Structure–Function Studies of Muscarinic Acetylcholine Receptors

Katie Leach, John Simms, Patrick M. Sexton, and Arthur Christopoulos

Abstract There has been great interest in the structure–function relationships of the muscarinic acetylcholine receptors (mAChRs) because these prototypical Family A/class 1 G protein-coupled receptors (GPCRs) are attractive therapeutic targets for both peripheral and central nervous system disorders. A multitude of drugs that act at the mAChRs have been identified over the years, but many of these show minimal selectivity for any one of the five mAChR subtypes over the others, which has hampered their development into therapeutics due to adverse side effects. The lack of drug specificity is primarily due to high sequence similarity in this family of receptor, especially in the orthosteric binding pocket. Thus, there remains an ongoing need for a molecular understanding of how mAChRs bind their ligands, and how selectivity in binding and activation can be achieved. Unfortunately, there remains a paucity of solved high-resolution structures of GPCRs, including the mAChRs, and thus most of our knowledge of structure–function mechanisms related to this receptor family to date has been obtained indirectly through approaches such as mutagenesis. Nonetheless, such studies have revealed a wealth of information that has led to novel insights and may be used to guide future rational drug design campaigns.

Keywords Allosteric • Molecular modeling • Muscarinic receptor • Mutagenesis • Orthosteric • Structure-function

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1 Introduction

GPCRs comprise the largest family of membrane proteins in mammalian organisms (Fredriksson et al. 2003) and respond to a wide range of endogenous and exogenous ligands. Mammalian GPCRs are divided into three main classes based on similarities in their amino acid sequence (Foord et al. 2005). Family A (or class 1) GPCRs, which include the mAChRs, share sequence similarity to rhodopsin; Family B (class 2) GPCRs to secretin receptors; Family C (class 3) GPCRs to metabotropic glutamate receptors. Less than 10% of these three GPCR families already constitute the targets of approximately 30% of all drugs on the market (Harmar et al. 2009; Hopkins and Groom 2002) and, thus, there is ongoing incentive in understanding how the amino acid sequence of these proteins relates to their function and three-dimensional structure in order to facilitate drug discovery. The characteristic structural feature of all these receptors is the presence of an extracellular N-terminal region, intracellular C-terminal region and seven transmembrane (TM)-spanning α-helical domains connected by three extracellular and three intracellular loops. However, until very recently, detailed three-dimensional structural information on GPCRs has been hampered by difficulties in obtaining high-resolution crystal structures of these receptors. This is because they are highly unstable upon removal from their membranous environment and also because they dynamically isomerize between multiple conformations, both of which hinder the crystallization process (Congreve and Marshall 2010). To date, crystallization efforts have been successful for only a few GPCRs; rhodopsin (Palczewski et al. 2000), the β_1 (Warne et al. 2008) and β_2 (Cherezov et al. 2007; Rasmussen et al. 2007; Rosenbaum et al. 2007) adrenergic receptors (β-ARs), and the A_{2A} adenosine receptor (Jaakola et al. 2008).[1] As a consequence, computational approaches, such as homology modeling and associated methods, are the mainstay of rationalizing structural information derived at other GPCRs, such as the mAChR family. However, molecular models, in and of themselves, have only limited utility if not used in conjunction with molecular and biophysical techniques that can help to refine our structural and functional understanding of a protein. In this regard, the mAChRs remain a prototypical Family A GPCR model system that has been extensively explored by site-directed mutagenesis and related approaches, which is the focus of this chapter.

2 Amino Acids That Are Essential for Stabilization of the Receptor Structure

Substitution of amino acids that are essential for the structural stability and folding of a protein can lead to impairment in its assembly, maturation, and/or trafficking. In the mAChRs, substitutions of certain amino acid residues that are conserved

[1] At the time of writing, the crystal structures of antagonist-bound chemokine CXCR4 and dopamine D_3 receptors have been solved but not published.

across all five mAChR subtypes cause a reduction in receptor expression, in particular mutation of $Asp^{2.50}$, $Leu^{3.43}$, $Asp^{3.49}$, $Tyr^{3.51}$, $Trp^{4.50}$, and $Pro^{7.50}$ (Hulme et al. 2001, 2003a; Lu et al. 1997, 2001; Lu and Hulme 1999) [numbering in superscript corresponds to the Ballesteros–Weinstein system (Ballesteros et al. 1995)]. In fact, these residues are highly conserved throughout the TM domains of Family A GPCRs and thus likely serve an important role in maintaining the overall helical structure of these receptors. For instance, in the rhodopsin X-ray crystal structure, $Asp^{2.50}$ interacts with $Asn^{1.50}$ and $Asn^{7.49}$ in a hydrogen-bonded network of residues mediated by water molecules that may assist in the initial folding of the receptor, whilst at later stages of the receptor lifetime these residues are implicated in signaling cascades. $Asp^{3.49}$ and $Tyr^{3.51}$ are two additional residues that are essential for the function of the majority of Family A GPCRs. In the mAChRs, only His, Asn, or Glu substitutions are tolerated at the position of $Asp^{3.49}$, although even in these instances receptor expression levels are reduced. Substitution with any other amino acid at this position generally results in undetectable levels of radioligand binding (Lu et al. 1997), suggesting that this residue is critical for maintaining a receptor conformation able to bind ligand.

In addition to traditional approaches that have relied on rationally guided systematic mutagenesis of the mAChR, a more recent, higher-throughput, random mutagenesis study identified a number of additional mutations that profoundly affected the expression of the M_3 mAChR (Li et al. 2007a), suggesting that there remains much to be learned about the structural determinants of mAChR stability

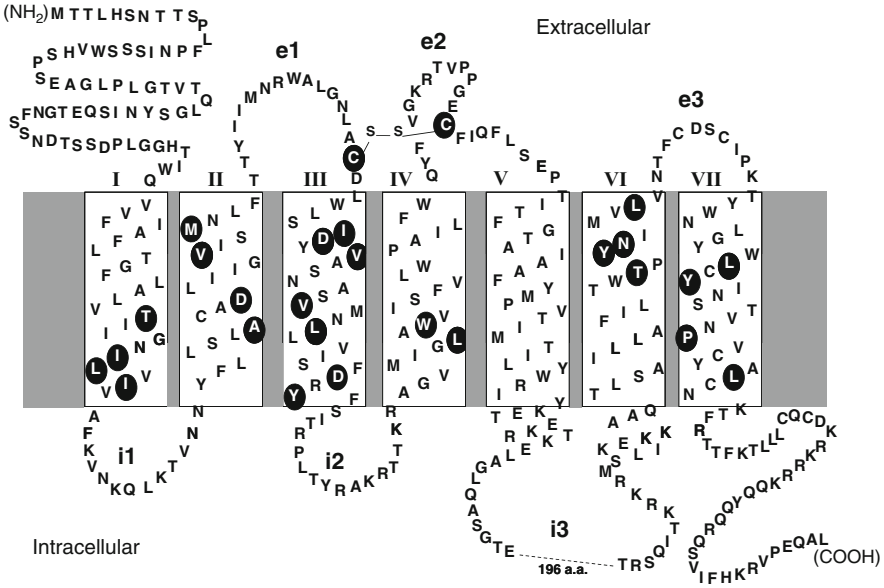

Fig. 1 Snake diagram of the M_3 mAChR. Residues labeled within the *black circles* indicate amino acids that have been implicated in the control of receptor expression

and expression. Many of these substitutions were for proline or charged amino acids, which are likely to disrupt important interactions that are essential for the folding of the receptor, including Thr$^{1.46}$Pro, Ile$^{1.51}$Asn, Leu$^{1.52}$Pro, Ile$^{1.54}$Asn, Ala$^{2.47}$Pro, Val$^{2.55}$Asp, Cys^{140}Gly, Ile$^{3.31}$Asp, Val$^{3.34}$Glu, Leu$^{4.48}$Pro, Cys^{220}Ser, Thr$^{6.49}$Asn, Leu$^{6.56}$Pro, Leu$^{6.56}$Gln, Leu$^{7.41}$Pro, and Leu$^{7.55}$Pro (Fig. 1). Interestingly, mapping these positions onto models of the mAChR revealed that they are adjacent, in either 2D or 3D space, to conserved residues and may affect their local environment. In contrast to the above mutations, another series of positions (Met$^{4.43}$, Leu$^{4.46}$, Leu$^{4.49}$, Ala$^{4.58}$, Phe$^{4.61}$, and Thr$^{7.47}$) have also been shown to contribute to receptor conformation(s) that can result in an increase in the amount of cell surface expression.

