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Allosteric activators of muscarinic receptors as novel approaches for treatment of CNS disorders^{†,‡}

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Abstract

Muscarinic acetylcholine receptors (mAChRs) represent exciting therapeutic targets for the treatment of multiple CNS disorders. The high degree of conservation of amino acids comprising the orthosteric acetylcholine (ACh) binding site between individual mAChR subtypes has hindered the development of subtype-selective compounds that bind to this site. As a result, many academic and industry researchers are now focusing on developing allosteric activators of mAChRs including both positive allosteric modulators (PAMs) and allosteric agonists. In the past 10 years major advances have been achieved in the discovery of allosteric ligands that possess much greater selectivity for individual mAChR subtypes when compared to previously developed orthosteric agents. These novel allosteric modulators of mAChRs may provide therapeutic potential for treatment of a number of CNS disorders such as Alzheimer's disease and schizophrenia.

Introduction

Cholinergic projection neurons in the basal forebrain extend from the medial septum through the nucleus basalis of Meynert (nbM) and provide the primary source of ACh to brain regions including the neocortex and hippocampus.^{1–4} These neurons influence several aspects of CNS function including information processing, learning and memory, cognition, motor control and attention.^{5,6} Several lines of evidence implicate impaired cholinergic neurotransmission in these brain regions as contributing to the symptoms of a wide variety of CNS disorders. Pharmacological blockade or physical lesions in basal forebrain neurons in animal models^{7–15} and humans^{16,17} mimic symptoms commonly observed in patients suffering from Alzheimer's disease (AD); furthermore, degeneration of cholinergic inputs to the forebrain is one of the earliest pathological changes observed in AD patients.^{4,18}

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Decreased mAChR abundance and impaired mACh receptor coupling have also been observed in post mortem forebrain tissues from individuals with schizophrenia.^{19–22} These findings support the hypothesis that dysfunction of the cholinergic system, at least in part, underlies behavioral impairments associated with multiple CNS disorders. Because of this, many efforts have been made to develop clinically useful compounds that enhance cholinergic signaling in the brain.

One class of cholinergic drugs commonly used to ameliorate symptoms of AD is acetylcholinesterase (AChE) inhibitors. These compounds inhibit the activity of the enzyme AChE which degrades synaptic ACh not only in the brain but at nerve terminals in the periphery. In doing so, they cause increased ACh-mediated signaling and have been shown to enhance memory formation and decrease the occurrence of dementia-related behavioral disturbances. For these reasons, they remain the primary treatment for patients suffering from CNS disorders such as AD. Indeed, dose-related improvements in cognitive performance and quality of life are routinely observed in AD patients using AChE inhibitors;²³ however, activation of cholinergic receptors in the heart, GI tract, and secretory glands induces unwanted, poorly tolerated side effects such as vomiting and nausea that limit the therapeutic index of these drugs.²⁴ Consequently, there is a need for agents that increase cholinergic signaling in a more selective manner than AChE inhibitors.

Two families of receptors mediate the action of ACh in target tissues: nicotinic acetylcholine receptors (nAChRs), which function as ligand-gated cation channels, and muscarinic acetylcholine receptors (mAChRs), which are members of the G protein-coupled receptors (GPCRs) superfamily. Historically, most CNS drug development efforts have focused on mAChR subtypes because these receptors have more established roles in attention mechanisms and learning and memory.^{4,18} Molecular cloning studies have identified five mAChR subtypes denoted M₁–M₅.^{25–29} Each subtype is a single seven-transmembrane protein, which when stimulated by ACh, activates GTP-binding proteins (G proteins) that couple to various downstream effectors. M₁, M₃, and M₅ receptors have been shown to activate phospholipase C *via* pertussis toxin (PTX)-insensitive G proteins whereas M₂/M₄ receptors inhibit adenylyl cyclase *via* PTX-sensitive G proteins²⁹ (Fig. 1). All subtypes can activate extracellular signal-regulated kinase (ERK), a signaling protein involved in cell growth, differentiation, cell survival, and multiple CNS functions.³⁰

