how oligomerization impact receptor function is critical to GPCR pharmacology.[8] Fluorescent probes used by FRET have begun a new and productive way to study GPCR oligomerization.

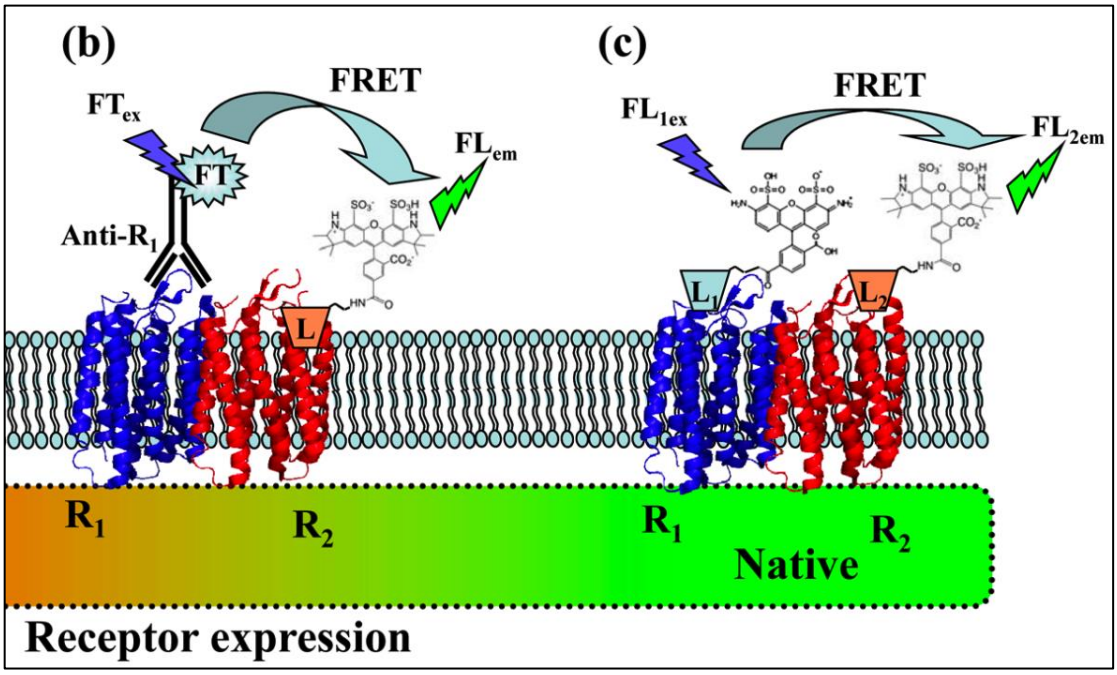

Figure 8. Schematic representation of FRET-based methods using fluorescent ligands applied to the study of GPCR oligomerization on the cell surface of living cells.[8]

The method seen in Figure 8, represents the FRET based mechanism of studying GPCR oligomerization. This process includes a receptor with a fluorescent tag (FT) in the extracellular

region involved in a FRET process with the fluorescent ligand (FL) of the second receptor (R2). The receptors forming the oligomer can be detected with FRET fluorescent ligands (FL1 and FL2), which after the receptors bind to each other the FRET process is engaged. Interestingly, the approach shown in b and c of Figure 8, find ability to detect oligomeric complexes on the plasma membrane not only in heterologous systems but also in native tissues.[8] This is verified by a study with the oxytocin receptor in rats, where europium and another fluorescent ligand tag discovered the oligomerization of the receptor in both transfected cells and in the rat's mammary gland.[5] Receptor visualization is important not only for the development of new drug targets in which receptor-receptor interactions are involved, but also in examining GPCR trafficking, functionality, and biosynthesis/degredation.[8] The possibility that GPCR oligomers have unique targets, opens new possibilities for precisely targeting individual pathologies. Chabre *et al*., has also noted that the heterodimerization could upset the pharmacological specificity of the receptors.[15] The growth and elaboration of new and groundbreaking tools will stimulate many advances in modern pharmacology.[8]

