Abstract

Schizophrenia is a psychiatric disorder affecting approximately 1% of the world population and is characterised by the presence of positive and negative symptoms and cognitive deficits. Whilst current therapeutics ameliorate positive symptoms, they are largely ineffective in improving negative symptoms and cognitive deficits. The cholinergic neurotransmitter system heavily influences cognitive function and there is evidence that implicates disruption of the central cholinergic system in schizophrenia. Historically, targeting the cholinergic system has been impeded by poor selectivity leading to intolerable side effects warranting the need to develop more targeted therapeutic compounds. In this review we will summarise evidence supporting the roles of the cholinergic system, particularly the muscarinic M₁ receptor, in the pathophysiology of schizophrenia and discuss the potential of a promising new class of candidate compounds, allosteric ligands, for addressing the difficulties involved in targeting this system. The body of evidence presented here highlights the dysfunction of the cholinergic system in schizophrenia and that targeting this system by taking advantage of allosteric ligands is having clinically meaningful effect on cognitive deficits.

Key words: Central nervous system; Antipsychotic; Allosteric; Cholinergic; Schizophrenia; Mutagenesis; Cognition; Muscarinic
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INTRODUCTION
Cognitive impairment is associated with multiple disease states and usually has a big impact on quality of life. Schizophrenia is a psychiatric disorder with a predominant age of onset from late teens to early adulthood that affects approximately 1% of the world population. It is diagnosed by the presence of what are characterised as positive and negative symptoms and cognitive deficits. Unlike neurological disorders no major lesion was apparent in the central nervous system (CNS) from subjects with schizophrenia and therefore many of the hypotheses as to its cause have been based on the actions of drugs that either worsen or ameliorate the symptoms of the disorder.

The dopamine hypothesis
With the serendipitous discovery that chlorpromazine had unexpected therapeutic benefit when given to psychotic individuals there was a significant effort to develop a range of what became known as first generation neuroleptic drugs[2]. Subsequently, it was determined that the ability of these drugs to reduce the severity of positive symptoms in some people with schizophrenia was directly related to the ability of first generation neuroleptic drugs to act as antagonists at the dopamine D2 receptor[3].

The dopamine hypothesis was the first attempt to encapsulate the biological mechanisms underpinning schizophrenia. This hypothesis grew from an understanding that the early antipsychotics block the dopamine D2 receptor and that drugs which increase CNS dopamine function cause or worsen psychosis. The original dopamine hypothesis proposed that widespread hyperactivity of the dopamine neurotransmitter system contributes to the pathophysiology of schizophrenia (reviewed in[4]).

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The serotonin hypothesis
Clozapine is the archetypal drug that led to the development of a family of drugs termed second-generation neuroleptics[5]. Whilst the second-generation drugs were developed to bind to some of the receptors targeted by clozapine it is notable that none of these drugs have achieved the extended therapeutic reach of clozapine such as improving the cognitive deficits[6]. An early second generation neuroleptic drug, risperidone, was suggested to have significant benefits compared to first generation neuroleptic drugs[7,8] which were presumed to be due, at least in part, to its ability to antagonise the serotonin 2A receptor[9]. The clinical benefit obtained by risperidone, added to the observation that drugs such as lysergic acid diethylamide could cause psychotic symptoms by stimulating the serotonergic system[10], became the evidence to support the serotonin hypothesis of schizophrenia.

Initial interest in the serotonergic system stems from the observation that drugs affecting serotonin receptors produce psychotomimetic effects[11] and the discovery that second generation neuroleptics bind to various serotonin receptors[12,13]. These observations have led to investigation of the serotonin system both post-mortem and in vivo; a recent meta-analysis[14] demonstrated a moderately higher level of the serotonin receptor subtype 5-HT1A and a substantially lower levels of the 5-HT2A receptor subtype in the prefrontal cortex of post-mortem subjects with schizophrenia.