3 Elucidation of the mAChR Orthosteric Binding Site

A wide range of structurally diverse ligands bind to the orthosteric site of GPCRs and, as such, a number of different domains may potentially form the ligand binding pocket, depending on the receptor. To date the high-resolution X-ray crystal structures for orthosteric inverse agonist-bound rhodopsin, the β_1- and β_2-ARs, and the A_{2A} adenosine receptor, have all been solved (Palczewski et al. 2000; Cherezov et al. 2007; Rasmussen et al. 2007; Jaakola et al. 2008). Rhodopsin is not a typical GPCR in that its ligand, 11-*cis*-retinal, is covalently bound to Lys$^{7.43}$ via a Schiff base in the inactive form of the receptor. 11-*cis*-retinal also makes a number of additional contacts within the receptor that contribute to a binding pocket that shares similarities with the orthosteric binding site in the β-ARs (Rasmussen et al. 2007), being comprised of residues positioned predominantly in TMIII, TMV, and TMVI. Residues that form the binding crevice in rhodopsin and the β-ARs include 3.28, 3.29, 3.32, 3.33, 3.35, 3.36, 3.37, 5.41, 5.42, 5.43, 5.46, 5.47, 6.44, 6.48, 6.51, 6.52, 6.55, 7.35, 7.39, and 7.40. In contrast, the binding site for the A_{2A} receptor antagonist, ZM241385, is somewhat different and involves residues predominantly located in TMII, TMVI, and TMVII (Jaakola et al. 2008). Mutagenesis data support the hypothesis that the orthosteric binding site in mAChRs closely resembles that of rhodopsin and the β-ARs. Some of the first studies that investigated the location of the ACh binding site in mAChRs involved propylbenzilylcholine and acetylcholine mustards. These alkylating agents were used to highlight an important interaction that occurs between the common ammonium moiety that exists in all biogenic amines and Asp$^{3.32}$ (Curtis et al. 1989; Spalding et al. 1994; Kurtenbach et al. 1990), conserved within TMIII of the biogenic amine receptors. A series of site-directed mutagenic studies have since identified additional amino acids that are equally critical for the binding of ACh to the mAChRs (Lu et al. 2001; Wess et al. 1991; Ward et al. 1999). Generation of a homology model of the M_1 mAChR based on the structure of bovine rhodopsin has predicted residues that most probably form direct contact points for ACh (Fig. 2a), including five residues in particular: Tyr$^{3.33}$, Thr$^{5.39}$, Thr$^{5.42}$, Tyr$^{6.51}$, and Tyr$^{7.39}$

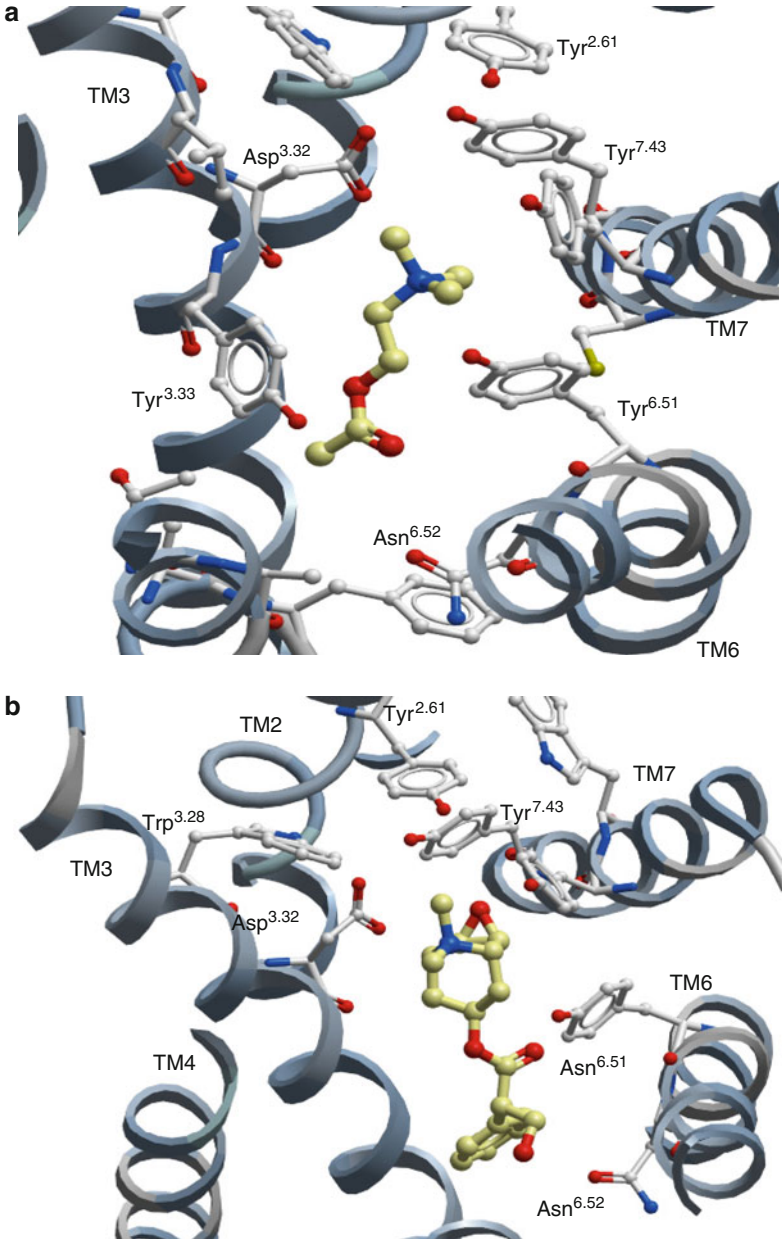

Fig. 2 The orthosteric binding pocket of mAChRs. A homology model of the M_2 mAChR was constructed using the crystal structure of the inactive-state β_2 adrenergic receptor as a template. Docking of (**a**) ACh or (**b**) NMS was performed and the key residues contributing to the respective pockets are also indicated

(Hulme et al. 2003a, b). Further residues that have been implicated in the binding of ACh have also been identified as $Trp^{3.28}$, $Leu^{3.29}$, $Ser^{3.36}$, $Asn^{3.37}$, $Trp^{4.50}$, $Ser^{4.53}$, $Trp^{4.57}$, $Ala^{5.46}$, $Leu^{6.56}$, $Cys^{7.42}$, and $Tyr^{7.43}$ (Hulme et al. 2003a, b; Lu et al. 2001; Hulme and Lu 1998). However, visual inspection of homology models of the mAChR suggests that some of these amino acids lie outside of the orthosteric site and may thus affect the route of entry for the ligand into its main binding site crevice.

Many of the residues that are essential for ACh binding are equally as important for the binding of inverse agonists/antagonists such as *N*-methyl scopolamine (NMS), quinuclidinyl benzilate (QNB) and atropine to the mAChRs (Fig. 2b), although some subtle differences have been observed with regards to amino acids that contribute to the binding of these different ligands. For instance, although $Asn^{6.52}$ is predicted to face into the ligand binding pocket and is important for the binding of atropine and NMS (Ward et al. 1999; Bluml et al. 1994a), it has a lesser role in ACh and QNB binding (Bluml et al. 1994a). Similarly, the binding of QNB is not significantly affected by substitution of $Tyr^{6.51}$ (Ward et al. 1999), $Tyr^{7.39}$, $Cys^{7.42}$, or $Tyr^{7.43}$ (Lu et al. 2001). $Phe^{5.47}$, on the other hand, which does not appear to interact with ACh and QNB, has been predicted to lie in close proximity to NMS and may be positioned at the very bottom of the NMS binding site, which extends deeper into the helical bundle than the ACh binding site (Goodwin et al. 2007). Thus, different ligands clearly form molecular interactions with different amino acid residues.