Another use of fluorescently labeled receptors is in examining the conformational dynamics of GPCRs, and is completed by Bockenhauer *et al* to study the dynamics of β_2-adrenergic receptors from class C.[10] The conformational plasticity of GPCRs is very important, and has been investigated in many studies involving conformational dynamics or molecular dynamics over long time periods.[3] Manglik *et al*, also mention the importance of noting that even though receptors fall into classes of active and inactive states, structural data can only represent a small percentage of receptor conformations, and that additional states do exist but cannot be characterized structurally.[3] The researchers under Bockenhauer *et al*., developed a unique apparatus to observe the single labeled adrenergic receptor for a short period of time. This

apparatus is called the Anti-Brownian Electrokinetic (ABEL) trap.[10] The ABEL trap uses fast electrokinetic feedback through a microfluid to excite the fluorescently tagged receptor (Figure 9).

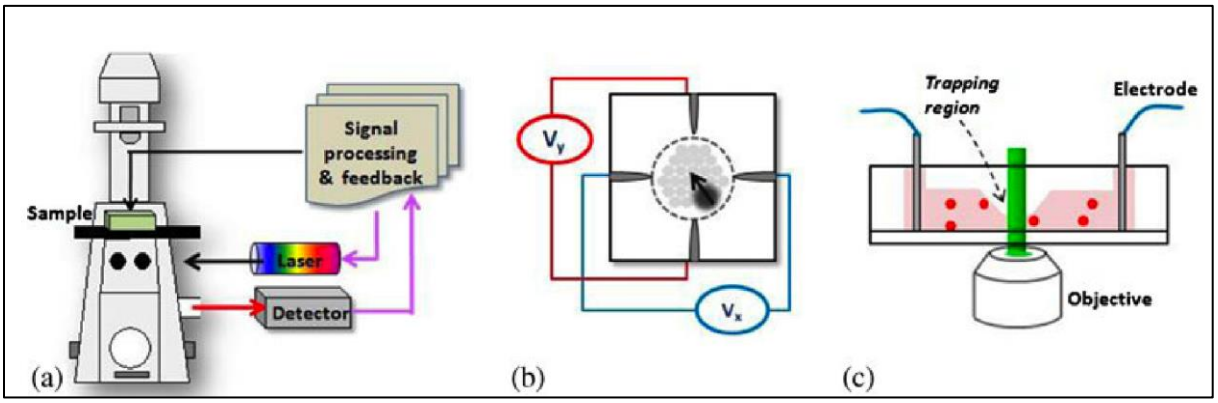

Figure 9. a) Layout of the feedback-controlled ABEL trap, b) top view of trapping cell, c) side view of the microfluidic cell.[16]

Essentially, the ABEL trap combines a fluorescence-based estimation of the target object with fast electrokinetic feedback to counter the Brownian motion of the object.[16] In a feedback- controlled ABEL device, confocal microscopy tracks the position of the molecule from the fluorescent signal, and engages feedback-controlled displacement to ensure minimal movement of the molecule. Consequently, the ABEL trap works better when trapping smaller objects. After choosing a fluorescent dye and labeling the site of ligand binding, a readout of discrete fluorescence intensity and lifetime levels in single β_2-adrenergic receptors (Figure 10).[10] The discrete intensity steps/peaks seen in Figure 10 (blue peaks) of ligand free receptor indicate multiple states existence for GPCRs. From this data we can

conclude that GPCRs are extremely dynamic transmembrane proteins that exist in multiple atomic-scale conformations. Even before an agonist binds to the receptor, they are constantly switching between the inactive and active states with many possible intermediate states.[10] To further understand how agonist binding influences conformational change in GPCRs, Bockenhauer *et al.* used the ABEL trap to study the agonist bound receptors. The data from this experiment can be

seen in Figure 11 and illustrates the mean dwell times with ligand free or with agonist bound receptors. The conclusions from this graph show that agonist binding does increase the dwell time of the active state of the receptor, however, without G-proteins to stabilize the active state the receptor will switch back to the inactive conformation.[10]