Studies using highly specific antibodies to each of the individual mAChR subtypes have revealed differential but partially overlapping localization of these subtypes in areas of the brain known to be affected in multiple disorders of the CNS. For example, in neocortex and hippocampus, M₁ ACh receptors are the most abundantly expressed muscarinic subtype ranging from 35–60% of all mAChR binding sites.^{31–33} M₁ receptors are also expressed postsynaptically on the somatodendritic regions of pyramidal neurons in the striatum.^{31,34} Their postsynaptic localization on asymmetric synapses is consistent with M₁ having a general role in cholinergic modulation of glutamatergic neurotransmission.^{31,35} M₄ receptors, on the other hand, are modestly expressed in the hippocampus and cortex, but highly enriched in the striatum.^{31,32,34} M₂ receptors are expressed throughout the brain including hippocampus and neocortex, but are also abundant on noncholinergic neurons that project to these areas.^{31,34} M₃ receptors are expressed in the hippocampus whereas M₅

receptors are expressed at relatively low levels in the brain.^{31–33} These localization patterns suggest that each mAChR likely has differential roles in cholinergic modulation of brain circuits. Furthermore, as M₁ and M₄ expression patterns are abundant in brain regions known to be affected in CNS disorders such as AD and schizophrenia, M₁ and M₄ have generally been viewed as promising drug targets. Consequently, considerable efforts have been made to develop selective ligands for these subtypes. However, to date, there are no truly selective ligands for M₁ or M₄ in clinical trials. In the following review, we will explore the advances in development of subtype-selective ligands for M₁ and M₄ receptors, with a special emphasis on development of allosteric agents which show unprecedented subtype selectivity for these two mAChR subtypes.

The role of M₁ receptors in AD highlights the need for subtype-selective muscarinic activators

AD, the most common form of dementia in the elderly, affected 25 million individuals in 2006.³⁶ It is characterized by several distinct neuropathological features including extraneuronal amyloid plaques, intracellular neurofibrillary tangles, and neocortical atrophy as well as an enlargement of ventricles.³⁷ Several findings indicate that failed neurotransmission at cholinergic synapses is responsible, at least in part, for the clinical manifestations associated with AD. For example, decreased levels of choline acetyltransferase (ChAT), the enzyme that synthesizes acetylcholine from acetyl CoA, are routinely observed in post mortem brain tissue from AD patients.^{38,39} Furthermore, up to 90% degeneration of cholinergic neurons in the basal forebrain has been observed in these same tissues.⁴⁰ Localized infusion of the muscarinic antagonist scopolamine into parahippocampal structures of primates has also revealed a role of cholinergic receptors in visual memory as tested by recognition of sample stimuli.⁴¹ Additionally, the phenotypes of rodents in which muscarinic receptors have been genetically deleted suggest that mAChRs contribute to the cognition-enhancing effects of ACh.⁴² These experiments, as well as others, have established that muscarinic cholinergic receptors are the most likely receptors for mediating the cholinergic involvement in learning and memory.⁷

As discussed above, M₁ receptors are the most abundantly expressed mAChR in the neocortex and hippocampus, two regions where cholinergic innervation is lost in AD. For this reason, M₁ receptors have been viewed as the most attractive muscarinic therapeutic target for treatment of AD. Recent studies suggest that activation of M₁ receptors not only enhances cognition but also decreases multiple pathological features commonly seen in AD patients.^{43–46} For example, stimulation of M₁ has been shown to increase formation of secreted amyloid precursor protein (sAPP) by modulating α -secretase,⁴⁷ thereby inhibiting the formation of β -secretase products, the precursors of amyloid plaques.^{48–52} Furthermore, M₁ activation reduces neurofibrillary tangles by decreasing tau phosphorylation *via* GSK-3 β inhibition.⁵³ Signaling through the M₁ receptor also increases activation of ERK and potentiates NMDA receptor currents, two cellular events considered important for memory formation.^{54–56} Together, these studies suggest that M₁ receptors modulate multiple aspects of AD including learning and memory and cellular neuropathology. Based on this, selective

activators of M₁ may not only enhance memory but also have disease modifying potential by decreasing the progression of cellular pathologies that develop with AD.