In addition to the role of the TM domains in binding orthosteric ligands, there is some evidence that the extracellular domains of the mAChRs may contribute structural stability to the orthosteric binding site. Family A GPCRs possess two conserved cysteine residues that form a disulfide bond between the extracellular portion of TMIII and the second extracellular loop of the receptors. In rhodopsin, part of the second extracellular loop folds into the center of the helical bundle, with Glu^{181} (residues that lie outside the TM domains are indicated by their amino acid position) orientated toward 11-*cis*-retinal (Palczewski et al. 2000). Similarly, $Thr^{5.34}$ at the junction of TMV and the second extracellular loop in the β_1-AR is directed toward the ligand binding pocket, suggesting that this extracellular region may form a "cap" to that pocket. Thus, in most mAChR structural models that are based on homology with rhodopsin or the β-ARs, the second extracellular loop of these receptors defines a boundary of the orthosteric binding site that forms a lid-like structure over the top of the crevice. Although substitution of amino acid residues in the second extracellular loop does not significantly alter the binding affinity of prototypical orthosteric ligands, restriction of flexibility of this region in the M_2 mAChR (via engineering of an additional disulfide bond) was shown to substantially hinder the access of ligands such as NMS and ACh to the orthosteric binding site (Avlani et al. 2007). Residues that lie in close proximity to the cysteine residues responsible for the conserved disulfide bond have additionally been implicated in regulating the access of orthosteric ligands into the binding pocket. For instance, substitution of $Asp^{3.26}$ reduces the binding of orthosteric ligands such as ACh, QNB, and NMS (Goodwin et al. 2007). It has been speculated that this

residue may be involved in the initial contact of positively charged orthosteric muscarinic ligands with their receptors before they enter into the main binding site (Lu et al. 2001; Goodwin et al. 2007; Jakubik et al. 2000). Structural predictions of the M_1 mAChR suggest that $Ser^{4.53}$, $Trp^{4.57}$, and $Ile^{4.61}$ lie in close proximity to $Asp^{3.26}$ and may also act to form this "peripheral" binding site (Lu et al. 2001).

The boundary between the top extracellular portion of TMII and the first extracellular loop in the mAChRs has also been implicated in the binding affinity of orthosteric ligands, with substitution of Trp^{99} in the M_1 mAChR for Ala or Phe, or the equivalent Trp^{133} for Gly in the M_3 mAChR, significantly reducing the binding affinity of ACh, NMS, and QNB (Li et al. 2007a; Matsui et al. 1995; Avlani et al. 2010). Similarly, mutation of $Asn^{2.68}$ Ile at the junction of TMII and the first extracellular loop in the M_3 mAChR results in a reduction in the binding of [^{3}H]NMS (Li et al. 2007a). Interestingly, recent in silico studies have suggested that the extracellular loops can have a strong influence on how TM helices pack together and, as such, perturbation of the extracellular loops may have an additional effect on the fine packing in the TM helices; it is thus possible that effects of extracellular loop mutations on orthosteric ligand binding may reflect such indirect perturbations of the orthosteric pocket.

4 Elucidation of mAChR Allosteric Binding Sites

In addition to the orthosteric binding site, it is now well established that GPCRs can possess topographically distinct allosteric sites (May et al. 2007a). Indeed, studies of the phenomenon at the mAChRs represent the earliest known examples in the field, dating back to the late 1960s and early 1970s when investigators described noncompetitive interactions between orthosteric mAChR agonists and the neuromuscular blocking agent, gallamine, or certain alkane-bis-ammonium compounds, exemplified by C_7/3-phth (Clark and Mitchelson 1976; Lullmann et al. 1969). Since that time, the actions of additional allosteric mAChR modulators have been characterized (Stockton et al. 1983; Lazareno and Birdsall 1995; Lazareno et al. 1998). Although beyond the scope of this chapter, it should be noted that there now exists a relatively rich, and expanding, allosteric pharmacology around the mAChRs, including prototypical negative allosteric modulators, such as gallamine and C_7/3-phth, as well as positive modulators of ACh, such as brucine and BQCA at the M_1 mAChR, LY2033298 at the M_4 mAChR, and VU0238429 at the M_5 mAChR (Lazareno et al. 1998; Birdsall et al. 1999; Chan et al. 2008; Gharagozloo et al. 1999; Leach et al. 2010; Ma et al. 2009; May et al. 2007b). In recent years, a number of putative allosteric agonists, which can activate the receptor in their own right, have also been identified (Chan et al. 2008; Leach et al. 2010; Ma et al. 2009; May et al. 2007b; Nawaratne et al. 2008; Jones et al. 2008; Langmead et al. 2006; Spalding et al. 2006; Sur et al. 2003; Thomas et al. 2008; Bridges et al. 2009).

There is compelling pharmacological evidence indicating that there are at least two allosteric binding sites on the mAChRs that can be targeted by small

molecule ligands. The best characterized site, referred to herein as the "prototypical modulator site," recognizes compounds such as gallamine, C_7/3-phth, brucine and alcuronium, whereas the "second" allosteric site binds certain indolocarbazoles and the benzimidazole analogs, WIN 51,708 and WIN 62,577 (Lazareno et al. 2000; Lanzafame et al. 2006). To date, all mutagenesis studies of mAChR allosteric binding sites have focused on the prototypical modulator site, which is believed to comprise epitopes that are more extracellularly located than those within the TM-bound orthosteric pocket. The location of the "second" allosteric site is currently unknown, although a molecular modeling study has suggested an intracellular location (Espinoza-Fonseca and Trujillo-Ferrara 2005, 2006).

Given that many prototypical modulators interact with all five mAChR subtypes, it is likely that some conserved residues may be involved in their actions. An early study at the M_1 mAChR proposed that $Trp^{3.28}$ and $Trp^{7.35}$, which lie at the extracellular end of TMIII and TMVII, respectively, may serve such a role, at least with respect to the binding of gallamine (Matsui et al. 1995). However, another key aspect of allosteric modulator action is that these compounds typically display greater degrees of selectivity across mAChR subtypes than do orthosteric ligands, and thus nonconserved amino acids must also contribute to modulator binding and/or actions. For example, gallamine binds with higher affinity to the M_2 mAChR than to the other mAChR subtypes and this preference has been attributed, in part, to the interaction of gallamine with residues located in the second extracellular loop of the M_2 mAChR, predominantly Tyr^{177} and to a lesser degree ^{172}Glu-Asp-Gly-Glu^{175}, as well as residues at the junction of the third extracellular loop and the top of TMVII, namely $Asn^{7.32}$, $Trp^{7.35}$, and $Thr^{7.36}$ (May et al. 2007b; Voigtlander et al. 2003; Huang et al. 2005; Prilla et al. 2006; Valant et al. 2008). Similarly, in the M_4 mAChR, $Ser^{7.36}$ has been implicated in gallamine binding (Buller et al. 2002), whilst $Glu^{7.32}$ in the M_1 mAChR has been implicated in the transmission of positive cooperativity between brucine and ACh (Stewart et al. 2010).

The binding site for the allosteric modulator/agonist, LY2033298, may also overlap with the prototypical allosteric site, because the interaction between LY2033298 and C_7/3-phth appears competitive (Leach et al. 2010). In support of this hypothesis, alanine substitution of Phe^{186} in the second extracellular loop of the M_4 mAChR, which corresponds to Tyr^{177} in the M_2 mAChR, markedly attenuates the binding of LY2033298 (Nawaratne et al. 2010). Interestingly, the equivalent position in the M_1 and M_3 mAChRs is also an aromatic residue, suggesting that aromaticity is an important characteristic in this region of the second extracellular loop of most mAChRs. Moreover, alanine substitution of the Tyr in this position of the M_1 mAChR extracellular loop greatly diminished the potency of BQCA as an allosteric modulator of ACh (Ma et al. 2009). Also in agreement with prior studies on the M_1 mAChR that focused on gallamine, substitution of the conserved $Trp^{3.28}$ and $Leu^{3.29}$ in the M_4 mAChR with alanine decreased the affinity of both C_7/3-phth and LY2033298 (Leach et al. 2011). However, alanine substitution of $Asp^{7.32}$ in the M_4 mAChR to the corresponding $Asn^{7.32}$ in the M_2 mAChR had no significant effect on the binding affinity of LY2033298 (Chan et al. 2008). Similarly, mutation

of $Ser^{7.36}$ (M_4 mAChR) to the corresponding $Thr^{7.36}$ in the M_2 mAChR did not alter the interaction between LY2033298 and ACh (Chan et al. 2008). Thus, as with orthosteric ligands, it appears that allosteric ligands can recognize a common site but, nonetheless, adopt different poses within that site such that they display differential sensitivity to specific mutations.