The glutamate hypothesis
The glutamate hypothesis is somewhat unique as it is founded in the observation that phencyclidine could cause a broad range of symptoms in normal individuals[15] and exacerbate psychotic symptoms in patients with schizophrenia but no drug targeting the glutamatergic system has proved useful in treating the disorder[16]. Further investigation has revealed elevated glutamate activity particularly in the medial prefrontal cortex and basal ganglia of un-medicated and first episode patients, demonstrated by magnetic resonance spectroscopy studies (reviewed in[17]). Of studies in post-mortem brain tissue, the most robust finding has been widespread differences in glutamatergic pyramidal neuron morphology, particularly lower numbers[18] and lengths of dendrites and lower numbers of dendritic spines in deep cortical layer III of the dorsolateral prefrontal cortex. There have also been mixed reports of differences in mRNA and protein levels of receptor subunits, transporters, and synthesis enzymes (post-mortem glutamatergic differences in schizophrenia reviewed in[19]).

The cholinergic hypothesis
Preceding all of these neurotransmitter hypotheses was the cholinergic hypothesis which was founded on the observation that some subjects with schizophrenia had remission or an improvement in symptom severity...
after coma induced by a dose of acetylcholine (ACh)\textsuperscript{27}. Since those early experiments components of the cholinergic system, the muscarinic receptors, have been targeted by drugs such as clozapine and olanzapine that reduce the symptoms of schizophrenia\textsuperscript{28} and drugs such as benzatropine which were used to control the extrapyramidal side effects associated with treating with first generation antipsychotic drugs\textsuperscript{29}. Whilst these neuropsychopharmacological findings are important to consideration of the role of the cholinergic system in schizophrenia, other studies which have advanced our understanding of the cholinergic system and how it may be affected in the disorder have been important in refining the cholinergic hypotheses of schizophrenia.

The cholinergic system consists of several distinct pathways; the most well studied being the pathway that projects from the basal forebrain to most of the CNS\textsuperscript{30}. This pathway extensively modulates the dopamine system by affecting striatal dopamine release\textsuperscript{31}; the glutamate system by potentiating NMDA receptors\textsuperscript{32}, and the serotonergic system via projections to the dorsal raphe nucleus where ACh inhibits the release of serotonin\textsuperscript{33} (cholinergic interactions in schizophrenia reviewed in\textsuperscript{34}). Whilst the modulatory interactions of the cholinergic system with other neurotransmitter systems provide mechanisms which can implicate that system in schizophrenia there are also several lines of evidence to suggest that the cortical muscarinic M\textsubscript{1} receptor, a component of the cholinergic system, is particularly implicated in the pathophysiology of schizophrenia.

Although there have been several reviews discussing the cholinergic system and its role in schizophrenia\textsuperscript{35,36}, there have recently been some exciting advances in developing drugs to target the cholinergic system\textsuperscript{37-39} that make a review of the area timely. This is particularly the case because the cognitive deficits of schizophrenia are largely non-responsive to current drug treatments\textsuperscript{40} whereas preliminary data suggest that the new drugs targeting the cholinergic system will improve cognition\textsuperscript{41}. Cognitive deficits are a core feature of schizophrenia and are made up of deficits in the domains of speed of processing, attention/vigilance, working memory, verbal learning and memory, visual learning and memory, reasoning and problem solving, and social cognition (reviewed in\textsuperscript{42}). The impact of these deficits in people with schizophrenia is now considered the most debilitating of all the symptom domains associated with the disorder. Therefore, this review will first summarize the cholinergic system, then briefly review evidence supporting its role, particularly the muscarinic M\textsubscript{1} receptor, in cognition and the pathophysiology of schizophrenia and finally discuss the potential of a promising new class of candidate compounds and how they target the muscarinic M\textsubscript{1} receptor for addressing the difficulties involved in targeting this system.