Although M₁ receptors are the most abundant mAChR subtype expressed in the hippocampus, their role as being the primary muscarinic subtype essential for learning and memory is less clear. For example, studies employing mice in which the M₁ receptor was genetically deleted showed normal or enhanced memory using tasks that involved matching-to-sample problems, but were impaired on non-matching-to-sample tasks.⁵⁷ Based on this, the authors of this study suggested that M₁ mutant mice have impaired hippocampal–cortical interactions causing memory dysfunction specific to working memory and remote reference memory.⁵⁷ M₁ knockout mice also displayed profound hyperactivity making it unclear if some of the cognitive impairments were actually due to the hyperactivity.^{58,59}

Despite this uncertainty, numerous attempts have been made to generate compounds which are selective for the M₁ receptor subtype. However, many of these efforts have failed, in part, because these first generation muscarinic agents bind to the highly conserved orthosteric ACh binding site. For example, compounds such as AF102B, AF267B, SB202026, and WAY-132983 were designed to orthosterically bind M₁. These agents initially provided important proof of concept evidence that agonism at M₁ receptors is a viable approach for treatment of AD and were used in clinical trials. However, they were all subsequently discontinued because of cholinergic side effects.^{44,60–62} In many cases, these agents were initially thought to be selective for M₁ as they displayed selectivity for this mAChR subtype in cell based assays. However, upon further characterization in more native tissues, these agents were subsequently found to be without absolute M₁ selectivity. To this end, there is still no truly selective M₁ orthosteric agonist available for treatment of AD.

Activation of muscarinic receptors as a novel treatment strategy for schizophrenia

Schizophrenia is characterized by a broad spectrum of symptoms including positive (hallucinations), negative (flattened affect), and cognitive dysfunction (attention and working memory deficits).⁶³ Dysregulation of glutamate and dopamine systems in the brain have been implicated as the main causes for this complex disease. However, it is increasingly evident that the pathology of schizophrenia also involves other neurotransmitter systems. Many sets of data from studies using neuroimaging and animal models suggest a role for the cholinergic system.^{63–65} Furthermore, decreased levels of mAChR expression, as well as functional impairments in mAChR coupling, have been observed in post-mortem brain tissue from schizophrenic patients.^{19–22}

In the clinic, a recent study using the M₁/M₄ preferring agonist xanomeline (Xan) (Structure A; Fig. S1‡) has confirmed that targeting the muscarinic cholinergic system is a viable treatment for schizophrenia.^{66,67} Originally developed as a drug for treatment for AD, Xan induced modest improvements in cognitive function; however, its most robust therapeutic effects were on psychotic symptoms. In a double blind placebo-controlled study, Shekhar *et al.* showed that Xan induced a highly significant improvement in positive symptoms in schizophrenic patients including vocal outbursts, suspiciousness, agitation, and

hallucinations.⁶⁸ In addition, Xan subjects showed mild improvements in cognitive measures of verbal learning and short-term memory formation suggesting Xan is somewhat efficacious for cognitive dysfunction. Interestingly, the response to Xan was superior to the effects observed with traditional antipsychotics, and therapeutic effects were seen in less than one week, as opposed to the multi week delay for traditional antipsychotics.⁶⁸ Unfortunately, Xan was not a truly selective M₁/M₄ agonist and had off-target activity at M₂, M₃, and M₅ receptor subtypes.^{69–71} Further, Xan had poor brain penetration and required high plasma concentrations to achieve sufficient activation of M₁. For these reasons, Xan suffers from the same (mainly GI) side effects as those seen with the use of AChE inhibitors and is not a viable drug candidate for long-term clinical use. Despite this, Xan's positive effects provide strong clinical validation that targeting of M₁/M₄ mAChRs is a valid therapeutic strategy for treatment of psychosis and behavioral disturbances in patients suffering from a broad range of disorders including schizophrenia.