More recently, a novel class of ligand has been described that can bridge *both* orthosteric and allosteric sites concomitantly. Such ligands have been termed "bitopic," and it is possible that a number of putative "allosteric agonists" may actually fall into this category (Voigtlander et al. 2003). A good example of this phenomenon has been noted with the functionally selective mAChR agonist, McN-A-343. Although exhibiting many properties commensurate with a competitive (orthosteric) mode of action, there have been provocative examples in the literature to suggest that McN-A-343 can also interact allosterically with the M_2 mAChR (May et al. 2007b; Birdsall et al. 1983; Waelbroeck 1994). A subsequent study revealed that the molecule is actually a hybrid composed of orthosteric (trimethylammonium) and allosteric (3-chorophenylcarbamate) moieties (Lanzafame et al. 2006), thus providing a possible explanation of previous studies; depending on the experimental conditions, McN-A-343 can adopt a binding pose that bridges both orthosteric and allosteric sites (Fig. 3) or a second pose that only interacts allosterically with a prebound orthosteric ligand. Importantly,

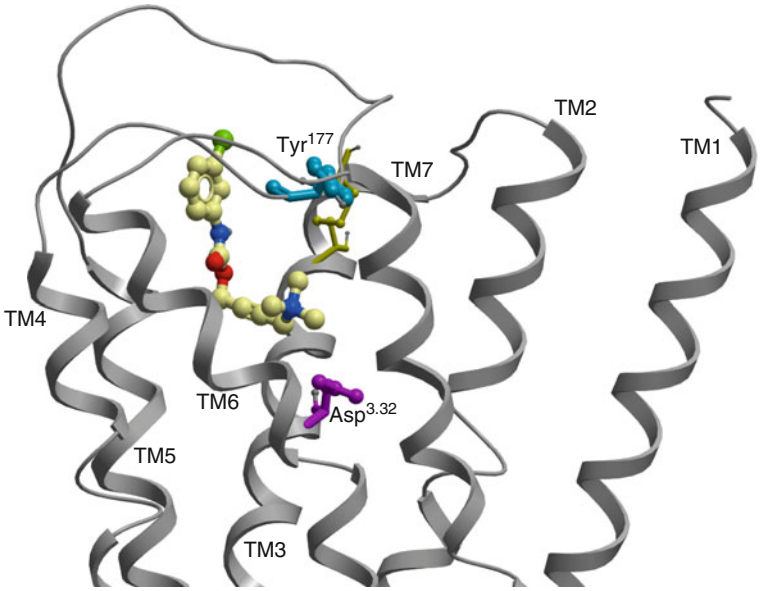

Fig. 3 A possible bitopic binding mode for McN-A-343 at the M_2 mAChR. Two key residues affecting the actions of the agonist in the orthosteric ($Asp^{3.32)}$ and allosteric (Tyr^{177}) pockets are highlighted. Coordinates taken from Valant et al. (2008)

the binding of McN-A-343 is sensitive to mutation of both the key orthosteric site residue, $Asp^{3.32}$, and key allosteric site residue, Tyr^{177}, in the M_2 mAChR (May et al. 2007b; Valant et al. 2008).

Similarly to McN-A-343, there is some evidence that the putative allosteric agonists, AC-42 and its congener 77-LH-28-1, may also bridge both the orthosteric and allosteric binding sites at the M_1 mAChR, and thus are better classed as bitopic, rather than allosteric, agonists. Although the binding of AC-42 is relatively insensitive to orthosteric site mutations at $Tyr^{3.33}$ and $Tyr^{6.51}$ (Spalding et al. 2002, 2006), recent studies have suggested that AC-42 and 77-LH-28-1 could still interact with the key orthosteric site residue, $Asp^{3.32}$, but bind with a significantly different pose to prototypical orthosteric agonists (Lebon et al. 2009). In agreement with this model, the binding affinity of AC-42 is decreased by mutation of $Leu^{3.29}$, whilst that of AC-42 and 77-LH-28-1 is increased by Ala substitution of $Trp^{3.28}$ (Avlani et al. 2010; Spalding et al. 2006; Gregory et al. 2010), indicating that they interact with a region that may border the orthosteric and allosteric binding sites. To accommodate this binding mode, it has been proposed that $Trp^{3.28}$, which would normally face toward the center of the helical bundle, may "flip" outwards and be stabilized by $Phe^{2.56}$ in the M_1 mAChR, which is located on the external side of TMII (Avlani et al. 2010).

Collectively, these recent studies of bitopic ligands highlight a number of important considerations. First, it is possible that ligands previously classed as "functionally selective" may achieve such selectivity as a consequence of a bitopic mechanism of action. Second, caution must be exercised when classifying any novel agonist as "allosteric," unless rigorous pharmacological data are available to suggest that such a compound's agonism indeed arises directly from an interaction with an allosteric site (and not the orthosteric site, as would be expected for a bitopic ligand). Third, it should be possible to rationally design bitopic ligands by purposefully utilizing appropriate orthosteric and allosteric fragments joined together by an optimal linker. A number of recent elegant studies have indeed provided proof of concept for this approach (Disingrini et al. 2006; Steinfeld et al. 2007; Antony et al. 2009).

5 Effects of Mutations on Signaling

The molecular details underlying the activation mechanisms of GPCRs remain largely unknown, and thus represent a major ongoing field of research. Much of the problem lies with the fact that GPCRs are highly dynamic proteins that can adopt multiple active states, each associated with different intracellular interacting partners and functional outcomes. Thus, any interpretation of mutational studies on GPCR signaling must be tempered by the fact that it will be influenced by the choice of functional assay used as a measure of receptor activation.

In general, ACh binding is predicted to initially elicit conformational changes in the mAChRs that result in a reduced pocket volume between key residues,

specifically $Tyr^{3.33}$, $Thr^{5.39}$, $Thr^{5.42}$, $Tyr^{6.51}$, and $Tyr^{7.39}$, that surround the bound ligand (Lu et al. 2002). Not surprisingly therefore, substitutions of these amino acids result in reduced ACh efficacy (Lu et al. 2001; Nawaratne et al. 2010; Gregory et al. 2010; Spalding et al. 1998). However, the efficacy of other agonists need not be affected by mutation of some or all of these residues, as they are likely to adopt differing poses to ACh, depending on their structures. Recent disulfide cross-linking experiments performed on the rat M_3 mAChR also indicated that $Ser^{3.36}$ and $Cys^{7.42}$ lie in close proximity when the receptor is in an active conformation (Han et al. 2005a), consistent with movement of residues that point into the orthosteric binding pocket toward one another. $Ser^{3.36}$ is predicted to face directly into the core of the orthosteric binding pocket thus it may serve as a secondary contact point for ACh when receptor activation is triggered. Indeed, substitution of $Ser^{3.36}$ for Ala in the M_1 (Lu and Hulme 1999), M_2 (Gregory et al. 2010), and M_4 mAChRs (Leach et al. 2011) leads to a large attenuation in the signaling of ACh and other agonists.

The movement of key "inner shell" residues toward ACh causes a reorientation of amino acids located in TMVII, in particular those located in the highly conserved $Asn^{7.49}$-$Pro^{7.50}$-X-$Cys^{7.52}$-$Tyr^{7.53}$ motif, which mediates a large conformational change at the intracellular end of TMVII. Agonist binding triggers movement of $Tyr^{7.53}$ toward $Val^{1.53}$, whilst residues in TMVII that are predicted to face the lipid bilayer move opposite TMI, suggesting a rotational movement of the cytoplasmic end of TMVII (Han et al. 2005b) and concomitant movement of helix VIII away from TMI (Li et al. 2007b). An M_1 model based on homology with rhodopsin predicted that the $Asn^{7.49}$-$Pro^{7.50}$-X-$Cys^{7.52}$-$Tyr^{7.53}$ motif constrains the inactive receptor conformation by forming a network of hydrogen bonds that connect TMVII to TMI, TMII, and TMIII (Lu et al. 2001). In support of this, Ala substitution of $Asn^{7.49}$, $Pro^{7.50}$, and $Tyr^{7.53}$ increases the affinity of ACh for the M_1 mAChR (Lu et al. 2001). However, although $Asn^{7.49}$ interacts with $Thr^{6.43}$ and $Asp^{6.44}$ in the inactive state of rhodopsin, an interaction between $Asn^{7.49}$ and $Asp^{2.50}$ is observed in opsin through crystal waters (Urizar et al. 2005), suggesting that this residue forms new contacts upon receptor activation that are important for the stability of the active receptor state. An identical interaction seems likely in the M_1 mAChR (Bee and Hulme 2007), and the significant reduction in agonist efficacy following mutation of $Asn^{7.49}$ in the mAChRs confirms that this residue is indeed important for stabilizing an active receptor conformation.

