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Muscarinic acetylcholine receptors: novel opportunities for drug development

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Abstract

The muscarinic acetylcholine receptors are a subfamily of G protein-coupled receptors that regulate numerous fundamental functions of the central and peripheral nervous system. The past few years have witnessed unprecedented new insights into muscarinic receptor physiology pharmacology and structure. These advances include the first structural views of muscarinic receptors in both inactive and active conformations, as well as a better understanding of the molecular underpinnings of muscarinic receptor regulation by allosteric modulators. These recent findings should facilitate the development of new muscarinic receptor subtype-selective ligands that could prove to be useful for the treatment of many severe pathophysiological conditions.

The muscarinic acetylcholine receptors (mAChRs) comprise a family of five related G protein-coupled receptors (GPCRs) belonging to the α -branch of class A GPCRs¹. The mAChR family consists of five distinct subtypes, denoted M₁ to M₅ (and encoded by the genes *CHRM1* to *CHRM5*). Three of these receptor subtypes (M₁, M₃ and M₅) have been shown to couple to G proteins of the G_{q/11} family, whereas the remaining two subtypes (M₂ and M₄) preferentially signal through the G_{i/o} family of G proteins². The mAChRs have a central role in human physiology, regulating heart rate, smooth muscle contraction, glandular secretion and many fundamental functions of the central nervous system (CNS)³.

Currently, drugs targeting muscarinic receptors are used for the treatment of several pathophysiological conditions, including chronic obstructive pulmonary disease, overactive bladder and Sjögren's syndrome³. Despite the powerful and diverse pharmacological actions of muscarinic receptor agonists and antagonists, the development of these drugs for other

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clinical applications has probably been held back, at least in part, by the lack of small-molecule ligands that can inhibit or activate specific mAChRs with high selectivity. As a result, the precise physiological and pathophysiological roles of the individual mAChR subtypes have, until recently, remained poorly defined. However, during the past 15 years the generation and phenotypic analysis of *Chrm1*- to *Chrm5*-knockout mice has been instrumental in improving our understanding of the biology of the individual mAChRs³.

Recent studies with novel mAChR mouse models have provided additional insights into the physiological roles of the different mAChR subtypes⁴. Moreover, during the past few years, several laboratories have succeeded in developing ligands that show high selectivity for specific mAChR subtypes^{5,6}. In contrast to conventional muscarinic receptor ligands, which bind to the orthosteric receptor site, most of these new ligands bind to distinct allosteric sites. Such allosteric ligands can influence the potency and efficacy of orthosteric ligands, and they may possess agonistic or inverse agonist activity in their own right. Interestingly, bitopic muscarinic receptor ligands with preference for certain subtypes have also been developed recently⁷⁻⁹. Such agents, which can interact with both allosteric and orthosteric receptor sites simultaneously, offer new opportunities to target specific mAChR subtypes for therapeutic purposes.

The recent determination of the first mAChR structures (M_2 and M_3 subtypes) represents a milestone in the mAChR field¹⁰⁻¹². These studies have provided the first molecular views of mAChRs in both their inactive^{10,11} and active conformations¹², revealing the molecular nature of the binding sites for orthosteric muscarinic receptor ligands. Moreover, structural and computational studies have identified the mechanisms by which drug-like allosteric modulators bind to the M_2 subtype^{12,13}.

In this Review, we summarize and discuss a series of recent studies that have advanced our knowledge of mAChR biology, structure and pharmacology. In particular, we emphasize the potential therapeutic implications of these novel findings. As mAChRs are prototypic class A GPCRs, the topics covered herein have implications for other members of this receptor superfamily.

Novel mAChR mouse models

Since the late 1990s, studies with *Chrm1*- to *Chrm5*- knockout mice have elucidated many important physiological functions of the individual mAChR subtypes³. The outcome of this work suggested that modulating the activity of specific mAChR subtypes by selective ligands might prove to be beneficial for the treatment of many CNS disorders and other clinical conditions³. Until recently, essentially all phenotyping studies were carried out with conventional, constitutive mAChR-knockout mice, which lack one or more of the genes encoding mAChRs throughout development in all cells of the body. The proper interpretation of the phenotypes observed with conventional mAChR-knockout mice is often complicated because individual mAChR subtypes are expressed in many different tissues and cell types, and compensatory molecular or physiological changes can occur during development.

To circumvent these difficulties, novel conditional knockout mice have been created that lack specific mAChR subtypes only in certain tissues or cell types. For these studies, mice containing ‘floxed’ versions of specific genes encoding mAChRs were crossed with transgenic mice expressing Cre recombinase in a cell type- or tissue-selective fashion (TABLE 1). mAChR knock-in mice have also been reported, in which the native genes encoding M₃ or M₄ receptors — *CHRM3* or *CHRM4*, respectively— were replaced with mutant versions (TABLE 1). Here, we summarize several recent studies that have used novel mAChR-mutant mice as well as conventional mAChR-knockout mice. These studies are discussed in the context of selected human diseases in which the development of novel classes of drugs targeting muscarinic receptors might be beneficial.