**OVERVIEW OF THE CHOLINERGIC SYSTEM**

ACh is a modulatory neurotransmitter\textsuperscript{43} in the CNS and peripheral autonomic and somatic nervous systems. It is essential to the function of all branches of the peripheral nervous system\textsuperscript{44} and has been associated with a large number of cognitive processes in the CNS. Two classes of integral membrane receptor mediate signal transmission: The ionotropic nicotinic receptors (nAChR), and the metabotropic muscarinic receptors.

Within the CNS, cholinergic neurons are predominantly located in the basal forebrain as cortical projection neurons (reviewed in\textsuperscript{45}) and striatal interneurons (Figure 1). The projection neurons arise from four cell groups (Ch1-4) in the basal forebrain\textsuperscript{46} and from the pedunculopontine-lateral dorsal tegmental area in the brainstem. These cell groups are not exclusively composed of cholinergic neurons, but contain a diverse mix of other neurotransmitter systems including GABAAergic, peptide transmitter, and catecholaminergic neurons. These projection neurons modulate all regions of cortex including the hippocampal formation\textsuperscript{46}. In addition to the projection nuclei of the basal forebrain, striatal cholinergic interneurons interact heavily with the dopaminergic projections from the substantia nigra pars compacta and the ventral tegmental area (VTA)\textsuperscript{47} (The human cholinergic brain architecture is extensively reviewed in\textsuperscript{48}).

ACh acts in part via the family of five muscarinic (M\textsubscript{1-5}) receptors; they have a differential expression pattern throughout the human CNS\textsuperscript{49}. Point mutation and other studies suggest that the structural position of the muscarinic receptor transmembrane domains relative to one another affect the differential binding specificity of ligands to these receptors\textsuperscript{49,50}. Significantly, the orthosteric site on the five muscarinic receptors is highly conserved and this has presented a challenge to the development of receptor-specific drugs.

**Muscarinic M\textsubscript{1} receptors**

Of the five muscarinic receptors, the muscarinic M\textsubscript{1} receptor is the predominant muscarinic receptor in all cortical areas\textsuperscript{49} where it is located on excitatory neurons to modulate their firing, for example, by potentiating NMDA receptors\textsuperscript{51} and at cholinergic synapses\textsuperscript{52}. Mouse knockout and knock down studies have elucidated some of its roles in CNS function. For example, in muscarinic M\textsubscript{1} receptor knockout mice, mitogen activated protein kinase (MAPK) signalling is impaired in cortical neuronal cultures\textsuperscript{53} and hippocampal slices\textsuperscript{54}. The role of MAPK in hippocampal long term potentiation\textsuperscript{54} and neuronal plasticity\textsuperscript{55} demonstrate a potential mechanism by which muscarinic M\textsubscript{1} receptors modulate learning and memory. Gamma oscillations (20-80 Hz) of neuronal firing patterns are associated with memory\textsuperscript{56}, hippocampal gamma oscillations induced by muscarine, a muscarinic agonist, but not those induced by kainite, a glutamatergic kainite receptor agonist, are completely absent from muscarinic M\textsubscript{1} receptor knockout mice\textsuperscript{57}. Another study reported that muscarinic M\textsubscript{1} receptor knockout mice had fewer and shorter dendrites and disrupted cortical tonotopic maps in the auditory cortex\textsuperscript{58}. Additionally,
muscarinic M\(_1\) receptor knockout mice had impaired experience dependent plasticity in the auditory cortex\(^{[59]}\), implicating the muscarinic M\(_1\) receptor in auditory cortical organisation, sensory processing, and learning. Additionally, muscarinic M\(_1\) receptor knockout mice are deficient in working memory and memory consolidation processes where the hippocampus and cerebral cortex interact\(^{[60]}\). These studies together implicate muscarinic M\(_1\) receptors in various aspects of learning and memory.