In conjunction with the conformational changes associated with TMVII, relocation of the bottom of TMVI away from the helical bundle and toward TMV takes place upon receptor activation. The crystal structures of rhodopsin and opsin show significant differences in the position of TMVI relative to TMIII (Palczewski et al. 2000; Park et al. 2008), particularly within the regions that comprise the retinal binding pocket. The movement of TMVI is driven, in part, by alterations in a region that contains an aromatic cluster of amino acids ($Cys^{6.47}$-$Trp^{6.48}$-$Leu^{6.49}$-$Pro^{6.50}$-$Tyr^{6.51}$-$Ala^{6.52}$ in rhodopsin) located toward the extracellular portion of TMVI (Ruprecht et al. 2004). This leads to the development of a kink at the highly conserved $Pro^{6.50}$, which causes the cytoplasmic end of TMVI to tilt away from

the center of the helical bundle (Park et al. 2008). In the mAChRs, mutations in the Thr$^{6.47}$-Trp$^{6.48}$-Thr/Ala$^{6.49}$-Pro$^{6.50}$-Tyr$^{6.51}$-Asn$^{6.52}$ motif lead to reduced agonist efficacy or nonfunctional receptors (Spalding et al. 1998; Wess et al. 1992), as do substitutions at surrounding residues, including Lys$^{6.29}$, Ala$^{6.34}$ and Ser$^{6.38}$ in the M_3 mAChR and Val$^{6.55}$ and Val$^{6.57}$, Val$^{6.59}$ in the M_3 and M_5 mAChRs (Spalding et al. 1998; Schmidt et al. 2003). In contrast, mutations at other residues in TMVI, including Glu$^{6.30}$ in the M_1 mAChR, Gln$^{6.35}$ in the M_3 mAChR and Ile$^{6.40}$, Ala$^{6.43}$, Phe$^{6.44}$, and Ser$^{6.58}$ in the M_5 mAChR, result in constitutive activity (Spalding et al. 1998; Schmidt et al. 2003; Hogger et al. 1995), suggesting that these residues help to stabilize the ground state of the receptor. Overall, these observations highlight the importance of the cytoplasmic end of TMVI in mAChR activation.

Substitution of conserved amino acids throughout TMIII, TMIV, and TMV, including Asp$^{3.26}$, Asp$^{3.32}$, Ile$^{3.46}$, Trp$^{4.57}$, Pro$^{4.59}$, Thr$^{5.37}$, Ile$^{5.38}$, Ala$^{5.46}$, and Ile$^{5.61}$ can have particularly detrimental effects on agonist efficacy (Lu and Hulme 1999; Nawaratne et al. 2010; Spalding et al. 1998; Schmidt et al. 2003; Lu and Hulme 2000; Page et al. 1995). However, Pro$^{4.59}$ is predicted to face into the lipid bilayer (Wess et al. 1991), thus the effect of mutations at this position may be indirect. In the M_1 mAChR, Ala substitution of Trp$^{3.28}$ also greatly reduces the signaling efficacy of ACh (Lu and Hulme 1999).

More recently, a number of mutations that disrupt the function of the M_3 mAChR were identified in TMI and TMII, including mutations at Thr$^{1.46}$, Ile$^{1.47}$, Asn$^{1.50}$, Val$^{1.53}$, Asn$^{1.60}$, Asn$^{2.38}$, Asn$^{2.39}$, Ser$^{2.45}$, Ala$^{2.49}$, Asp$^{2.50}$, Leu$^{2.51}$, Ser$^{2.57}$ Met$^{2.58}$, Asn$^{2.59}$, Phe$^{2.61}$ Ile$^{2.66}$, and Asn$^{2.68}$ (Li et al. 2007a). Furthermore, residues in the second extracellular loop of the M_3 mAChR are critical for the functional activity of the receptor, including Gln^{207}, Gly^{211}, Arg^{213}, Gly^{218}, Ile^{222}, Phe^{224}, Leu^{225}, and Pro^{228} (Scarselli et al. 2007). This is consistent with observations that conformational changes in the second extracellular loop occur upon activation of rhodopsin, whereby movement of TMV and disruption of the proposed ionic lock between TMIII and TMVI causes rearrangement of the hydrogen bond network that connects the extracellular ends of TMIV, TMV, and TMVI to the second extracellular loop (Ahuja et al. 2009).

In addition to inactivating mutations, amino acid substitutions in TMIII can result in increased constitutive activity of mAChRs, including Leu$^{3.43}$ and Ser$^{3.47}$ in the M_1 mAChR (Lu and Hulme 1999), suggesting a role for these residues in constraining the inactive receptor state. In particular, the highly conserved $^{3.49}$Glu/Asp-Arg-Tyr$^{3.51}$ motif, which is found in approximately 70% of Family A GPCRs, has been implicated in stabilizing the inactive receptor state and enabling a switch to an active receptor conformation. In rhodopsin, the β-ARs and the A_{2A} adenosine receptor, Arg$^{3.50}$ forms a hydrogen bond with the adjacent Glu/Asp$^{3.49}$ (Cohen et al. 1993; Ballesteros et al. 2001; Scheer et al. 1996). In rhodopsin, Arg$^{3.50}$ also forms a key salt bridge with Glu$^{6.30}$ (although this interaction was not present in the β-ARs or A_{2A} adenosine receptor structures) that is broken upon receptor activation as TMVI moves apart from TMIII. In opsin, Arg$^{3.50}$ interacts instead with Tyr$^{5.58}$ and potentially with Tyr$^{7.53}$. Accordingly, mutation of Arg$^{3.50}$ in the M_1 mAChR leads to significant reductions in agonist

efficacy (Jones et al. 1995), suggesting that this residue is essential for stabilizing the active receptor state in the mAChRs. However, and in contrast to observations at other GPCRs, significant effect on the signaling efficacy of ACh is not observed in the M_1 or M_5 mAChRs upon mutation of $Asp^{3.49}$ (Lu et al. 1997; Burstein et al. 1998), suggesting that the postulated hydrogen bond between $Asp^{3.49}$ and $Arg^{3.50}$ does not necessarily exist in the mAChRs.

The bulk of the studies described above have focused on activation mechanisms thought to be "universal" to agonists of the mAChRs. However, recent studies of allosteric and other novel functionally selective agonists of these receptors have begun to identify residues that contribute to receptor activation in a more ligand or pathway-specific manner. For instance, $Phe^{2.56}$ in the M_1 mAChR is essential for the activity of AC-42 and 77-LH-28-1 but not ACh or pilocarpine (Avlani et al. 2010), whilst the efficacy of the allosteric agonist, LY2033298, but not that of ACh or McN-A-343, at the M_4 mAChR is selectively sensitive to mutations of extracellular loop 1 residues Ile^{93} and Lys^{95} (Nawaratne et al. 2010). Conversely, a recent study of the M_2 mAChR identified $Tyr^{3.33}$ as a key residue selectively linking activation of the receptor to the phosphorylation of ERK1/2, irrespective of the nature of the activating ligand (Gregory et al. 2010). These findings indicate that there are likely to be a number of agonist and pathway-specific mechanisms that contribute to receptor signaling, consistent with the hypothesis that mAChRs can adopt multiple active states that are differentially stabilized by various classes of ligand and/or intracellular interacting proteins.

6 The G Protein Binding Interface

The only crystallographic evidence of the interaction between a GPCR and its G protein comes from the structure of opsin and metarhodopsin II in combination with a synthetic peptide composed of the first 11 amino acids of transducin, the cognate Gα subunit for this receptor (Scheerer et al. 2008). This study indicated that the second and third intracellular loops, the cytoplasmic ends of TMIII, TMV, and TMVI and the amino-terminal segment of helix 8 are all involved in G protein binding events. Specifically, an interaction between $Arg^{3.50}$ and a Cys residue in the Gα peptide corresponding to Cys^{347} in transducin was observed, confirming the importance of $Arg^{3.50}$ in receptor signaling and underlining its importance in stabilizing an active receptor state. Likewise, interactions were observed between the transducin peptide and Leu^{226}, Val^{230}, Ala^{233}, Thr^{242}, Thr^{243}, Ala^{246}, and Val^{250} in the third intracellular loop of the receptor.