Targeting mAChRs in disease

Alzheimer's disease

Alzheimer's disease, which is the most common form of dementia, is recognized as a major public health crisis¹⁴. At present, there are no drugs available that are highly effective in preventing Alzheimer's disease or slowing its progress. Based on behavioural, pharmacological, anatomical and neurochemical evidence, M₁ receptor-selective agonists seem to have the potential to ameliorate the symptoms of Alzheimer's disease and related cognitive disorders¹⁵. In fact, several recent studies support the concept that M₁ receptor-selective agonists or positive allosteric modulators (PAMs) may prove to be useful as cognition-enhancing drugs. Davis *et al.*¹⁶ demonstrated that M₁ receptor deficiency increases amyloidogenic processing of amyloid precursor protein (APP) — a key feature of Alzheimer's disease — in a mouse model of Alzheimer's disease. In a related study, Medeiros *et al.*¹⁷ reported that the lack of M₁ receptors further exacerbated cognitive processes and Alzheimer's disease-related pathological features in a mouse model of Alzheimer's disease. In addition, *Chrm1*-knockout mice exhibited an age-dependent cognitive decline in tasks that they performed normally at a younger age¹⁷. Most importantly, several recent studies have shown that compounds that act as PAMs at M₁ receptors have cognition-enhancing activity in rodents and are able to improve impaired cognition in mouse models of Alzheimer's disease^{18,19}. Taken together, these new findings provide a rational basis for the development of M₁-selective drugs (PAMs or agents that can activate M₁ receptors directly) for the treatment of Alzheimer's disease and related disorders.

Schizophrenia

Schizophrenia, which is a chronic disabling brain disorder that affects ~1% of the general population²⁰, is characterized by enhanced central dopa-minergic signalling. Studies with conventional mAChR-knockout mice have indicated that the lack of central M₁ and/or M₄ receptors leads to a ‘dopamine hypersensitivity phenotype’, which suggests that agents that can enhance signalling through these receptor subtypes may be endowed with antipsychotic activity³. In agreement with this concept, two clinical trials demonstrated that xanomeline, an M₁- and M₄-preferring muscarinic receptor agonist²¹, was effective in ameliorating psychosis-like symptoms^{22,23}. A recent study with cell type-specific *Chrm4*-knockout mice strongly suggests that a distinct subpopulation of neuronal M₄ receptors expressed by D₁ dopamine receptor-expressing neurons may be of particular importance in mediating the

antipsychotic actions triggered by M₄ receptor activation²⁴. In addition, Dencker *et al.*²⁵ reported that the antipsychotic-like effects of xanomeline were almost completely abolished in two mouse models of psychosis in which mice lacked M₄ receptors selectively in D₁ receptor-expressing neurons. In agreement with these findings, several recent preclinical studies suggest that PAMs of M₁ and/or M₄ receptors may prove to be clinically useful for the treatment of schizophrenia (reviewed in REFS 18,26–28).

Drug addiction

Acetylcholine has a key role in modulating the neurochemical and behavioural CNS responses to drugs of abuse²⁹. Phenotypic analysis of *Chrm5*-knockout mice suggested that centrally active M₅ receptor blockers might prove to be useful for the treatment of drug abuse, including cocaine or opioid addiction³. More recent studies with other mAChR-knockout lines indicate that signalling through central M₁ and M₄ receptors may also modulate drug-seeking behaviour. For example, recent work demonstrated that allosteric M₁ receptor agonists and xanomeline can attenuate the reinforcing and discriminative stimulus effects of cocaine^{30,31}. These effects were greatly reduced or abolished in *Chrm1*-knockout mice or in mice that were deficient in both M₁ and M₄ receptors (*Chrm1*^{−/−} *Chrm4*^{−/−} mice). Moreover, Schmidt *et al.*³² reported that the reinforcing effects of chronic cocaine self-administration are significantly increased in *Chrm4*-knockout mice. These findings indicate that centrally active drugs that can selectively stimulate M₁ and/or M₄ receptors may prove to be useful in the treatment of drug addiction.

Type 2 diabetes

Type 2 diabetes has emerged as a major threat to public health worldwide³³. One of the key patho-physiological features of type 2 diabetes is that pancreatic β -cells are unable to secrete sufficient insulin to overcome peripheral insulin resistance. Studies with β -cell-selective *Chrm3*-mutant mice^{34,35} showed that strategies aimed at enhancing the activity of β -cell M₃ receptors may prove to be useful for the treatment of type 2 diabetes. To further test this hypothesis, two recent studies^{36,37} analysed transgenic mice that expressed an M₃ receptor-based designer receptor (G_q DREADD; where DREADD stands for ‘designer receptor exclusively activated by a designer drug’) selectively in pancreatic β -cells. This modified M₃ receptor is unable to bind acetylcholine, the endogenous ligand, but can be selectively activated by clozapine N-oxide (CNO), a drug that is otherwise pharmacologically inert^{36,38}. Systematic metabolic analysis of this mouse line confirmed that CNO-dependent activation of this designer M₃ receptor has numerous beneficial effects on β -cell function and is able to prevent experimentally induced diabetes^{36,37}. Although there is evidence that the ability of β -cell M₃ receptors to promote insulin release depends on the presence of G proteins of the G_q family³⁹, two recent studies suggest that M₃ receptor-mediated activation of arrestin-dependent β -cell pathways may further enhance insulin secretion^{40,41}. Currently, ligands that are able to selectively promote signalling through M₃ receptors are not available, and the development of such agents would greatly facilitate studies aimed at testing the potential usefulness of targeting β -cell M₃ receptors for the treatment of type 2 diabetes and related metabolic disorders.