Allosteric activators of mAChR show promise in achieving subtype selectivity

As discussed above, previous attempts to achieve selective activation of mAChRs with orthosteric ligands have failed. While there have been reports of achieving this in patent and primary literature, subsequent studies across multiple systems revealed that previously developed orthosteric agents are not truly selective. This phenomenon is sometimes attributed to an overestimation of mAChR selectivity resulting from artificial receptor-G protein coupling in recombinant cell lines.⁷⁰ Furthermore, several compounds are chosen as selective agonists based on functional selectivity or selectivity based on efficacy rather than selective (*bona fide*) activation of individual mAChR subtypes. Because of this, a weak partial agonist at multiple mAChR subtypes may seem to be selective in cells lines where there is little or no receptor reserve but might have robust agonist activity at these same mAChR subtypes *in vivo* where high receptor reserves are common. As a result, these agents often activate multiple mAChR subtypes in animal models and humans.^{24,72} This apparent loss in subtype-selectivity upon evaluation in multiple systems was recently reported for the purported M₁ agonist AF267B. AF267B was suggested to have selective agonist activity at M₁ during its initial screening.⁴⁴ However, when evaluated across multiple systems, AF267B was found to have off-target binding and activity at M₃ and M₅ receptor subtypes.^{47,72}

Previously developed orthosteric ligands have been designed to bind to mAChRs at amino acids that are highly conserved between M₁–M₅ mAChR subtypes. As a result, these ligands are unable to select for an individual mAChR subtype. Using an alternative approach, many academic and industrial laboratories are now screening for and developing compounds that bind these receptors at less conserved allosteric or ectopic binding sites that are topographically distinct from the orthosteric binding site (Fig. 1). These ligands, termed allosteric activators or modulators, provide excellent subtype-selectivity for individual subtypes of several GPCR families including metabotropic glutamate receptors, adenosine and serotonin receptors (for review see ref. 73 and 74). For muscarinic receptors, several allosteric compounds including both positive allosteric modulators (PAMs) and allosteric agonists of M₁ and M₄ receptors have been identified. PAMs are unable to activate receptors

directly, but their binding is thought to modify receptor conformation which changes the binding and/or functional properties of orthosteric ligands for the targeted muscarinic receptor subtype. Because of these unique properties, these compounds may have theoretical advantages for treatment of various CNS disorders when compared to orthosteric agents in that they are inactive in the absence of neurotransmitter at a given synapse and only exert their effects in the presence of endogenous neurotransmitter. Thus, they maintain activity dependence in both temporal and spatial aspects of endogenous physiological signaling. The functional properties of a positive allosteric modulator can be seen in Fig. 2 where it elicits no response when added alone (A) but causes enhancement of the response to ACh resulting in a leftward shift in the concentration-response-curve of ACh (B). Allosteric agonists, on the other hand, do not require the presence of an endogenous ligand but activate mAChRs directly. This type of activation may prove advantageous for CNS disorders because these agonists could selectively drive activation of muscarinic receptors in neurons where presynaptic ACh release is attenuated due to degeneration of cholinergic inputs.

For several years, agents with negative effects on affinity modulation for orthosteric compounds were the only types of allosteric agents for muscarinic receptors, a classic example being the inhibitory effect of gallamine.^{75–77} However, through subsequent studies by many laboratories, several compounds with positive effects on affinity of orthosteric ligands for receptors (PAMs), as well as direct allosteric agonists for muscarinic receptors, were identified. The first of these reported was brucine, an alkaloid isolated from *Strychnos nux-vomica*.⁷⁸ Brucine is an allosteric potentiator at M₁ receptors with a binding profile that is consistent with a ternary allosteric complex model in which both orthosteric and allosteric ligands bind receptors simultaneously and modify the affinities of each other. However, brucine induced a modest 2-fold increase in ACh affinity and required relatively high concentrations (micromolar) to elicit these effects. For these reasons, brucine is an unlikely candidate as an allosteric potentiator suitable for use *in vivo*.⁷⁹ Regardless, brucine's selectivity for M₁ provides an important proof of concept that it is possible to develop agents with absolute selectivity for muscarinic subtypes.