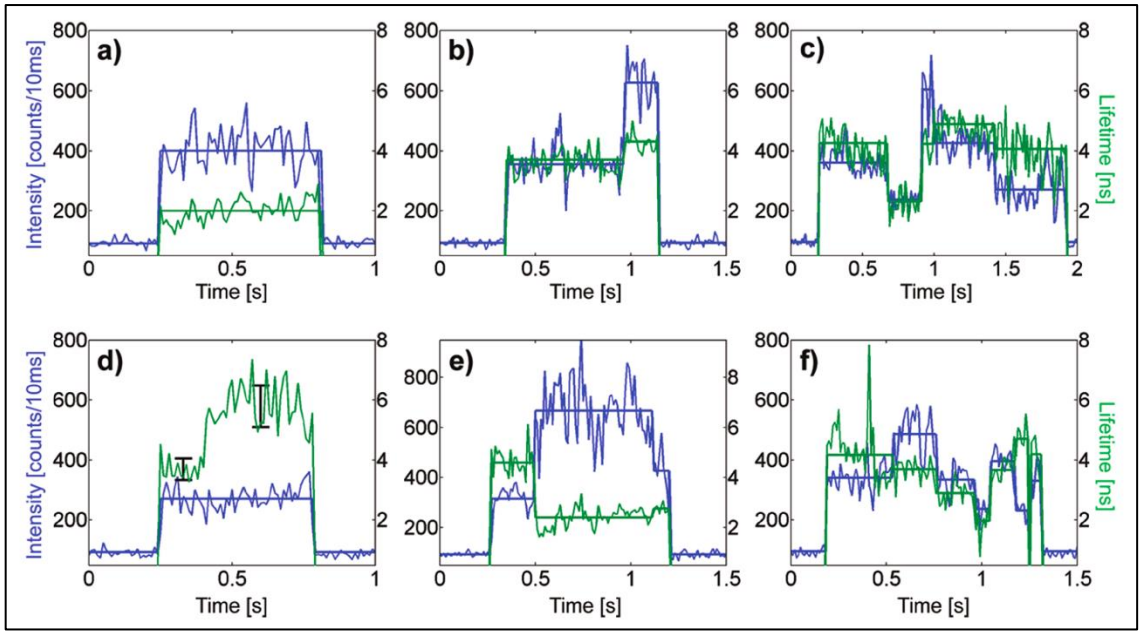

Figure 10. Graph of intensity-lifetime traces from single trapped ligand free-receptors. Real-time intensity (blue) and fluorescence lifetime (green). b,c) correlated intensity, d) uncorrelated, e) anticorrelated[10]

This is verified by the fact that active state GPCR structures were determined largely with help from proteins to stabilize the active conformation.[3] Furthermore, in the absence of G-proteins agonist bound GPCRs were crystallized in the inactive state.[10]

There are many challenges involved in novel drug design and development for modern pharmacology. As GPCRs are the target of over 30% of drug screening targets[14], data from FRET/TR-FRET, radioactive ligands, and conformational dynamics will continue to stimulate breakthroughs in the development of novel therapeutic compounds. One thing is certain, the development and improvement of innovative and inventive tools will lead to greater progress in

the field.[10] An understanding of the receptor-ligand binding affinity for possible drug targets is one of the largest hurdles for researchers in the drug design field.[14] Based on current knowledge of GPCRs such as serotonin, β_2-adrenergic, or angiotensin indicates that receptor dynamics are decisive to understanding their function.[17]