Cancer

Accumulating evidence suggests that mAChR-dependent signalling pathways can promote cell proliferation and cancer progression^{42,43}. Interestingly, Raufman *et al.*⁴⁴ demonstrated that *Chrm3*-knockout mice displayed reduced epithelial cell proliferation, tumour number and size in a mouse model of colon neoplasia (azoxymethane-induced colon cancer). In a related study⁴⁵, M₃ receptor deficiency was associated with a pronounced reduction in tumour number and volume in a genetic model of intestinal neoplasia (*Apc*^{Min/+} mice). Magnon *et al.*⁴⁶ reported that M₁ receptor deficiency inhibited mAChR-mediated prostate cancer invasion and metastasis in two mouse models of prostate cancer. These findings support the concept that subtype-selective mAChR antagonists may prove to be clinically useful for the treatment of certain forms of cancer.

Novel mAChR pharmacology

A major shift in drug discovery has been the recognition that most — if not all — GPCRs possess spatially distinct allosteric sites that can be exploited by small molecules to modulate the activity of orthosteric ligands^{6,47,48}. The mAChRs are arguably the pre-eminent models for contributing to our understanding of GPCR allostery^{5,49-51} (see BOX 1 for a representative list of muscarinic allosteric modulators).

Pharmacological characteristics of allosteric modulators

Allosteric ligands promote conformational changes in the receptor that manifest as an alteration in the properties of a ligand bound to the classical, orthosteric site, and by the potential for direct activation of receptor signalling via the allosteric site⁵². PAMs enhance orthosteric activity, negative allosteric modulators (NAMs) inhibit it, and agents that occupy an allosteric site but do not change the activity of orthosteric ligands are referred to as neutral allosteric ligands (NALs). Allosteric compounds that directly activate GPCRs are called allosteric agonists. However, these designations are contextual, as they are dependent on the receptor, orthosteric ligand and the assay system used⁵²⁻⁵⁴. For example, the M₁ receptor-selective modulator BQCA (REF. 55) acts solely as a PAM of acetylcholine activity when assayed in a cell line with low M₁ receptor expression⁵⁶ and/or at signalling pathways that are weakly coupled to the M₁ receptor⁵⁷, but it acts as both a full allosteric agonist and as a PAM in a system with a high M₁ receptor reserve⁵⁷.

Characteristically, the effects of allosteric modulators are saturable: that is, no further activity is observed above a certain limit, irrespective of modulator dose. This phenomenon is driven by the cooperativity between orthosteric and allosteric sites, and can range from subtle fine-tuning to very large modulation. For instance, *N*-chloromethyl brucine potentiates acetylcholine affinity by threefold at the M₃ receptor⁵⁸, whereas BQCA potentiates acetylcholine affinity at the M₁ receptor by ~100-fold⁵⁷. A second property is probe dependence⁵⁹, whereby the magnitude and direction of the allosteric effect mediated by the same modulator acting at the same receptor varies depending on the orthosteric ligand used to probe receptor function. A striking example is LY2033298, which is a PAM of oxotremorine-M signalling at the M₂ receptor, but a NAM of xanomeline at the same receptor⁶⁰.

The third characteristic of GPCR allostery is biased agonism, which refers to the ability of different ligands to stabilize a subset of functionally relevant GPCR conformations such that different signalling outputs are achieved at the exclusion of others^{54,61,62}. Biased agonism can be imposed on the signalling of orthosteric agonists by co-bound modulators. One example is the compound VU0029767, which potentiates acetylcholine-mediated phospholipase C activity via the M₁ receptor, but does not affect acetylcholine-induced phospholipase D activation⁶³. Finally, a crucial property of allosteric modulators is potential mAChR-subtype selectivity. This can be attained either by targeting a less conserved site on the receptor or via selective cooperativity with the orthosteric ligand, even if the allosteric site is shared between subtypes⁶⁴. Indeed, selective cooperativity is the major mechanism by which compounds such as thiochrome and LY2033298 show high selectivity for the M₄ receptor^{64–66}.