After brucine, several subsequent studies led to the discovery of other allosteric modulators of mAChR subtypes. For example, thiochrome (2,7-dimethyl-5*H*-thiachromine-8-ethanol), an oxidation product and metabolite of thiamine, was reported as an M₄ PAM, and SCH-202676 was shown to be a PAM at multiple GPCR subtypes including M₁.^{80,81} In equilibrium binding assays, thiochrome had little or no effect on radiolabeled [³H]NMS binding at any mAChR. However, it inhibited [³H]NMS dissociation from M₁ and M₄ and increased the affinity of ACh three- to five-fold for inhibiting [³H]NMS binding at M₄ while having little to no effect on ACh affinity at the other four mAChR subtypes.⁸¹ From these data, the authors concluded that the selective nature of thiochrome is due to selective cooperativity rather than selective binding.⁸¹

In addition to the discovery of early M₁ and M₄ PAMs, a major breakthrough in mAChR pharmacology came when Spalding *et al.* identified AC-42 (Structure B: Fig. S1‡) as the first in a novel class of compounds that bind to an allosteric site on the M₁ receptor. Using a series of chimeric receptors, AC-42 was demonstrated to activate M₁ receptors at an region of the receptor that is clearly distinct from the orthosteric ACh site and involves

transmembrane domains one and seven⁸² (but not 3, 5, and 6 which contribute to the orthosteric site^{83–85}). This region of the receptor is not conserved among mAChR subtypes likely explaining its unprecedented selectivity. Subsequent studies monitoring intracellular calcium release and inositolphosphate accumulation confirmed that AC-42 was active in cell lines; however, the *in vivo* use of AC-42 has remained in question.⁷⁰ In addition to AC-42, the biologically active metabolite of the atypical antipsychotic clozapine, *N*-desmethyloclozapine (NDMC) was also shown have antagonistic properties at M₁ mAChRs.⁸⁶ NDMC is highly selective for M₁ relative to other mAChR subtypes and activates M₁ receptors containing a mutation in the orthosteric binding site. Furthermore, NDMC potentiates NMDA receptor activity, crosses the blood–brain barrier, and induces *c-fos* expression in rat forebrain suggesting that it may contribute to the unique therapeutic profile of clozapine *via* selective activation of M₁.^{86–88}

These discoveries have broad implications for our understanding of muscarinic receptor structure and function as they suggest that allosteric agents activate M₁ and M₄ mAChRs with a high degree of subtype selectivity. However, previously developed mAChR allosteric agonists and PAMs such as brucine do not have the pharmacological profiles and physicochemical properties required to be useful tools to assess the effects of allosteric activation in more complex native systems. For example, many of the earlier agents have substantial off-target activities and have poor solubility in physiological buffer systems, preventing their use *in vivo*. Furthermore, compounds such as AC-42 failed to activate M₁ receptors in native tissue systems such as brain slices.⁷⁰ For these reasons, many industrial and academic laboratories are now developing newer allosteric molecules that have the required properties such as true subtype selectivity, favorable pharmacokinetic profiles, and CNS penetration.

Advances in the development of novel selective activators of M₁

Over the past few years, several novel selective M₁ agonists and allosteric potentiators have been identified. These compounds are providing important new tools to evaluate the potential utility of selective activators of M₁ for treatment of AD and other CNS disorders. For instance, TBPB,⁷² 77-LH-28-1,^{70,89} and AC-260584^{90–92} (Structure E, C, and D; see Fig. S1‡) have been reported as agonists of M₁. These compounds provide an exciting new approach for developing therapeutic agents to selectively activate M₁. In addition, they provide new tools that can be used to determine the physiological roles of M₁ in the central nervous system and test the hypotheses that activation of M₁ has effects that would predict antipsychotic efficacy or cognitive enhancements in humans. All compounds are systemically active and are proving to be useful for *in vivo* studies of M₁ activation.