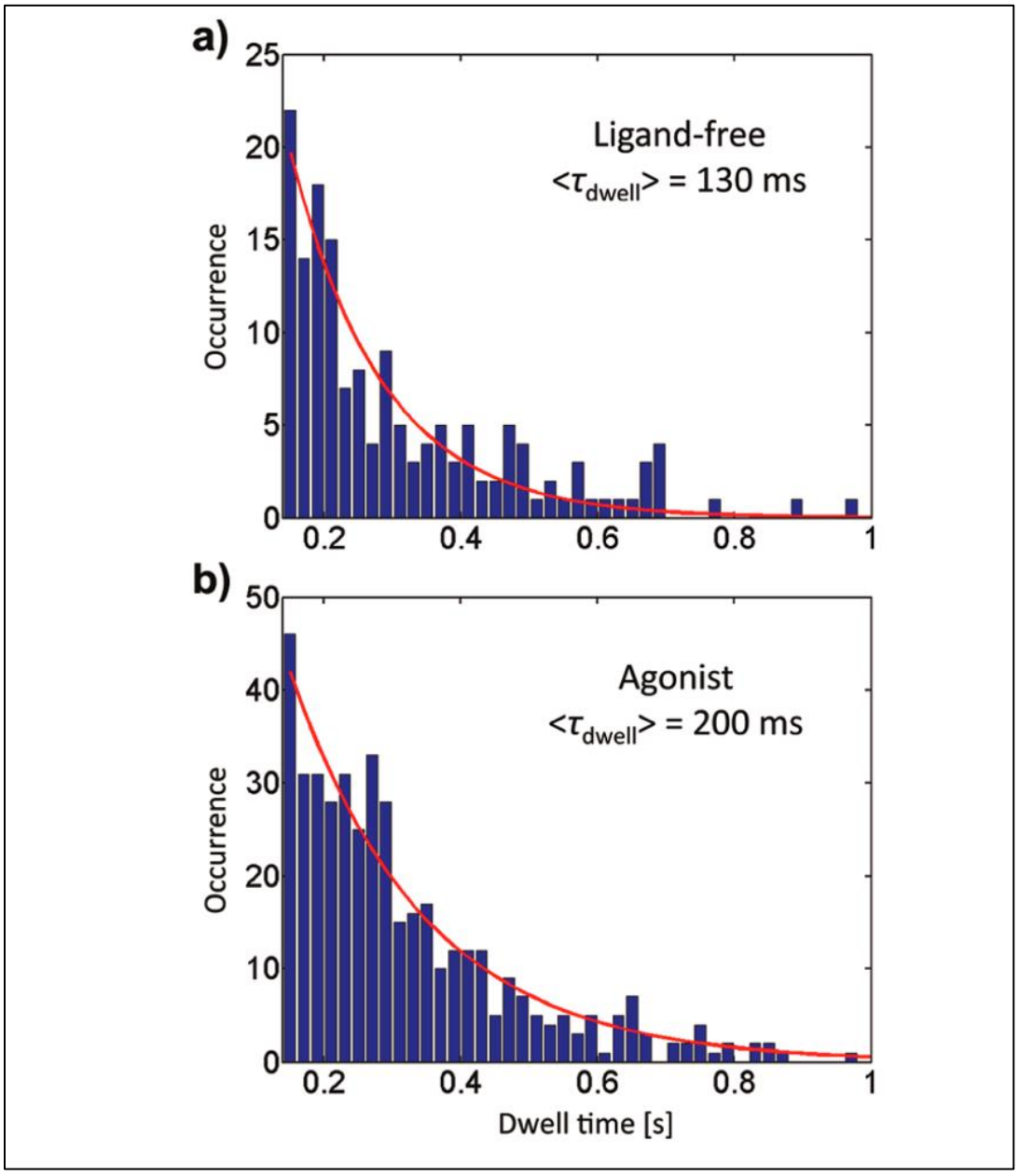

Figure 11. Histograms of dwell times for ligand free (a) and agonist bound receptors (b).[12]

Ch. 3: Allosteric Modulators

When developing any drug, studying the effects of this drug in a system directly, or in association with another drug, is the best approach for discovering the mechanism of action of the drug.[9] Researchers can then compare the responses to a mathematical model of drug-receptor interaction. Once the data and the model agree the researchers have found one possible mechanism of action for the drug. When the data and the model, do not agree the model can be rejected or improved until the mechanism matches the prediction by the model.[9] The various physiological roles that GPCRs are involved in, and the evidence of signaling in several pathological conditions explain why they are the therapeutic target of more than 30% of drugs on the market today.[13,18] Studies of GPCRs have highlighted two sites of possible ligand binding: the orthosteric site and the allosteric site (Figure 12).