In addition to their cognitive effects, muscarinic M\(_1\) receptors are implicated in the response to a number of brain-penetrant drugs in mice. Muscarinic M\(_1\) receptor knockout mice are highly resistant to seizures induced by pilocarpine, a muscarinic agonist, which does not suppress the voltage dependent K\(^+\) M current in these animals. Conversely, muscarinic M\(_2\)–4 receptor knockout mice display wild-type seizure response and M current suppression when administered pilocarpine\(^{[61]}\). As M current suppression is considered the mechanism by which pilocarpine seizures are produced\(^{[62]}\), the muscarinic M\(_1\) receptors mediate this drug induced seizure response by modulating M currents in sympathetic neurons\(^{[61]}\). Muscarinic M\(_1\) receptor knockout mice displayed an elevated striatal level of extracellular dopamine and increased locomotor activity, which neuroleptic treatment attenuated\(^{[63]}\). In addition, muscarinic M\(_1\) receptor knockout mice had an increased sensitivity to amphetamine administration, in both the locomotor response and striatal dopamine levels\(^{[63]}\) demonstrating an interaction between the cholinergic and dopaminergic systems. This interaction is supported by the report of an increase in morphine-induced analgesia and lower rates of self-administration of morphine and cocaine in muscarinic M\(_1\) receptor knockout mice\(^{[64]}\). Taken together, these studies implicate the muscarinic M\(_1\) receptor in the both the response to these drugs and as a mechanism by which the cholinergic system interacts with the dopamine reward pathway.

THE CHOLINERGIC SYSTEM IN THE PATHOPHYSIOLOGY OF SCHIZOPHRENIA

The cholinergic system’s role in schizophrenia is supported by research involving neuropsychopharmacological agents, post-mortem brain tissue, and neuroleptic drug treatments. In addition to these three areas of research, a landmark single photon emission computed tomography (SPECT) study showed widespread lower levels of muscarinic receptor binding in the cerebral cortex in patients with schizophrenia who were medication free compared to healthy control subjects\(^{[65]}\).

Neuropsychopharmacology

Several neuropsychopharmacological studies provide evidence to support the role of muscarinic receptors in schizophrenia. Administering a muscarinic antagonist, scopolamine, to healthy control subjects produced deficits in spatial and working memory as well as sustained visual attention\(^{[66]}\). In another report this group demonstrated that scopolamine produced deficits in spatial and object working memory using the n-back paradigm\(^{[67]}\), which is used to assess working memory function in patients with schizophrenia. Additionally, acute administration of scopolamine to healthy subjects produced deficits in maintenance of working memory\(^{[68]}\). Another study demonstrated higher doses of scopolamine as well as atropine and Ditran, two other muscarinic antagonists, produced hallucination, and delirium in addition to cognitive deficits in healthy
control subjects\(^{[69]}\). Thus blocking muscarinic receptor activity mimics both positive and cognitive symptoms observed in patients with schizophrenia.

Sleep disturbances, including impaired sleep continuity, abnormal REM latency\(^{[70]}\) and decreased slow wave sleep\(^{[71]}\) observed in patients with schizophrenia have been associated with impaired sleep dependent memory consolidation, as well as negative symptom severity\(^{[71]}\). Administration of cholinergic agonists to healthy volunteers causes shortening of REM latency during sleep\(^{[72,73]}\) and altered REM latency in a subset of patients with schizophrenia more severely than healthy control subjects\(^{[24]}\). These findings demonstrate that pharmacological manipulation of the cholinergic system can mimic and worsen the sleep disturbances observed in patients with schizophrenia. These studies add support to the body of evidence implicating the cholinergic system in the pathology of schizophrenia.