Bitopic muscarinic ligands

In recent years, research has turned to the rational design of hybrid molecules, which are designed to simultaneously bridge orthosteric and allosteric sites within a single receptor; such compounds have been termed ‘bitopic’, ‘dualsteric’ or ‘multivalent’^{7,67,68}. As summarized in FIG. 1, each of the different modes of targeting mAChRs has its pros and cons. The orthosteric site can provide high affinity and is supported by decades of studies of structure–activity relationships (SARs), but mAChR-subtype selectivity remains largely unattainable. Allosteric sites provide the opportunity to achieve greater receptor selectivity and promote more physiological response patterns. However, high-affinity modulators have not been reported to date, and certain modulator scaffolds — like many orthosteric drugs — show minimal improvements in activity despite numerous modifications⁶⁹. The bitopic ligand approach attempts to target the allosteric site to achieve selectivity and the orthosteric site to provide high affinity.

Despite some challenges^{7,68,70}, the pursuit of bitopic ligands has yielded interesting pharmacological findings. Disingrini *et al.*⁷¹ were the first to combine a nonselective, high-affinity orthosteric agonist (iperoxo) with an M₂ receptor-selective allosteric modulator to generate an M₂ receptor-selective agonist. This concept was extended by Mohr and colleagues^{72–74}. Interestingly, although bitopic agonists with mAChR-subtype selectivity and signalling bias have been described, substantial gains in affinity were generally not observed⁷. By contrast, the bitopic muscarinic receptor antagonist THRX-160209 demonstrated remarkable gains in both affinity and selectivity for the M₂ receptor⁷⁵, which highlights that the appropriate combination of an orthosteric antagonist, NAM and linker can yield ligands with the desired pharmacological properties.

These findings raise the possibility that previously described selective and/or biased agonists may represent unappreciated bitopic ligands. Consistent with this concept, a study of the biased M₂ receptor partial agonist McN-A-343 unmasked a pure orthosteric agonist and pure NAM of agonist efficacy following reverse engineering of McN-A-343 into fragments⁷⁶. A more recent study of the M₁ receptor-selective agonist TBPB also identified orthosteric and allosteric fragments of this molecule⁵³. This latter study is of particular relevance because TBPB, together with other novel M₁ receptor-selective agonists such as AC-42 and 77-

LH-28-1, was originally classified as an allosteric agonist^{77–80}. However, like McN-A-343, these agents display mixed modes of orthosteric or allosteric pharmacology depending on the experimental assay conditions^{48,53,81}. It is thus likely that such compounds adopt novel poses within the mAChRs that bridge both orthosteric and allosteric sites, hence accounting for their unique pharmacology⁸¹.

Towards the molecular mechanisms of allostery

The simplest mechanism to account for allosteric behaviour is the Monod–Wyman–Changeux (MWC) model^{82,83}, which postulates that receptors exist in an equilibrium between different states, and that orthosteric and allosteric ligands select one or more of these states over others⁸⁴. Because this occurs via topographically distinct sites, the state that is stabilized by one class of ligand can present a modified binding surface for the other class, which is manifested as the allosteric interaction. If this mechanism were restricted to two states, it would be expected that any ligand favouring the active state would display some degree of agonism, whereas compounds stabilizing the inactive state would show some degree of inverse agonism. Moreover, allosteric ligands that favoured the active state would be PAMs for agonists but NAMs for inverse agonists; this is the simplest molecular explanation for probe dependence. Interestingly, these predictions were confirmed in a recent study of BQCA activity at the M₁ receptor⁵⁷, which indicates that some mAChR modulators can mediate their effects predominantly through a simple conformational selection mechanism.

However, this simple model does not explain the probe dependence observed with compounds such as LY2033298 at the M₂ receptor, which can show PAM or NAM activity with different agonists⁶⁰. It also does not explain biased allosteric modulation, or why compounds such as gallamine are NAMs for agonists and antagonists⁸⁵. Thus, additional mechanisms seem to be involved, most obviously involving multiple active receptor states⁸⁶. Prior studies have also shown that compounds such as staurosporine and WIN62577 interact with a second allosteric site on mAChRs that is distinct from the common site^{87,88}. The location of this site remains unidentified, but it may be intracellular⁸⁹. If so, one can envisage more complex scenarios that involve an allosteric mechanism with respect to the orthosteric site, but potentially a steric mechanism with respect to receptor–transducer interactions. Similarly, it has been postulated that gallamine may utilize a two-step binding process, involving a peripheral domain that is distinct from the common allosteric site, in its interaction with the receptor⁸⁵. Finally, the simplest allosteric models do not explicitly incorporate receptor oligomers as the key functional units; a tetrameric arrangement has been proposed, at least for the M₂ receptor, to account for the observed cooperative effects of both orthosteric and allosteric ligands^{90,91}. Thus, despite the identification of a vast repertoire of behaviours for muscarinic allosteric ligands, the molecular mechanisms underlying these behaviours are only starting to be elucidated. It is particularly exciting, therefore, that recent structural and computational biology breakthroughs have shed new light on this matter.