TBPB selectively activates M₁ in cell lines and has no agonist activity at any other mAChR subtypes. Mutations that reduce the activity of orthosteric agonists have no effect on the response to TBPB, and Schild analysis of the blockade of TBPB effects with the orthosteric antagonist atropine revealed that TBPB does not interact with the orthosteric site in a competitive manner. These data are consistent with the predictions of an allosteric ternary complex model for the actions of two molecules that interact with distinct sites^{73,93} and are consistent with the hypothesis that TBPB acts as an allosteric M₁ agonist. Interestingly,

TBPB induces M_1 -dependent potentiation of NMDA currents in hippocampal pyramidal cells, an action that is thought to be important for the cognition-enhancing effects of mAChR activation. By contrast, TBPB does not reduce inhibitory or excitatory synaptic transmission at the Schaffer Collateral-CA1 synapse, effects thought to be mediated by M_2 and M_4 mAChRs, respectively.⁷² Of particular relevance to AD pathology, early studies in cell lines indicated that TBPB induces a shift in processing of APP towards the non-amyloidogenic pathway and decreases A β production *in vitro*.⁷² This is consistent with previously discussed studies indicating that mAChR activation has favorable effects on APP processing in animal models and in humans^{94–96} and provides further support for the hypothesis that selective activation of M_1 might have disease-modifying potential in the treatment of AD. TBPB is also systemically active and crosses the blood–brain barrier, making this a useful tool for studies of the possible cognition-enhancing effects of M_1 -selective agonists. These encouraging results provide strong support for the utility of M_1 allosteric agonists in activating this crucial mAChR subtype in the CNS without inducing the adverse effects associated with less selective mAChR agonists.

A second systemically active M_1 agonist, 77-LH-28-1, was discovered as a structural analog of AC-42.^{70,89} 77-LH-28-1 is selective for M_1 relative to M_2 , M_4 , and M_5 subtypes but also has weak agonist activity at M_3 at higher concentrations. Interestingly, in contrast to the effects of atropine on the response to TBPB, the orthosteric antagonist scopolamine induces parallel rightward shifts in the 77-LH-28-1 concentration response curve (CRC) suggesting that 77-LH-28-1 may bind the orthosteric site of M_1 .⁷⁰ However, extensive mutagenesis studies combined with functional studies and analysis of the effects of 77-LH-28-1 on binding of an orthosteric antagonist are consistent with an allosteric mode of agonist action of this compound. Together, these studies raise the possibility that 77-LH-28-1 might be what has been referred to as a ‘bi-topic’ ligand that binds to a site that overlaps with the orthosteric site but also includes an allosteric site that modulates orthosteric site affinity.⁹⁷ Electrophysiological studies indicate that 77-LH-28-1 increases activity of hippocampal CA1 pyramidal cells *in vitro* and *in vivo* and induces synchronous network activity in the hippocampus.⁷⁰ Interestingly, unlike the orthosteric agonist oxotremorine, which activates G_{α_s} , G_{α_i} , and G_{α_q} G proteins, 77-LH-28-1 selectively activates the coupling of M_1 to G_{α_q} and G_{α_s} signaling without activating coupling to G_{α_i} ;⁸⁹ this indicates that 77-LH-28-1 differentially activates distinct M_1 -mediated responses. Such differential effects of allosteric agonists on various responses to M_1 activation could prove to be crucially important in determining the *in vivo* and ultimately therapeutic potential of allosteric M_1 agonists.

An additional structural analog of AC-42, AC-260584 was also recently reported as an orally bioavailable M_1 allosteric agonist with antipsychotic and cognitive enhancing effects.^{90,98} Acute systemic administration of AC-260584 significantly reversed amphetamine and MK-801-induced hyperlocomotion and apomorphine-induced climbing, two animal models predictive of antipsychotic efficacy. Additionally, AC-260584 did not affect spontaneous locomotor activity or step down latency and produced no stereotypic behaviors, sedation, or ataxia in the dose ranges tested in mice and rats. Furthermore, administration of AC-260584 did not induce catalepsy in rats suggesting it has a lesser liability for causing extrapyramidal motor side-effects when compared to typical