**Post mortem studies**

A role for the muscarinic M\(_4\) receptor in schizophrenia is supported by a variety of post-mortem studies investigating radioligand binding and mRNA levels in multiple brain regions (Table 1), which report a decrease in muscarinic M\(_4/M_4\) receptor binding and muscarinic M\(_4\) receptor mRNA levels in multiple brain regions (reviewed in\(^{[81]}\)). Notably, we reported a decrease in muscarinic M\(_4\)\(^{[78]}\), but not M\(_3\), M\(_5\)\(^{[77]}\) or M\(_4\)\(^{[76]}\) receptor protein in the cortex of subjects who had schizophrenia confirming that the M\(_4\) receptor is selectively decreased in people with the disorder. More recently, we\(^{[78]}\) reported lower binding of \(^{[3]}\)H]pirenzepine (\(^{[3]}\)H]PRZ), a muscarinic M\(_4/M_4\) receptor selective antagonist, in the dorsolateral prefrontal cortices from people with schizophrenia to be limited to a subset (approximately 25%) of subjects; these subjects had 75% lower binding to muscarinic receptors in Brodmann area (BA) 9 when compared to both healthy control subjects and other subjects with schizophrenia. These data underpin the proposal of a subgroup within schizophrenia that have a muscarinic receptor deficit schizophrenia (MRDS). This hypothesis is pertinent to the growing acceptance that schizophrenia is a syndrome of disorders that may well have differing aetiologies\(^{[79]}\).

Although \(^{[3]}\)H]PRZ binds to both muscarinic M\(_3\) and M\(_4\) receptors, its selectivity for the muscarinic M\(_4\) receptor was increased under the conditions used for the study that identified MRDS\(^{[80]}\). Furthermore, we previously showed that muscarinic M\(_4\), but not M\(_3\), receptor mRNA and \(^{[3]}\)H]PRZ binding levels were significantly lower in BA 9 in subjects with schizophrenia\(^{[78]}\). These data, combined with the high abundance of muscarinic M\(_3\) receptors relative to the other muscarinic receptor types within the cerebral cortex\(^{[81]}\), strongly implicates muscarinic M\(_3\) receptors in the pathophysiology of the MRDS group. More recent work has demonstrated lower \(^{[3]}\)H]PRZ binding in BAs 6, 10, 24, 44 and 46 in the same cohort\(^{[81,82]}\); these findings are consistent with the widespread loss of cortical muscarinic receptors in people with schizophrenia reported in the SPECT study\(^{[83]}\).

### Table 1  Muscarinic receptors in post-mortem studies

| Ref. | Receptor Type | Regions | Results |
|------|---------------|---------|---------|
| [114] | M\(_4\)/M\(_4\) | CPu | Lower binding |
| [115] | M\(_4\)/M\(_4\) | CPu | Lower binding |
| [116] | M\(_4\)/M\(_4\) | CPu | M\(_3\): no difference; M\(_4\): below sensitivity in both SCZ and HC |
| [117] | M\(_4\)/M\(_4\) | PFC | Lower binding |
| [76] | M\(_4\)/M\(_5\) | DLPFC, PCx | Lower binding and M\(_3\): mRNA levels in DLPFC; no difference in binding, lower M\(_4\) and M\(_5\): mRNA levels in PCx |
| [118] | M\(_4\) | PMC | Lower levels |
| [119] | M\(_4\)/M\(_4\) | ACC | Lower binding |
| [120] | M\(_4\)/M\(_4\) | ACC | No difference |
| [121] | M\(_4\)/M\(_5\)/M\(_5\) | STG | Lower M\(_4\)/M\(_5\): binding non-significant; lower M\(_5\)/M\(_5\): binding |
| [77] | M\(_4\)/M\(_5\) | DLPFC, PCx | M\(_4\): and M\(_5\): binding and M\(_3\): mRNA no difference; M\(_3\): mRNA below sensitivity in both SCZ and HC |
| [122] | M\(_4\)/M\(_4\) | Hipp | Lower binding; lower M\(_4\): mRNA no difference in M\(_3\): mRNA levels |
| [123] | M\(_4\)/M\(_5\) | PMC | Lower M\(_4\)/M\(_5\): binding; no difference in M\(_3\): binding |
| [78] | M\(_4\) | DLPFC | Lower binding in subset of SCZ subjects |
| [81] | M\(_4\)/M\(_5\) | PMC | Lower M\(_4\): binding in subset of SCZ subjects; no difference in mRNA levels or M\(_3\): binding |
| [82] | M\(_4\) | DLPFC, ACC, BrA | Lower binding in subset of SCZ subjects |

* / * Indicates that the radioligand used in the study binds both receptor types listed. CPu: Caudate-putamen; SCZ: Subjects with schizophrenia; HC: Non-psychiatric healthy control; PFC: Prefrontal cortex; DLPFC: Dorsolateral prefrontal cortex; PCx: Parietal cortex; PMC: Premotor cortex; ACC: Anterior cingulate cortex; STG: Superior temporal gyrus; Hipp: Hippocampus; BrA: Broca’s area.