Structural insights into ligand binding mode

Although mAChRs are among the most extensively studied GPCRs in terms of their pharmacology and biological function, the molecular structures of these receptors have, until recently, remained poorly understood. In 2012 the first mAChR structures were published^{10,11}, revealing the molecular organization of the M₂ and M₃ receptor subtypes in inactive, antagonist (inverse agonist)-bound conformations. This work was made possible owing to advances in GPCR biochemistry and crystallography, principal among them the T4 lysozyme fusion method⁹², new detergents⁹³, the lipidic cubic phase crystallization method^{94,95} and micro-focus X-ray diffraction data collection⁹⁶.

The structures of the M₂ and M₃ receptors revealed that, like other biogenic amine receptors, members of the mAChR family share the seven-pass transmembrane topology and overall fold of other GPCRs, with a few features extending outside of the membrane plane (FIG. 2). The ligand-binding pocket is deeply buried within the membrane (FIGS 2, 3a), and is situated similarly to the orthosteric binding sites of the related histamine⁹⁷, dopamine⁹⁸, adrenaline^{99–101} and serotonin^{102,103} receptors. The ligands bound to the M₂ and M₃ receptors in the two crystal structures — quinuclidinyl benzilate (QNB) and tiotropium, respectively — are both antagonists (inverse agonists) with similar chemical structures. In each case, the ligand occupies a similar pose (FIG. 3b), engaging in extensive hydrophobic contact with the receptor. A smaller number of polar contacts are evident: a pair of hydrogen bonds between Asn^{6.52} and the ligand hydroxyl and ketone, and a charge–charge interaction between the cationic amine of the ligand and the conserved Asp^{3.32} (superscripts denote Ballesteros–Weinstein¹⁰⁴ GPCR numbering) (FIG. 3b). The latter interaction is seen in all biogenic amine receptor structures solved to date and has been shown to make a major energetic contribution to binding¹⁰⁵. The paired hydrogen bonding between the ligand and Asn^{6.52} seems to be a unique feature of the mAChR family and has been proposed to be an important factor in slow ligand dissociation from mAChRs¹⁰⁶.

Comparison of the orthosteric ligand-binding pockets of the M₂ and M₃ receptors reveals a probable underlying factor for the limited success in the development of mAChR-subtype-selective ligands. The residues lining the orthosteric binding site are conserved absolutely in sequence and, moreover, are positioned almost identically in space in both receptors (FIG. 3b). Although there are a few subtle differences between the structures of the M₂ and M₃ receptors, attempts to exploit these minor structural variations to obtain ligands with increased mAChR-subtype selectivity have thus far been unsuccessful¹⁰⁵.

Strikingly, in the structures of the M₂ and M₃ receptors, the orthosteric ligand is almost completely occluded from solvent, with a tyrosine ‘lid’ located directly above — that is, extracellular to — the ligand (FIG. 3). This lid divides a large, solvent-accessible cavity into two distinct regions, only one of which interacts with the bound antagonists (FIG. 3a). The upper portion of this cavity, termed the extracellular vestibule, is lined with residues that have been implicated in the binding of allosteric modulators¹⁰⁷. As discussed below, LY2119620, which is a PAM of mAChRs, binds to this outer cavity¹². Although the backbone fold of the structures surrounding this allosteric site is remarkably well conserved between the M₂ and M₃ receptors, the amino acid side chains lining it show more divergence¹⁰. Hence, the development of ligands that interact with non-conserved amino

acids in the allosteric site provides a possible means by which subtype-selective therapeutics might be developed.

Molecular dynamics studies of the M₃ receptor indicate that the allosteric site may have a role in facilitating the binding of orthosteric ligands. Simulations of tiotropium binding to the M₃ receptor suggested that this ligand may rapidly interact with the extracellular vestibule of the receptor, but in these simulations tiotropium failed to enter the orthosteric site¹⁰. This may be due to limitations in the timescale accessible to molecular dynamics simulations, as well as the slow binding kinetics of tiotropium¹⁰. Notably, it has been shown experimentally that orthosteric ligands can bind to an allosteric site on the M₂ receptor, albeit with low affinity⁹⁰. Interactions with the extracellular vestibule may initiate desolvation of the ligand, which is required for the occupation of the orthosteric pocket. If binding to the allosteric site is indeed a metastable state for orthosteric muscarinic ligands, deliberate exploitation of this feature could potentially facilitate the development of muscarinic ligands with increased mAChR-subtype selectivity and/or improved kinetic properties.

Molecular basis for NAM function at the M₂ receptor

Recently, the molecular mechanism of allosteric modulator binding to the inactive state of the M₂ receptor was investigated by computational molecular dynamics and site-directed mutagenesis studies¹³. Using the inactive crystal structure of the M₂ receptor as a starting model, the binding of a series of structurally diverse cationic small-molecule modulators was simulated using long-timescale molecular dynamics. These simulations suggested that modulator binding to the extracellular vestibule may be driven largely by interactions between cationic amines on the modulators and aromatic residues on the receptor. The allosteric pocket appears to contain two cation- π centres: one including Trp422^{7,35} and Tyr177^{ECL2} (extracellular loop 2), and the other bounded by Tyr80^{2,61} and Tyr83^{2,64}. The importance of these regions in modulator binding was confirmed by site-directed mutagenesis experiments, which showed that affinity for the NAM heptane-1,7-bis(dimethyl-3'-phthalimido-propyl)ammonium bromide (C₇/3-phth) could be enhanced by the addition of more aromatic residues or acidic residues to the extracellular vestibule¹³.