In agreement with the binding of the transducin peptide to opsin, mutagenic studies suggest an interaction between full-length Gαq and TMV, TMVI and helix 8 of the M_3 mAChR. Residues located at the junctions between the third intracellular loop and TMV and TMVI are particularly important for the recognition by Gαq proteins of the M_3 mAChR, including $Tyr^{5.64}$, $Ala^{6.32}$, $Ala^{6.33}$, $Leu^{6.36}$,

and Ser$^{6.34}$ (Bluml et al. 1994b, c; Blin et al. 1995; Kostenis et al. 1997). Similar observations have been made in the M_5 mAChR (Burstein et al. 1995, 1996; Hill-Eubanks et al. 1996). A recent cysteine cross-linking study additionally identified Leu^{173} and Arg^{176} in the second intracellular loop and Thr^{549}, Thr^{552}, and Thr^{556} in the amino-terminal segment of helix 8 of the M_3 mAChR as residues that directly interact with the carboxy-terminal portion of Gαq (Hu et al. 2010). These interactions were observed in the absence and presence of agonist and even in the presence of the inverse agonist, atropine, suggesting that inactive mAChRs can exist in complex with Gα proteins. However, upon agonist stimulation, an interaction between Ala$^{6.33}$ and the carboxy-terminal region of Gαq was promoted, whilst interactions between Thr^{549} and Thr^{552} and the G protein were enhanced. This is consistent with the concept that agonists trigger movement of the cytoplasmic end of TMVI away from the TM bundle, which enables the carboxy-terminus of Gα to interact with TMV and TMVI. A weaker interaction was detected between Lys^{548} at the junction of TMVII and helix 8 of the M_3 mAChR and Asp^{321} in the carboxy-terminus of Gαq, which was also enhanced by agonist treatment. Interestingly, the same study identified an interaction between Leu^{173} in the second intracellular loop of the M_3 mAChR and Arg^{31} in the amino-terminal region of the Gαq subunit, again in the absence of agonist or inverse agonist, indicating that multiple regions of the G protein are involved in coupling to the receptor.

7 Conclusions

A wealth of mutagenesis-derived information continues to provide insight into the structural and functional role of diverse receptor regions in the mAChRs. Coupled with direct crystallographic information obtained for other Family A GPCRs, we are starting to gain an understanding of the intramolecular interactions that exist in the mAChRs, how mAChR ligands bind to their receptors, and how ligand binding triggers conformational changes in the mAChR structure that ultimately lead to intracellular signaling events. No doubt, given the recent advances in the field of GPCR structural biology, high-resolution structures of ligand-bound mAChRs are a likely outcome in the not-too-distant future. Irrespective of the nature of the experimental paradigm, the most likely gage of success in the area of receptor structure–function analyses is the use of information gained to successfully explain and predict biological events, and to rationally design drugs that can alleviate diseases associated with the mAChRs.

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References

Ahuja S, Hornak V, Yan EC, Syrett N, Goncalves JA, Hirshfeld A, Ziliox M, Sakmar TP, Sheves M, Reeves PJ, Smith SO, Eilers M (2009) Helix movement is coupled to displacement of the second extracellular loop in rhodopsin activation. Nat Struct Mol Biol 16:168–175

Antony J, Kellershohn K, Mohr-Andra M, Kebig A, Prilla S, Muth M, Heller E, Disingrini T, Dallanoce C, Bertoni S, Schrobang J, Trankle C, Kostenis E, Christopoulos A, Holtje HD, Barocelli E, De Amici M, Holzgrabe U, Mohr K (2009) Dualsteric GPCR targeting: a novel route to binding and signaling pathway selectivity. FASEB J 23:442–450

Avlani VA, Gregory KJ, Morton CJ, Parker MW, Sexton PM, Christopoulos A (2007) Critical role for the second extracellular loop in the binding of both orthosteric and allosteric G protein-coupled receptor ligands. J Biol Chem 282:25677–25686

Avlani VA, Langmead CJ, Guida E, Wood MD, Tehan BG, Herdon HJ, Watson JM, Sexton PM, Christopoulos A (2010) Orthosteric and allosteric modes of interaction of novel selective agonists of the M1 muscarinic acetylcholine receptor. Mol Pharmacol 78(1):94–104

Ballesteros JA, Weinstein H, Stuart CS (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. Methods Neurosci 25:366–428

Ballesteros JA, Jensen AD, Liapakis G, Rasmussen SG, Shi L, Gether U, Javitch JA (2001) Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. J Biol Chem 276:29171–29177

Bee MS, Hulme EC (2007) Functional analysis of transmembrane domain 2 of the M1 muscarinic acetylcholine receptor. J Biol Chem 282:32471–32479

Birdsall NJ, Burgen AS, Hulme EC, Stockton JM, Zigmond MJ (1983) The effect of McN-A-343 on muscarinic receptors in the cerebral cortex and heart. Br J Pharmacol 78:257–259

Birdsall NJ, Farries T, Gharagozloo P, Kobayashi S, Lazareno S, Sugimoto M (1999) Subtype-selective positive cooperative interactions between brucine analogs and acetylcholine at muscarinic receptors: functional studies. Mol Pharmacol 55:778–786

Blin N, Yun J, Wess J (1995) Mapping of single amino acid residues required for selective activation of Gq/11 by the m3 muscarinic acetylcholine receptor. J Biol Chem 270:17741–17748

Bluml K, Mutschler E, Wess J (1994a) Functional role in ligand binding and receptor activation of an asparagine residue present in the sixth transmembrane domain of all muscarinic acetylcholine receptors. J Biol Chem 269:18870–18876

Bluml K, Mutschler E, Wess J (1994b) Functional role of a cytoplasmic aromatic amino acid in muscarinic receptor-mediated activation of phospholipase C. J Biol Chem 269:11537–11541

Bluml K, Mutschler E, Wess J (1994c) Identification of an intracellular tyrosine residue critical for muscarinic receptor-mediated stimulation of phosphatidylinositol hydrolysis. J Biol Chem 269:402–405

Bridges TM, Marlo JE, Niswender CM, Jones CK, Jadhav SB, Gentry PR, Plumley HC, Weaver CD, Conn PJ, Lindsley CW (2009) Discovery of the first highly M5-preferring muscarinic acetylcholine receptor ligand, an M5 positive allosteric modulator derived from a series of 5-trifluoromethoxy N-benzyl isatins. J Med Chem 52:3445–3448

Buller S, Zlotos DP, Mohr K, Ellis J (2002) Allosteric site on muscarinic acetylcholine receptors: a single amino acid in transmembrane region 7 is critical to the subtype selectivities of caracurine V derivatives and alkane-bisammonium ligands. Mol Pharmacol 61:160–168

Burstein ES, Spalding TA, Hill-Eubanks D, Brann MR (1995) Structure-function of muscarinic receptor coupling to G proteins. Random saturation mutagenesis identifies a critical determinant of receptor affinity for G proteins. J Biol Chem 270:3141–3146

Burstein ES, Spalding TA, Brann MR (1996) Amino acid side chains that define muscarinic receptor/G-protein coupling. Studies of the third intracellular loop. J Biol Chem 271:2882–2885

Burstein ES, Spalding TA, Brann MR (1998) The second intracellular loop of the m5 muscarinic receptor is the switch which enables G-protein coupling. J Biol Chem 273:24322–24327

Chan WY, McKinzie DL, Bose S, Mitchell SN, Witkin JM, Thompson RC, Christopoulos A, Lazareno S, Birdsall NJM, Bymaster FP, Felder CC (2008) Allosteric modulation of the muscarinic M4 receptor as an approach to treating schizophrenia. Proc Natl Acad Sci USA 105:10978–10983

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, Stevens RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science 318:1258–1265

Clark AL, Mitchelson F (1976) The inhibitory effect of gallamine on muscarinic receptors. Br J Pharmacol 58:323–331

Cohen GB, Yang T, Robinson PR, Oprian DD (1993) Constitutive activation of opsin: influence of charge at position 134 and size at position 296. Biochemistry 32:6111–6115

Congreve M, Marshall F (2010) The impact of GPCR structures on pharmacology and structure-based drug design. Br J Pharmacol 159:986–996

Curtis CA, Wheatley M, Bansal S, Birdsall NJ, Eveleigh P, Pedder EK, Poyner D, Hulme EC (1989) Propylbenzilylcholine mustard labels an acidic residue in transmembrane helix 3 of the muscarinic receptor. J Biol Chem 264:489–495

Disingrini T, Muth M, Dallanoce C, Barocelli E, Bertoni S, Kellershohn K, Mohr K, De Amici M, Holzgrabe U (2006) Design, synthesis, and action of oxotremorine-related hybrid-type allosteric modulators of muscarinic acetylcholine receptors. J Med Chem 49:366–372

Espinoza-Fonseca LM, Trujillo-Ferrara JG (2005) Identification of multiple allosteric sites on the M1 muscarinic acetylcholine receptor. FEBS Lett 579:6726–6732

Espinoza-Fonseca LM, Trujillo-Ferrara JG (2006) The existence of a second allosteric site on the M1 muscarinic acetylcholine receptor and its implications for drug design. Bioorg Med Chem Lett 16:1217–1220

Foord SM, Bonner TI, Neubig RR, Rosser EM, Pin JP, Davenport AP, Spedding M, Harmar AJ (2005) International Union of Pharmacology. XLVI. G protein-coupled receptor list. Pharmacol Rev 57:279–288

Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol 63:1256–1272

Gharagozloo P, Lazareno S, Popham A, Birdsall NJ (1999) Allosteric interactions of quaternary strychnine and brucine derivatives with muscarinic acetylcholine receptors. J Med Chem 42:438–445

Goodwin JA, Hulme EC, Langmead CJ, Tehan BG (2007) Roof and floor of the muscarinic binding pocket: variations in the binding modes of orthosteric ligands. Mol Pharmacol 72:1484–1496

Gregory KJ, Hall NE, Tobin AB, Sexton PM, Christopoulos A (2010) Identification of orthosteric and allosteric site mutations in M2 muscarinic acetylcholine receptors that contribute to ligand-selective signaling bias. J Biol Chem 285:7459–7474

Han SJ, Hamdan FF, Kim SK, Jacobson KA, Bloodworth LM, Li B, Wess J (2005a) Identification of an agonist-induced conformational change occurring adjacent to the ligand-binding pocket of the M(3) muscarinic acetylcholine receptor. J Biol Chem 280:34849–34858

Han SJ, Hamdan FF, Kim SK, Jacobson KA, Brichta L, Bloodworth LM, Li JH, Wess J (2005b) Pronounced conformational changes following agonist activation of the M(3) muscarinic acetylcholine receptor. J Biol Chem 280:24870–24879

Harmar AJ, Hills RA, Rosser EM, Jones M, Buneman OP, Dunbar DR, Greenhill SD, Hale VA, Sharman JL, Bonner TI, Catterall WA, Davenport AP, Delagrange P, Dollery CT, Foord SM, Gutman GA, Laudet V, Neubig RR, Ohlstein EH, Olsen RW, Peters J, Pin JP, Ruffolo RR, Searls DB, Wright MW, Spedding M (2009) IUPHAR-DB: the IUPHAR database of G protein-coupled receptors and ion channels. Nucleic Acids Res 37:D680–D685

Hill-Eubanks D, Burstein ES, Spalding TA, Brauner-Osborne H, Brann MR (1996) Structure of a G-protein-coupling domain of a muscarinic receptor predicted by random saturation mutagenesis. J Biol Chem 271:3058–3065

Hogger P, Shockley MS, Lameh J, Sadee W (1995) Activating and inactivating mutations in N- and C-terminal i3 loop junctions of muscarinic acetylcholine Hm1 receptors. J Biol Chem 270:7405–7410

Hopkins AL, Groom CR (2002) The druggable genome. Nat Rev Drug Discov 1:727–730

Hu J, Wang Y, Zhang X, Lloyd JR, Li JH, Karpiak J, Costanzi S, Wess J (2010) Structural basis of G protein-coupled receptor-G protein interactions. Nat Chem Biol 6:541–548

Huang XP, Prilla S, Mohr K, Ellis J (2005) Critical amino acid residues of the common allosteric site on the M2 muscarinic acetylcholine receptor: more similarities than differences between the structurally divergent agents gallamine and bis(ammonio)alkane-type hexamethylene-bis-[dimethyl-(3-phthalimidopropyl)ammonium]dibromide. Mol Pharmacol 68:769–778

Hulme EC, Lu ZL (1998) Scanning mutagenesis of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor. J Physiol Paris 92:269–274

Hulme EC, Lu ZL, Bee M, Curtis CA, Saldanha J (2001) The conformational switch in muscarinic acetylcholine receptors. Life Sci 68:2495–2500

Hulme EC, Lu ZL, Saldanha JW, Bee MS (2003a) Structure and activation of muscarinic acetylcholine receptors. Biochem Soc Trans 31:29–34

Hulme EC, Lu ZL, Bee MS (2003b) Scanning mutagenesis studies of the M1 muscarinic acetylcholine receptor. Receptors Channels 9:215–228

Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR, Ijzerman AP, Stevens RC (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. Science 322:1211–1217

Jakubik J, El-Fakahany EE, Tucek S (2000) Evidence for a tandem two-site model of ligand binding to muscarinic acetylcholine receptors. J Biol Chem 275:18836–18844

Jones PG, Curtis CA, Hulme EC (1995) The function of a highly-conserved arginine residue in activation of the muscarinic M1 receptor. Eur J Pharmacol 288:251–257

Jones CK, Brady AE, Davis AA, Xiang Z, Bubser M, Tantawy MN, Kane AS, Bridges TM, Kennedy JP, Bradley SR, Peterson TE, Ansari MS, Baldwin RM, Kessler RM, Deutch AY, Lah JJ, Levey AI, Lindsley CW, Conn PJ (2008) Novel selective allosteric activator of the M1 muscarinic acetylcholine receptor regulates amyloid processing and produces antipsychotic-like activity in rats. J Neurosci 28:10422–10433

Kostenis E, Gomeza J, Lerche C, Wess J (1997) Genetic analysis of receptor-Galphaq coupling selectivity. J Biol Chem 272:23675–23681

Kurtenbach E, Curtis CA, Pedder EK, Aitken A, Harris AC, Hulme EC (1990) Muscarinic acetylcholine receptors. Peptide sequencing identifies residues involved in antagonist binding and disulfide bond formation. J Biol Chem 265:13702–13708

Langmead CJ, Fry VA, Forbes IT, Branch CL, Christopoulos A, Wood MD, Herdon HJ (2006) Probing the molecular mechanism of interaction between 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine (AC-42) and the muscarinic M(1) receptor: direct pharmacological evidence that AC-42 is an allosteric agonist. Mol Pharmacol 69:236–246

Lanzafame AA, Sexton PM, Christopoulos A (2006) Interaction studies of multiple binding sites on m4 muscarinic acetylcholine receptors. Mol Pharmacol 70:736–746

Lazareno S, Birdsall NJ (1995) Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. Mol Pharmacol 48:362–378

Lazareno S, Gharagozloo P, Kuonen D, Popham A, Birdsall NJ (1998) Subtype-selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors: radioligand binding studies. Mol Pharmacol 53:573–589

Lazareno S, Popham A, Birdsall NJ (2000) Allosteric interactions of staurosporine and other indolocarbazoles with N-[methyl-(3)H]scopolamine and acetylcholine at muscarinic receptor subtypes: identification of a second allosteric site. Mol Pharmacol 58:194–207

Leach K, Loiacono RE, Felder CC, McKinzie DL, Mogg A, Shaw DB, Sexton PM, Christopoulos A (2010) Molecular mechanisms of action and in vivo validation of an M(4) muscarinic acetylcholine receptor allosteric modulator with potential antipsychotic properties. Neuropsychopharmacology 35:855–869

Leach K, Davey AE, Felder CC, Sexton PM, Christopoulos A (2011) The role of transmembrane domain 3 in the actions of orthosteric, allosteric, and atypical agonists of the M4 muscarinic acetylcholine receptor. Mol. Pharmacol. 79: 855–865

Lebon G, Langmead CJ, Tehan BG, Hulme EC (2009) Mutagenic mapping suggests a novel binding mode for selective agonists of M1 muscarinic acetylcholine receptors. Mol Pharmacol 75:331–341

Li B, Scarselli M, Knudsen CD, Kim SK, Jacobson KA, McMillin SM, Wess J (2007a) Rapid identification of functionally critical amino acids in a G protein-coupled receptor. Nat Methods 4:169–174

Li JH, Han SJ, Hamdan FF, Kim SK, Jacobson KA, Bloodworth LM, Zhang X, Wess J (2007b) Distinct structural changes in a G protein-coupled receptor caused by different classes of agonist ligands. J Biol Chem 282:26284–26293

Lu ZL, Hulme EC (1999) The functional topography of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor, revealed by scanning mutagenesis. J Biol Chem 274:7309–7315

Lu ZL, Hulme EC (2000) A network of conserved intramolecular contacts defines the off-state of the transmembrane switch mechanism in a seven-transmembrane receptor. J Biol Chem 275:5682–5686

Lu ZL, Curtis CA, Jones PG, Pavia J, Hulme EC (1997) The role of the aspartate-arginine-tyrosine triad in the m1 muscarinic receptor: mutations of aspartate 122 and tyrosine 124 decrease receptor expression but do not abolish signaling. Mol Pharmacol 51:234–241

Lu ZL, Saldanha JW, Hulme EC (2001) Transmembrane domains 4 and 7 of the M(1) muscarinic acetylcholine receptor are critical for ligand binding and the receptor activation switch. J Biol Chem 276:34098–34104

Lu ZL, Saldanha JW, Hulme EC (2002) Seven-transmembrane receptors: crystals clarify. Trends Pharmacol Sci 23:140–146