antipsychotics, as catatonic induction in rats has been shown to predict these effects in humans.^{98,99} Interestingly, AC-260584 also improved the performance of mice in the Morris water maze, a measure of hippocampal-dependent spatial memory. Together, these initial data suggest that M₁ activation may attenuate dopaminergic hyperfunction and dopamine mediated behavioral disturbances and that the M₁ receptor likely plays a role in hippocampal-mediated memory. More recent studies revealed that subcutaneous administration of AC-260584 induced ERK1/2 activation in the CA1 area of mouse hippocampus, an effect that was absent in M₁ knockout mice.⁹⁰ Furthermore, AC-260584 administration improved visual recognition memory in mice in the novel object recognition paradigm further supporting pro-cognitive effects of M₁ activation. While these data provide advancements in the development of bioavailable muscarinic agonists, the lack of true mAChR subtype selectivity⁹⁰ of AC-260584 and related compounds may limit their use as antipsychotic/pro-cognitive agents.

An exciting development in the field of muscarinic pharmacology has been the recent discovery of M₁ allosteric agonists VU0184670 and VU0357017¹⁰⁰ (Structure F and G; Fig. S1‡). These compounds are chemically optimized analogs of M₁ allosteric agonists identified in a primary high-throughput screen in the Vanderbilt Screening Center for GPCRs, Ion Channels and Transporters, initiated and supported by the NIH Molecular Libraries Roadmap. A diversity-oriented synthesis/iterative screening approach was used to rapidly explore structure–activity relationships (SAR) of novel compounds with the goal being to improve potency while maintaining subtype selectivity. Neither VU0184670 nor VU0357017 displayed any agonist or antagonist activity at M₂–M₅, and these allosteric agonists also possessed an exceptionally clean ancillary pharmacology profile when assessed in a screen of sixty-eight GPCRs, ion channels, and transporter targets.¹⁰⁰ Thus, these newer generation M₁ allosteric agonists represent a major breakthrough and are much more highly selective; they also have improved physicochemical properties compared to previous allosteric or orthosteric M₁ agonists. Mutagenesis studies revealed that residues located in the extracellular loop three (o3) and the first helical turn of transmembrane seven (TM7) of the M₁ receptor were critical for activity of VU0184670. This same region of M₄ was also implicated in the actions of M₄ PAM LY2033298.¹⁰¹ Both M₁ allosteric agonists lead compounds were found to possess a high degree of solubility in aqueous solutions and were centrally penetrant after systemic administration with VU0184670 possessing a superior pharmacokinetic profile. Similar to what was found with TBPB, VU0184670 also potentiates NMDAR currents evoked by NMDA application in hippocampal CA1 pyramidal cells. Excitingly, acute *i.p.* administration of VU0357017 reversed cognitive deficits induced by the orthosteric mAChR antagonist scopolamine in a contextual fear conditioning paradigm, a measure of hippocampal dependent learning.¹⁰⁰

In addition to the discovery of novel M₁-selective allosteric agonists, much progress has been made in the discovery of novel M₁ PAMs that act as allosteric potentiators of this receptor. For instance, multiple M₁ allosteric potentiators have recently been identified in a high-throughput functional-screening campaign.¹⁰² These molecules belong to several structurally distinct chemical scaffolds and include compounds that are selective for M₁ relative to other mAChR subtypes. None of the compounds identified had agonist activity;

each behaved as a pure PAM and induced parallel leftward shifts in the ACh CRC. Furthermore, none of the newer M₁ PAMs compete for binding at the orthosteric ACh-binding site. The two most selective compounds, VU0029767 and VU0090157 (Structure A and B; Fig. S2‡), were studied in detail and induced dose-dependent shifts in ACh affinity at M₁ that are consistent with their effects in functional assays; this indicates that they mechanistically enhance M₁ activity by increasing ACh affinity. However, these compounds were strikingly different in their ability to potentiate responses at a mutant M₁ receptor that exhibits decreased affinity for ACh and in their ability to affect responses to the allosteric M₁ agonist TBPB. Furthermore, VU0090157 induced similar potentiation of M₁ activation of PLC and PLD activity, whereas VU0029767 potentiated activation of PLC but not PLD signaling. These data provide additional examples of an allosteric compounds inducing differential coupling of M₁ to distinct signaling pathways.