Dror *et al.*¹³ also examined the interaction between the modulators and orthosteric antagonists to obtain insights into cooperativity. Two key mechanisms were proposed. The first was electrostatic repulsion between the modulator and the antagonist. The second was conformational coupling between orthosteric and allosteric sites. Specifically, molecular dynamics simulations indicated that bulky modulators (for example, alcuronium) are likely to stabilize an open extracellular vestibule conformation reminiscent of that seen in the crystal structure of the M₂ receptor bound to QNB¹¹. By contrast, smaller modulators (for example, C₇/3-phth) may preferentially stabilize a closed conformation of the extracellular vestibule¹³, similar to that seen in molecular dynamics simulations of the unliganded M₃ receptor¹⁰. Thus, in the inactive state the smaller modulators generally exhibit negative cooperativity with antagonists (that is, they destabilize antagonist binding), whereas the bulkier modulators typically enhance antagonist binding. Consistent with this model, the addition of bulky substituents to C₇/3-phth improved the binding of this NAM to the M₂

receptors occupied by radiolabelled ^3H -*N*-methylscopolamine¹³. Taken together, these data support the concept that orthosteric antagonist binding can be enhanced or diminished by a modulator that preferentially stabilizes a particular conformation of the extracellular vestibule.

Activation and positive allosteric modulation of the M₂ receptor

The initial structures of the M₂ and M₃ receptors were obtained in complex with high-affinity antagonists (inverse agonists) and consequently represent inactive receptor conformations. Obtaining crystals of active GPCRs has thus far proved to be extremely challenging; this is probably due in large part to the conformational heterogeneity induced by agonist binding¹⁰⁸. To date, these problems have been surmounted in the case of rhodopsin^{109–111}, the β_2 -adrenergic receptor (β_2 -AR)^{112,113} and, more recently, the M₂ receptor¹². In the last two cases, the first active-state structures have been obtained with the aid of conformationally selective antibody fragments, which mimic G proteins and stabilize the active conformation of the receptors to which they bind. This approach should prove to be useful for obtaining active-state structures of other GPCRs in the future.

The most striking feature of the active-state M₂ receptor structure, like that of active β_2 -AR and rhodopsin, is the outward rotation of transmembrane domain 6 to create a G protein-binding cavity on the intracellular surface of the receptor (FIG. 4a,b). The active-state structure of the M₂ receptor shows substantial similarity to the active states of both β_2 -AR and rhodopsin, with a slightly closer resemblance to the latter structure.

One of the most intriguing structural features of active β_2 -AR and rhodopsin is that both receptors show far more subtle rearrangements in the ligand-binding pocket and extracellular surface than on the intracellular side. This observation suggests that these regions are relatively fixed conformationally, with few changes associated with agonist binding or activation. By contrast, the active structure of the M₂ receptor revealed large changes throughout the receptor, including an unexpected contraction of the extracellular vestibule, in addition to changes on the surface of the intracellular receptor that closely parallel those seen in β_2 -AR and rhodopsin (FIG. 4c,d). This extracellular rearrangement results in a narrower allosteric site and a smaller orthosteric site that is occluded entirely from solvent (FIG. 4d). The much smaller size of the orthosteric site is reflected by the lower molecular weight of muscarinic agonists compared to antagonists and inverse agonists.

In the active state of the M₂ receptor, the small orthosteric agonist iperoxo engages Asp103^{3,32} with its cationic head group, whereas the polar isoxazoline tail contacts Asn404^{6,52} with a single hydrogen bond, paralleling the interactions of the inactive receptor with the antagonist QNB (FIG. 4c). These two interactions are bridged by an acetylene moiety in iperoxo, which runs perpendicular to transmembrane domain 3. Within the binding site, iperoxo exhibits a high degree of shape complementarity to the active conformation, filling the pocket almost entirely (FIG. 4d). By contrast, in the inactive conformation the M₂ receptor presents a large orthosteric site that is better suited to the binding of larger antagonists and inverse agonists. The exceptional complementarity of iperoxo with the active — but not inactive — orthosteric site of the receptor may account for its agonistic activity.