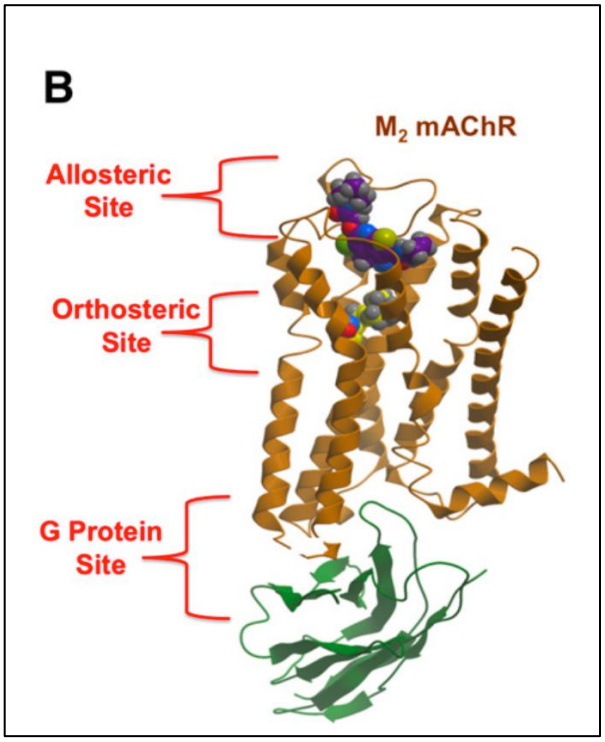

Figure 12. Schematic of the M_2-Acetylcholine receptor with identified allosteric, orthosteric, and G protein sites.[19]

Traditional GPCR drug discovery, has focused on targeting the orthosteric site.[12] Endogenous ligands, are compounds that are not introduced to the body, but are produced by the body such as: neurotransmitters, peptides, hormones, ions, odorants, etc. These ligands are bound at the orthosteric site, and are the targets of many therapeutic medications involving GPCRs.[12] Allosteric modulation of GPCRs by small molecules influencing the effects of endogenous agonists has been recognized for a long time.[11] The binding of the small sodium ion, established by the comparison of inactive and active state crystal structures, has shown allosteric stabilizing tendencies for inactive state receptors. When talking about an allosteric modulator they are mainly depicted as, any species that is capable of selecting a specific conformation and changing orthosteric ligand affinity/efficacy.[13] The idea of allosterism was officially accepted with the Monod-Wyman-Changeux (MWC) model. This model proposes a different mechanism of receptor conformational selectivity to account for the action of ligands in the presence of regulatory enzymes.[13] The mathematical model used in determining the mechanism of action for allosteric modulators can be seen in Figure 13. As the numbers of allosteric modulators being tested in humans increases, development of more accurate quantitative models, like equation 57, of are needed to decrease the rate of failure in new drugs.[9]

$$\text{response} = \frac{\frac{\tau_A[A]}{K_A}\left(1 + \frac{\alpha\beta[B]}{K_B}\right) + \frac{\tau_B[B]}{K_B}}{\frac{[A]}{K_A}\left\{1 + \frac{\alpha[B]}{K_B} + \tau_A\left(1 + \frac{\alpha\beta[B]}{K_B}\right)\right\} + \frac{[B]}{K_B}(1 + \tau_B) + 1} \quad (57)$$

Figure 13. Equation 57 describes the effects of an allosteric modulator. τ_B is the direct production of receptor response, the α term accounts for altering affinity of the agonist, and β models the efficacy of the agonist.[9]