### Neuroleptic drug treatment

Traditional antipsychotic therapies achieve their action by blockade of central dopamine D2 receptors (reviewed in\(^{[84]}\)); unfortunately, these drugs can cause extrapyramidal side effects (EPS) such as tremors and dyskinesia similar to those seen in people with Parkinson’s disease\(^{[84]}\). Adjunctive anti-cholinergic agents are often used to combat EPS, due to the interaction between the dopaminergic and cholinergic systems in the basal ganglia, particularly the VTA\(^{[85]}\). The non-specific muscarinic antagonist procyclidine, however, exacerbated positive symptoms in patients with schizophrenia treated
with the antipsychotic flupentixol\(^{[85]}\), while the muscarinic M\(_1\) preferring antagonist biperiden worsened positive symptoms and ameliorated the negative symptoms in patients who were otherwise drug free for at least 2 wk\(^{[87]}\). Patients with schizophrenia who were taking benzatropine, a muscarinic antagonist, as an adjunctive therapy had impaired semantic organisation\(^{[88]}\). These studies show that pharmacological interventions affecting the central cholinergic tone in patients with schizophrenia has a complex effect on symptomatology, and that more specific modulation of the system may be beneficial for ameliorating the symptoms of the disorder.

**Xanomeline: A proof of principle drug trial in schizophrenia**

A small, double-blind, placebo controlled trial of an agonist selective for the muscarinic M\(_1\) and M\(_3\) receptors, xanomeline, was conducted in a cohort of treatment resistant schizophrenia patients\(^{[39]}\). The xanomeline treated group showed significant improvement in both positive and negative symptoms as measured using the positive and negative syndrome scale and the brief psychiatric rating scale as well as improvements in a battery of cognitive tests, particularly in working memory and verbal and visual learning\(^{[39]}\). Unfortunately, peripheral adverse events were observed including vomiting, nausea and gastrointestinal distress similar to those observed in previous Alzheimer’s disease trials\(^{[89,90]}\); this led to the discontinuation of xanomeline. This compound highlighted two important points regarding muscarinic receptors as drug targets. Firstly, its efficacy in the schizophrenia trial\(^{[39]}\) supports the body of evidence suggesting that muscarinic receptors, particularly muscarinic M\(_1\) and M\(_3\) receptors, are viable targets for treatment of schizophrenia. Secondly, the problem of selectivity for specific muscarinic receptors. High selectivity for individual muscarinic receptors is difficult to achieve with orthosteric ligands, due to the high homology between their orthosteric binding pockets. The side effects observed in the xanomeline trials were considered to be the result of “off target” activation of muscarinic receptors, particularly muscarinic M\(_1\) and M\(_3\) receptors\(^{[89,90]}\). Clearly, there is a need for ligands that are more selective, both for drug development and investigating individual receptors both in vivo and in vitro; allosteric compounds present a possible solution.

**RECEPTOR ALLOSTERISM**

An allosteric site is a binding site on a receptor distinct from the orthosteric binding site of the endogenous ligand that can be activated by proteins or small molecules to elicit a biologic response (allosteric agonist) or modulate the response of an endogenous molecule, orthosteric agonist or orthosteric antagonist biding to the orthosteric site (allosteric modulator). Allosteric modulators increase [positive allosteric modulator (PAM)] or decrease [negative allosteric modulator (NAM)] the ability of orthosteric ligands to activate or inactivate the receptor. These terms describing allosterism are defined according to the guidelines set out by the International Union of Basic and Clinical Pharmacology\(^{[91]}\).