Lullmann H, Ohnesorge FK, Schauwecker GC, Wassermann O (1969) Inhibition of the actions of carbachol and DFP on guinea pig isolated atria by alkane-bis-ammonium compounds. Eur J Pharmacol 6:241–247

Ma L, Seager MA, Wittmann M, Jacobson M, Bickel D, Burno M, Jones K, Graufelds VK, Xu G, Pearson M, McCampbell A, Gaspar R, Shughrue P, Danziger A, Regan C, Flick R, Pascarella D, Garson S, Doran S, Kreatsoulas C, Veng L, Lindsley CW, Shipe W, Kuduk S, Sur C, Kinney G, Seabrook GR, Ray WJ (2009) Selective activation of the M1 muscarinic acetylcholine receptor achieved by allosteric potentiation. Proc Natl Acad Sci USA 106:15950–15955

Matsui H, Lazareno S, Birdsall NJ (1995) Probing of the location of the allosteric site on m1 muscarinic receptors by site-directed mutagenesis. Mol Pharmacol 47:88–98

May LT, Leach K, Sexton PM, Christopoulos A (2007a) Allosteric modulation of G protein-coupled receptors. Annu Rev Pharmacol Toxicol 47:1–51

May LT, Avlani VA, Langmead CJ, Herdon HJ, Wood MD, Sexton PM, Christopoulos A (2007b) Structure-function studies of allosteric agonism at M2 muscarinic acetylcholine receptors. Mol Pharmacol 72:463–476

Nawaratne V, Leach K, Suratman N, Loiacono RE, Felder CC, Armbruster BN, Roth BL, Sexton PM, Christopoulos A (2008) New insights into the function of M4 muscarinic acetylcholine receptors gained using a novel allosteric modulator and a DREADD (designer receptor exclusively activated by a designer drug). Mol Pharmacol 74:1119–1131

Nawaratne V, Leach K, Felder CC, Sexton PM, Christopoulos A (2010) Structural determinants of allosteric agonism and modulation at the M4 muscarinic acetylcholine receptor: identification of ligand-specific and global activation mechanisms. J Biol Chem 285:19012–19021

Page KM, Curtis CA, Jones PG, Hulme EC (1995) The functional role of the binding site aspartate in muscarinic acetylcholine receptors, probed by site-directed mutagenesis. Eur J Pharmacol 289:429–437

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. Science 289:739–745

Park JH, Scheerer P, Hofmann KP, Choe HW, Ernst OP (2008) Crystal structure of the ligand-free G-protein-coupled receptor opsin. Nature 454:183–187

Prilla S, Schrobang J, Ellis J, Holtje HD, Mohr K (2006) Allosteric interactions with muscarinic acetylcholine receptors: complex role of the conserved tryptophan M2422Trp in a critical cluster of amino acids for baseline affinity, subtype selectivity, and cooperativity. Mol Pharmacol 70:181–193

Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VR, Sanishvili R, Fischetti RF, Schertler GF, Weis WI, Kobilka BK (2007) Crystal structure of the human beta2 adrenergic G-protein coupled receptor. Nature 450:383–387

Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Yao XJ, Weis WI, Stevens RC, Kobilka BK (2007) GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. Science 318:1266–1273

Ruprecht JJ, Mielke T, Vogel R, Villa C, Schertler GF (2004) Electron crystallography reveals the structure of metarhodopsin. EMBO J 23:3609–3620

Scarselli M, Li B, Kim SK, Wess J (2007) Multiple residues in the second extracellular loop are critical for M3 muscarinic acetylcholine receptor activation. J Biol Chem 282:7385–7396

Scheer A, Fanelli F, Costa T, De Benedetti PG, Cotecchia S (1996) Constitutively active mutants of the alpha 1B-adrenergic receptor: role of highly conserved polar amino acids in receptor activation. EMBO J 15:3566–3578

Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, Hofmann KP, Ernst OP (2008) Crystal structure of opsin in its G-protein-interacting conformation. Nature 455:497–502

Schmidt C, Li B, Bloodworth L, Erlenbach I, Zeng FY, Wess J (2003) Random mutagenesis of the M3 muscarinic acetylcholine receptor expressed in yeast. Identification of point mutations that "silence" a constitutively active mutant M3 receptor and greatly impair receptor/G protein coupling. J Biol Chem 278:30248–30260

Spalding TA, Birdsall NJ, Curtis CA, Hulme EC (1994) Acetylcholine mustard labels the binding site aspartate in muscarinic acetylcholine receptors. J Biol Chem 269:4092–4097

Spalding TA, Burstein ES, Henderson SC, Ducote KR, Brann MR (1998) Identification of a ligand-dependent switch within a muscarinic receptor. J Biol Chem 273:21563–21568

Spalding TA, Trotter C, Skjaerbaek N, Messier TL, Currier EA, Burstein ES, Li D, Hacksell U, Brann MR (2002) Discovery of an ectopic activation site on the M(1) muscarinic receptor. Mol Pharmacol 61:1297–1302

Spalding TA, Ma JN, Ott TR, Friberg M, Bajpai A, Bradley SR, Davis RE, Brann MR, Burstein ES (2006) Structural requirements of transmembrane domain 3 for activation by the M1 muscarinic receptor agonists AC-42, AC-260584, clozapine, and *N*-desmethylclozapine: evidence for three distinct modes of receptor activation. Mol Pharmacol 70:1974–1983

Steinfeld T, Mammen M, Smith JA, Wilson RD, Jasper JR (2007) A novel multivalent ligand that bridges the allosteric and orthosteric binding sites of the M2 muscarinic receptor. Mol Pharmacol 72:291–302

Stewart GD, Sexton PM, Christopoulos A (2010) Prediction of functionally selective allosteric interactions at an M3 muscarinic acetylcholine receptor mutant using Saccharomyces cerevisiae. Mol Pharmacol 78(2):205–214

Stockton JM, Birdsall NJ, Burgen AS, Hulme EC (1983) Modification of the binding properties of muscarinic receptors by gallamine. Mol Pharmacol 23:551–557

Sur C, Mallorga PJ, Wittmann M, Jacobson MA, Pascarella D, Williams JB, Brandish PE, Pettibone DJ, Scolnick EM, Conn PJ (2003) *N*-desmethylclozapine, an allosteric agonist at

muscarinic 1 receptor, potentiates *N*-methyl-*D*-aspartate receptor activity. Proc Natl Acad Sci USA 100:13674–13679

Thomas RL, Mistry R, Langmead CJ, Wood MD, Challiss RA (2008) *G* protein coupling and signaling pathway activation by m1 muscarinic acetylcholine receptor orthosteric and allosteric agonists. J Pharmacol Exp Ther 327:365–374

Urizar E, Claeysen S, Deupi X, Govaerts C, Costagliola S, Vassart G, Pardo L (2005) An activation switch in the rhodopsin family of G protein-coupled receptors: the thyrotropin receptor. J Biol Chem 280:17135–17141

Valant C, Gregory KJ, Hall NE, Scammells PJ, Lew MJ, Sexton PM, Christopoulos A (2008) A novel mechanism of G protein-coupled receptor functional selectivity. Muscarinic partial agonist McN-A-343 as a bitopic orthosteric/allosteric ligand. J Biol Chem 283:29312–29321

Voigtlander U, Johren K, Mohr M, Raasch A, Trankle C, Buller S, Ellis J, Holtje HD, Mohr K (2003) Allosteric site on muscarinic acetylcholine receptors: identification of two amino acids in the muscarinic M2 receptor that account entirely for the M2/M5 subtype selectivities of some structurally diverse allosteric ligands in *N*-methylscopolamine-occupied receptors. Mol Pharmacol 64:21–31

Waelbroeck M (1994) Identification of drugs competing with d-tubocurarine for an allosteric site on cardiac muscarinic receptors. Mol Pharmacol 46:685–692

Ward SD, Curtis CA, Hulme EC (1999) Alanine-scanning mutagenesis of transmembrane domain 6 of the M(1) muscarinic acetylcholine receptor suggests that Tyr381 plays key roles in receptor function. Mol Pharmacol 56:1031–1041

Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R, Leslie AG, Tate CG, Schertler GF (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. Nature 454:486–491

Wess J, Gdula D, Brann MR (1991) Site-directed mutagenesis of the m3 muscarinic receptor: identification of a series of threonine and tyrosine residues involved in agonist but not antagonist binding. EMBO J 10:3729–3734

Wess J, Maggio R, Palmer JR, Vogel Z (1992) Role of conserved threonine and tyrosine residues in acetylcholine binding and muscarinic receptor activation. A study with m3 muscarinic receptor point mutants. J Biol Chem 267:19313–19319

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