Potentially all GPCRs contain topographically unique allosteric binding sites, targeting these sites presents an opportunity to increase subtype selectivity of drugs.[13] Allosteric modulators attached to allosteric binding sites of GPCRs provides a conceivable method of drugs with differential selectivity and better safety than traditional orthosteric ligands.[12] Allosteric modulators are not competition with endogenous ligands because they bind to a completely different site on the receptor. Traditional drugs that target orthosteric sites completely occupy the site when they are bound, causing complete shutoff of a receptor or complete activation of a receptor. Allosteric ligands influence receptors at the same as orthosteric ligands. Because they work in a concerted fashion, the allosteric ligand is able to regulate receptor activity and prevent the extremes of completely shutting off or activating a receptor. This modulation may lead to a decrease in the likelihood of side effects.[12] Allosteric modulators are also less prone to overdose because of the limitations of the modulatory effect.[13] One of the proposed solutions of the development of tolerance for the well-known analgesic morphine is the lack of ability to internalize the receptor caused by the drug. The non-competitive nature of allosteric modulators with ligands bound in the orthosteric site, could potentially allow them to induce or prevent receptor internalization that is compromised by morphine.[13] Two allosteric modulators are currently available to the public with FDA approval. Maraviroc[20] treats patients with HIV by targeting the chemokine receptor (Figure 14), and cinacalcet that targets the calcium sensing receptor to treat hyperthyroidism.[13]

Despite the approval of these two drugs, the therapeutic relevance of allosteric modulators in GPCRs still needs further investigation.[13] There are many challenges that involved in the finding of allosteric modulators.[12] A key part in any drug's is understanding the effects of drug in an acute vs chronic application. Assuming many of these allosteric modulators will be administered chronically, a better understanding of receptor regulation pathways is needed.[13] Other challenges

include low modulator receptor activity, low binding affinities, and the emergence of drug resistant mutations to name a few.[12] The progress made by crystallography has determined 14 distinct allosteric sites[12], however, there are hundreds of GPCRs and most of them are yet to be crystallized. One solution to the struggle of crystallographic allosteric site determination is the use of a computational identifier. Information of the three-dimensional structure of GPCRs is crucial to understanding how they function, ligand-receptor binding, and to improve the design and development of drugs like allosteric modulators.[21]

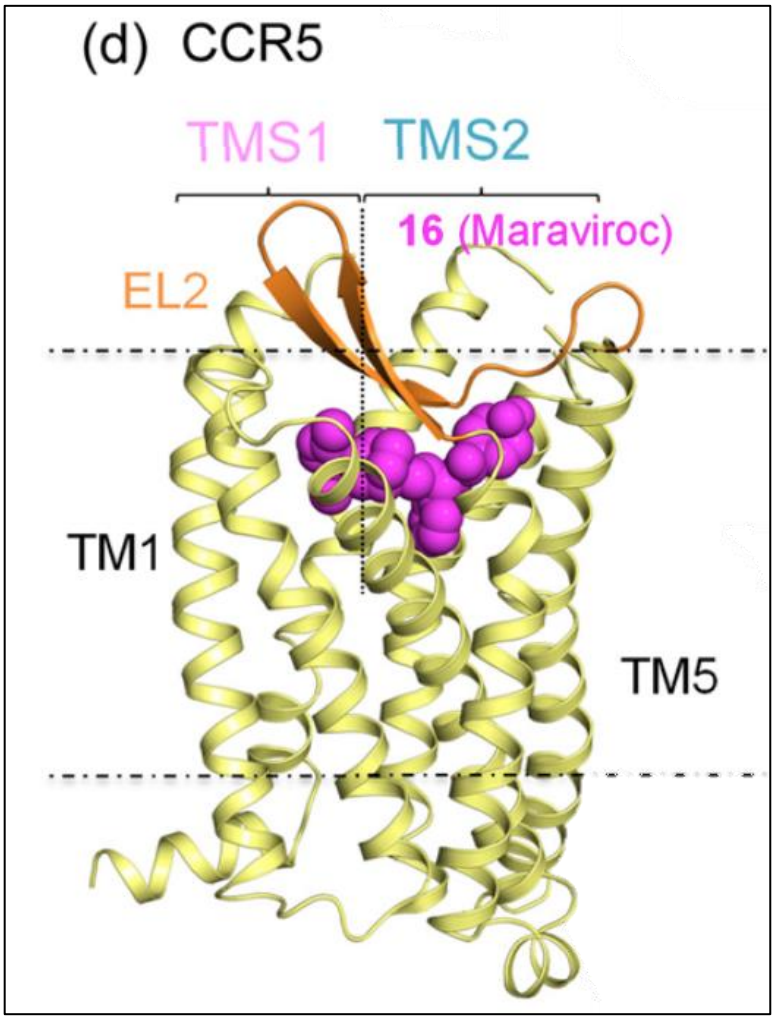

Figure 14. Structure of chemokine receptor CCR5 and the binding pocket of Maraviroc (purple).[20]