**Characterisation of putative M\(_1\) receptor allosteric site**

In an attempt to better understand the muscarinic M\(_1\) receptor and aid drug development, site-directed mutagenesis techniques have identified amino acid residues of the muscarinic M\(_3\) receptor implicated in the binding of compounds to the orthosteric site, the allosteric site, and in the functional cooperativity between the orthosteric and allosteric sites. From these studies, the allosteric site is considered to be a pocket deep in the transmembrane domains (TMs; reviewed in\(^{[92]}\)). Recent X-ray crystallographic determination of the structures of the muscarinic M\(_2\)\(^{[93]}\) and M\(_3\)\(^{[94]}\) receptors has confirmed the orthosteric site is located in a pocket that forms a channel between the TMs for these receptors. The differential binding specificity of ligands to the different receptors is thought to relate to the structural position of the transmembrane domains relative to one another\(^{[95,96]}\).

An early study characterising the allosteric site of the muscarinic M\(_1\) receptor performed alanine scanning of the extracellular loop (ECL) amino acid residues conserved across the five muscarinic receptors. The authors found that the only residue to have any appreciable effect on the binding characteristics of gallamine (NAM) was Tyr400, found in the 3\(^{rd}\) ECL, leading them to hypothesise that the allosteric site of gallamine is close in space to the orthosteric site\(^{[97]}\). More recently, other residues that are implicated in binding to the allosteric site (Figure 2A) and functional cooperativity between the allosteric and orthosteric sites (Figure 2B) of the muscarinic M\(_1\) receptor were identified in the 2\(^{nd}\), 3\(^{rd}\), 4\(^{th}\), 6\(^{th}\), and 7\(^{th}\) TMs and the 2\(^{nd}\) ECL\(^{[95,96]}\). Computer modelling of the residues identified in mutagenesis studies has predicted a putative binding site which is topographically distinct from the orthosteric site, mainly composed of residues from ECL2, TMII, and TMVI\(^{[96]}\). Notably, these studies using 1-(4-methoxybenzyl)-4-oxo-1, 4-dihydroquinoline-3-carboxylic acid (BQCA)\(^{[96]}\) and a structural derivative of BQCA (benzoquinazolinone 12)\(^{[95]}\) found that mutating the Tyr400 residue completely abolished binding to the allosteric site of the muscarinic M\(_1\) receptor; in agreement with the study using gallamine. Interestingly, the residues implicated in allosteric binding to the muscarinic M\(_1\) receptor are highly conserved across all five muscarinic receptors, while residues implicated in the functional cooperativity between the orthosteric and allosteric sites are not; leading the authors to hypothesise that these residues underlie the muscarinic M\(_1\) receptor specific cooperativity of these ligands\(^{[96]}\).

**Muscarinic M\(_1\) receptor allosteric compounds**

There has been considerable interest in using allosteric ligands to target GPCRs implicated in a variety of disease
Allosteric affinity reversed amphetamine induced hyperlocomotion in 1-[4-(pyridine-2-yl)piperidin-1-yl]-4-oxo-4-H-quinolinizine-3-carboxylic acid (PQCA) [37], attenuated scopolamine deficits in novel object recognition, self-ordered spatial search, and delayed alternation Y-maze tasks in rats, cynomolgus macaques, and rhesus macaques, respectively [111]. The efficacy of PQCA in these three models of cognitive function further supports the role of the muscarinic M1 receptor in cognition and demonstrates the potential of this class of compound as useful therapeutics. Additionally, Merck have taken MK-7622, a muscarinic M1 receptor PAM based on the BQCA scaffold, 1-[(1'-2-methylbenzyl)-1,4'-bipiperidin-4-yl]-1H-benzo[d]imidazol-2(3H)-one (TBPPB), which reversed amphetamine induced hyper-locomotion in rats [103], a model predictive of antipsychotic-like activity; LuAE51090, which improved mouse performance on the delayed alternation Y-maze [104], a model of working memory; and GSK1034702, which improved episodic memory in humans on the nicotine withdrawal model of cognitive dysfunction [105]. Additionally, [11C]GSK1034702 was clinically trialled as a positron emission tomography (PET) ligand (clinicaltrials.gov identifier: NCT00937846) to assess its blood brain barrier penetration and distribution in vivo; unfortunately [11C]GSK1034702 was deemed unsuitable as a PET ligand for quantification of muscarinic M1 receptors in vivo [105].