In addition to the substantial contraction of the orthosteric site upon activation, the extracellular vestibule is similarly much smaller in the active conformation of the M₂ receptor, even in the absence of a bound allosteric modulator. Isomorphous crystals were grown in the presence of LY2119620, a PAM of mAChRs¹², and inspection of electron density maps revealed binding of this ligand to the extracellular vestibule (FIG. 4d). Strikingly, structures of the active M₂ receptor with and without LY2119620 were highly similar, even in the extracellular vestibule¹². Indeed, the only substantial change in this pocket was a reorientation of Trp422^{7,35}, which directly interacts with the bound LY2119620. In addition, Phe181^{ECL2} was poorly resolved in the allosteric complex, which suggests that this residue may become more mobile upon binding of the receptor to this modulator. As Phe181^{ECL2} is a leucine residue in all other mAChR subtypes, interaction with this residue may allow modulators to have different effects on M₂ receptors compared to other mAChR subtypes.

The remarkable similarity of the extracellular vestibule conformation in the active M₂ receptor with and without bound LY2119620 suggests a mechanism for allosteric modulation. PAMs may achieve their effects by preferentially binding to and stabilizing the closed (that is, active) conformation of the extracellular vestibule. In doing so, PAMs may shift the receptor conformational equilibrium towards active conformations not only of the allosteric site but also of the receptor as a whole (FIG. 5). Analogously, NAMs may preferentially bind the open conformation of the extracellular vestibule seen in the inactive structures, thereby skewing the conformational ensemble of the receptor in favour of inactive states.

In total, the mAChRs contain at least three binding sites that have been defined pharmacologically and structurally: the extracellular vestibule, the orthosteric binding pocket and the G protein-binding site (FIG. 5). Comparison of the active- and inactive-state structures of the M₂ receptor shows that all three sites can undergo substantial conformational rearrangements upon receptor activation. However, it is important to recognize that the crystal structures present only static views of a single receptor conformation and thus provide, at best, an incomplete picture.

The structures solved to date are suggestive of a concerted conformational change throughout all three sites, but it remains to be seen whether changes in each site can occur independently of one another. Such a possibility is suggested by structures of the A_{2A} adenosine receptor bound to agonists^{114,115}. Conformational changes in the orthosteric site are indicative of receptor activation, but the intracellular surface remains in a closed, inactive state. Activation of the mAChRs might similarly involve separable conformational changes at each of the three sites rather than a single concerted activation throughout the entire receptor. Spectroscopic and other biophysical assays will probably be required to definitively establish the mechanism of receptor activation and the means through which ligands stabilize active receptor conformations.

Conclusions and future challenges

The past few years have witnessed unprecedented progress in our understanding of the biology, pharmacology and structure of mAChRs. The use of novel mutant mouse models, including mAChR knock-in mice with altered signalling properties and mutant mice in which distinct mAChR subtypes can be deleted in a temporally and spatially controlled fashion, should provide more detailed insights into mAChR biology.

From a structural point of view, the most obvious future goal is to obtain high-resolution structures for all five mAChR subtypes, both in their inactive and active conformations, as well as in complex with different classes of ligands (orthosteric, allosteric or bitopic ligands). Such studies may reveal a broad spectrum of mAChR conformations depending on the receptor subtype and activation state, as well as receptor-bound ligands. Identifying the structural basis that underlies the ability of mAChRs, as well as other GPCRs, to interact with G proteins, arrestins, GPCR kinases (GRKs) and other GPCR-associated signalling molecules should also be of great general interest. Taken together, the outcome of these studies should considerably facilitate the development of novel muscarinic receptor agents and tools that show a high degree of mAChR subtype selectivity and hopefully cause fewer side effects. Finally, lessons learned from work in the mAChR field may be applicable to GPCRs in general, offering new insights into how to design better therapeutic agents targeting this important class of cell surface receptors.

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Box 1**Selected allosteric modulators of mAChRs**

The allosteric modulators listed here have been used to provide important insights into muscarinic acetylcholine receptor (mAChR) allostery. Note that this list is by no means exhaustive. Some of the modulators bind to all five mAChR subtypes but are listed under the mAChR subtype for which they have been most extensively studied.

M₁ receptor

- Tacrine
- MT7
- Brucine
- Staurosporine
- BQCA
- VU0119498
- VU0029627
- ML169

M₂ receptor

- Gallamine
- Alcuronium
- C₇/3-phth
- W84
- DUO3
- Tacrine
- LY2033298
- LY2119620

M₃ receptor

- *N*-chloromethyl brucine
- WIN62577
- VU0119498

M₄ receptor

- MT3
- Thiochrome
- LY2033298

- LY2119620
- VU0010010
- VU0152100

M₅ receptor

- VU0119498
- VU0238429
- ML375

Figure 1. Modes of targeting mAChRs (GPCRs) by different classes of ligands
Orthosteric ligands (green) bind to the site recognized by the endogenous agonist (acetylcholine) for the receptor. Allosteric ligands (yellow) bind to a topographically distinct site. Bitopic ligands (blue) concomitantly interact with both orthosteric and allosteric sites. The key properties generally associated with each mode of receptor targeting are also indicated. GPCR, G protein-coupled receptor; mAChR, muscarinic acetylcholine receptor; SAR, structure–activity relationship.