It is important to note that without proper preparation and storage of GPCRs studying the function and drug delivery processes is impossible. The trouble of studying membrane proteins like GPCRs is how they are located in the plasma membrane.[17] Extraction of GPCRs from their native environment and stabilizing the proteins by placing them in micelles that mimic the cell membrane. The protein is fragile once removed from its native environment and if done improperly the integrity of the protein will be compromised. One of the techniques to counter this fragility is lyophilization (freeze-drying), where a sample is placed in a vacuum at cryogenic temperatures and dehydrated. This method can turn an aqueous solution of proteins into a powder by removing water via a sublimation process.[17] Freeze-drying eludes the potentially detrimental thawing of the proteins because the water is removed from the solution. Applications of these powdered GPCRs potentially involve; solid state NMR, studies of receptor signaling, dry powder inhaling, and drug design.

Discussion:

The amount of physiological regulation that GPCR signals is most likely the reason that they are the target of so many therapeutic drugs. They are targeted in many different drugs on the market today, for example, antihistamines, antidepressants, and many others. I believe that the reason that researchers are so interested in GPCRs is because of the possibilities that they offer. G protein coupled receptors are not well enough understood to truly appreciate just how helpful they could be in developing unique therapeutic compounds. The difficulty in understanding GPCRs has made it harder for researchers to fully understand how they work in the cell.

Researchers are trying hard to get as much data as possible about GPCR structure, conformations, and interactions on the cell membrane. I believe that only with a combination of static structural data and dynamic interaction data will researchers truly be able to develop better

drugs. I am convinced that, of the many methods of determining protein three-dimensional structure the most valuable regarding GPCRs is X-ray crystallography. Based on number of receptors determined, no other technique could match X-ray crystallography. While, the computational techniques mentioned in some papers do show promise, but they are lacking to be used efficiently unless improved on.

Arguably the most important focus of pharmaceutical companies funding research of GPCRs should be studies involved ligand-receptor or receptor-receptor interactions in vitro. While the crystallographic data is also extremely important; however, I believe that studies involving FRET or similar methods are more valuable to drug development. Simply because there is a such a risk of not understanding how receptors and ligands behave in cellular conditions. Both structural and dynamics data are needed in drug design and improvement, but if deciding where to prioritize funding on I would choose the dynamic data over the structural data.

Concerning allosteric modulators, I can definitely see the reason why millions of dollars is being spent on their research. When they function properly they can alleviate side effects and make overdose highly unlikely. However, the problem arises when we consider their binding affinity to the receptor. Binding in the allosteric site of GPCRs is not easy. The reason that traditional drug techniques target the orthosteric site is because if functions to bind to endogenous ligands. The drugs developed hijack this orthosteric site and ensure a high binding affinity. The allosteric site of GPCRs functions in a regulative way. It monitors receptor behavior and can control deviations with small allosteric ligands that can communicate with the cell. If researchers can find a way to ensure allosteric site binding, then their improved safety over traditional orthosteric ligands is significant. However, until then I believe pharmaceutical companies will stick with orthosteric ligands because they work.

Overall, the study of G protein coupled receptors continues to be of major interest for modern pharmacology. The impact of GPCRs in cellular communication and other regulatory roles they play emphasizes the fundamental biological and clinical importance of this superfamily of proteins. Optimizing structural determination processes like X-ray crystallography will lead to an improvement of the ligands chosen as therapeutic medications. Increasing our understanding of ligand-receptor and receptor-receptor interactions using FRET or TR-FRET methods will continue to stimulate advances in drug-receptor interactions and drug development as a whole. Receptor internalization has also become a major topic in the tolerance of analgesic drugs like morphine and heroin. Better understanding of this receptor internalization could hold to key to the problem of addiction to detrimental narcotics.

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