Notably, Researchers at Merck Laboratories identified the first highly selective PAM of the muscarinic M1 receptor: BQCA [106]. BQCA increases ACh affinity for human cloned muscarinic M1 receptors demonstrating a selective, dose dependant potentiation of ACh's ability to displace [3H]n-methyl scopolamine ([3H]NMS), a pan muscarinic antagonist, binding to muscarinic M1 receptors expressed in Chinese hamster ovary cells [107]. Further, BQCA has been demonstrated to potentiate muscarinic M1 receptors cloned from rhesus, dog, mouse and rat muscarinic M1 receptors as well as selectively potentiate native mouse muscarinic M1 receptors in vivo [107,108]. Recently, we demonstrated that BQCA dose dependently potentiates ACh's ability to displace [3H]NMS in human brain homogenate and that a subset of individuals with schizophrenia with a loss of cortical muscarinic M1 receptors [108] have a decreased response to BQCA's modulation of ACh binding [109]. Modification of this compound by chemical motif substitution [97,110] is ongoing. A selective muscarinic M1 receptor PAM based on the BQCA scaffold, 1-[(1'-2-methylbenzyl)-1,4'-bipiperidin-4-yl]-4-oxo-4-H-quinolinizine-3-carboxylic acid (PQCA) [37], attenuated scopolamine deficits in novel object recognition, self-ordered spatial search, and the object retrieval detour tasks in rats, cynomolgus macaques, and rhesus macaques, respectively [111]. The efficacy of PQCA in these three models of cognitive function further supports the role of the muscarinic M1 receptor in cognition and demonstrates the potential of this class of compound as useful therapeutics. Additionally, Merck have taken MK-7622, a muscarinic M1 receptor PAM to phase II clinical trial (clinicaltrials.gov identifier: NCT01852110) as an adjunct therapy to an acetylcholinesterase inhibitor (donepezil, rivastigmine or galantamine) for the treatment of cognitive impairment and disease progression in Alzheimer's disease. This highlights the promise of allosteric modulators as pro-cognitive agents.

CONCLUSION

Allosteric modulators of muscarinic M1 receptors provide a promising method for developing effective, well-tolerated therapies to redress cognitive impairment, particularly in schizophrenia and other diseases characterised by significant cognitive impairment. Historically, treating any disorder with drugs targeting muscarinic receptors has been hampered by the difficulties associated with targeting the orthosteric binding site, particularly the propensity for "off target" side effects, due to the high degree of homology between the orthosteric binding sites of the five muscarinic receptors. Fortunately, the discovery of highly selective allosteric ligands provides a
potential solution to this problem and provides a unique opportunity to maintain physiological firing patterns, unattainable using orthosteric ligands. However, allosteric modulation comes with its own host of idiosyncrasies to be considered when developing ligands targeting this region of the receptors. At the level of preclinical pharmacology, allosteric compounds provide an exciting opportunity to tease out specific downstream signalling pathways, selectively targeting them to achieve highly specific fine-tuning of receptor response. Additionally, by iterative chemical substitutions of the base compound, multiple parameters can be modified to tailor compounds to specific needs, enhancing some aspects of signalling while inhibiting or not affecting others. Although these aspects of allosteric provide unique opportunities, they also highlight a need for care when testing compounds and appropriate modelling paradigms, as particular effects could be overlooked or masked by classical drug screening methods. The emergence of allosteric ligands provides us with the exciting opportunity to develop well-tolerated, highly selective therapies with the ability to fine tune distinct pathways addressing subtle pathological changes in complex disease states.

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