Figure 2. Overall structure of the M₂ and M₃ receptors

The structures of the inactive, antagonist-bound M₂ (blue) and M₃ (yellow) receptors are highly similar to each other in overall fold. For the sake of clarity, only the M₃ receptor ligand (tiotropium) is shown. The overall architecture of the M₂ and M₃ muscarinic acetylcholine receptors (mAChRs) is very similar to that of other biogenic amine G protein-coupled receptors (GPCRs), with similar orthosteric ligand binding sites (orange spheres).

Figure 3. Structure of the orthosteric mAChR ligand binding site

a | A cross-section through the M₂ receptor structure reveals a large, solvent-accessible cavity extending through the receptor. The QNB ligand (orange spheres) is bound within the receptor transmembrane core (black, receptor protein; blue, receptor surface). **b** | The orthosteric ligand (antagonist)-binding sites for the M₂ and M₃ receptors are almost identical in both structure and sequence. Polar contacts (red dotted lines) between the receptor and bound antagonist (M₂, QNB; M₃, tiotropium) are identical for the two receptors. Residues are numbered according to the human M₂ receptor sequence. mAChR, muscarinic acetylcholine receptor.

Figure 4. Activation and allosteric modulation of the M₂ receptor

As shown in part **a** and part **b**, the intracellular tip of transmembrane domain 6 (TM6) rotates outwards in the active M₂ receptor structure (orange) relative to the inactive state (blue). As shown in part **c**, the orthosteric binding site contracts upon M₂ receptor activation, enclosing the agonist iperexo (yellow) in a smaller binding site, as compared to the antagonist (QNB; green) binding cavity. Residues are numbered according to the human M₂ receptor sequence. As shown in part **d**, LY2119620 (magenta), a muscarinic positive allosteric modulator, binds to the extracellular vestibule of the M₂ receptor directly above the orthosteric agonist iperexo (yellow). ICL2, intracellular loop 2.

Figure 5. Hypothetical mechanism for allosteric modulation of the M_2 receptor by a PAM

a | Scheme of the M_2 receptor with the orthosteric and allosteric binding sites highlighted in green and red, respectively. In the absence of a ligand, the receptor adopts inactive conformations, which are relatively more stable. **b** | Agonist binding shifts the equilibrium in favour of active receptor conformations. **c** | Binding of a positive allosteric modulator (PAM) such as LY2119620 to the active -state receptor enhances the affinity of the receptor for the agonist and shifts the equilibrium to further favour active receptor conformations.

Table 1
Novel mAChR-mutant mouse models

Mouse strain	Major phenotypes	Refs
<i>Chrm1</i> -mutant mice selectively lacking M ₁ receptors in the forebrain or hippocampal CA3 pyramidal neurons (use of floxed M ₁ receptor mice)	Lack of mGluR-mediated LTD in the hippocampus, accompanied by changes in presynaptic neurotransmitter release	116
Mice carrying a floxed <i>Chrm2</i> gene (floxed M ₂ receptor mice)	N/A	*
<i>Chrm3</i> -mutant mice selectively lacking M ₃ receptors in specific cell types (use of floxed M ₃ receptor mice)	Pancreatic β -cells: deficits in glucose tolerance, accompanied by impaired insulin release <i>in vivo</i>	34
	Brain (neurons and glial cells): reduced body size; hypoplasia of the anterior pituitary gland; reduced levels of pituitary and serum growth hormone, as well as prolactin	117
	Brain (neurons and glial cells): low bone mass, probably owing to enhanced central sympathetic outflow	118
	Hepatocytes: none (no changes in glucose homeostasis and hepatic glucose fluxes)	119
	Osteoblasts: none (no changes in bone formation and bone mass)	118
	Smooth muscle cells (airways): restoration of normal lung function in obese mice	120
<i>Chrm3</i> -knock-in mice containing 15 Ser→Ala point mutations within the i3 loop of the M ₃ receptor	Greatly reduced M ₃ receptor phosphorylation and impaired arrestin recruitment following M ₃ receptor activation; deficits in fear conditioning, learning and memory	121
	Impaired glucose tolerance, accompanied by reduced M ₃ receptor-mediated insulin release from pancreatic islets	40
<i>Chrm4</i> -mutant mice selectively lacking M ₄ receptors in D ₁ dopamine receptor-expressing neurons (use of floxed M ₄ receptor mice)	Increased dopamine efflux in the nucleus accumbens; modulation of dopamine-dependent behaviours	24
	Strong reduction of the antipsychotic-like effects of xanomeline in two mouse models of psychosis	25
<i>Chrm5</i> -knock-in mice containing an 18-amino-acid deletion within the M ₅ receptor-i3 loop (residues 369–386)	Reduction in evoked dopamine release from striatal slices	122

LTD, long-term depression; mAChR, muscarinic acetylcholine receptor (five distinct subtypes, denoted M₁ to M₅, which are encoded by the genes *Chrm1* to *Chrm5* in mice); mGluR, metabotropic glutamate receptor.

* J.W. & J. Jeon, unpublished observations.