In a recent publication, researchers reported the discovery of benzyl quinolone carboxylic acid (BQCA) (Structure C; Fig. S2‡) as a highly selective and efficacious allosteric potentiator of M₁.¹⁰³ Like the previously described M₁ PAMs VU0090157 and VU0029767, BQCA had no direct agonist activity but induced a robust leftward shift in ACh's concentration-response curve. BQCA had no activity at M₂, M₃, M₄, or M₅. Using *in situ* hybridization, the authors also showed that BQCA substantially increased two markers of neuronal activation, *c-fos* and *arc*, in multiple brain regions including cortex of wild type, but not M₁ knockout mice. Furthermore, BQCA did not compete for orthosteric [³H]-NMS binding but increased affinity of M₁ for ACh. Residues Y179 and W400 which lie in the second (o2) and third (o3) extracellular loop of the receptor were shown to be required for the potentiation of M₁ by BQCA as mutations of these residues completely eliminated potentiation. Interestingly, BQCA was shown to be systemically active and fully reversed the cognitive impairment induced by scopolamine in a contextual fear-conditioning model of episodic-like memory.¹⁰³ In addition, BQCA induced changes in electroencephalography oscillations in a manner that is consistent with a potential cognition-enhancing effect. These findings provide exciting new data in support of the hypothesis that it will be possible to develop highly selective M₁ PAMs that have cognition-enhancing effects *in vivo*.

Shirey *et al.*,¹⁰⁴ also extensively characterized the M₁ PAM BQCA and confirmed that this compound is highly selective for M₁ relative to other mAChR subtypes as well as to a panel of other GPCRs.¹⁰⁴ Since previous studies in M₁ knockout mice supported a role for this receptor in cognitive behaviors requiring normal functioning of the prefrontal cortex (PFC) (Anagnostaras *et al.*⁵⁷), we sought to examine the effect of selective M₁ activation in mPFC pyramidal cells. In these cells, carbachol (CCh) induced robust inward currents, an effect that was potentiated by BQCA.¹⁰⁴ BQCA also potentiated CCh-induced increases in excitatory drive to these cells as seen by an increase in spontaneous EPSCs. Furthermore, systemic administration of BQCA drastically increased the firing rate of mPFC pyramidal cells *in vivo* in multichannel single unit recordings and reversed impairments in PFC-dependent learning in a mouse model of AD.¹⁰⁴ Together, these studies support a pivotal role for the M₁ receptor in modulating PFC-dependent learning and suggest that this mAChR subtype represents an exciting potential therapeutic target for the treatment of CNS disorders characterized by memory and cognitive impairment.

BQCA was also recently optimized to yield a M₁-selective allosteric potentiator with improved potency and increased free fraction. Using an analog library synthesis approach, Yang *et al.*¹⁰⁵ showed that modifications to BQCA's quinolone core and N1-benzyl substituent yielded multiple compounds with potencies that were less than 100 nM while retaining greater than 5% free fraction. Later studies showed that the incorporation of pyridines and diazines into the biphenyl region of BQCA led to lower plasma protein binding.¹⁰⁶ Also, the addition of a C-ring pyrazole increased CNS exposure and improved performance in a mouse contextual fear conditioning assay.¹⁰⁷ Together, these studies suggest an optimized version of BQCA with possible improvements in its cognition-enhancing effects *via* increased CNS exposure.

In addition to M₁-selective allosteric agonists and PAMs, another major breakthrough in mAChR pharmacology came with the discovery of the novel highly selective allosteric potentiator of M₄, LY2033298¹⁰¹ (Structure D; Fig. S2‡). Like the previously described M1 PAMs, LY2033298 does not activate mAChRs directly but induces robust potentiation of ACh responses. It also showed exquisite selectivity at M₄ receptors with no discernable activity at the other mAChR subtypes. Site-directed mutagenesis studies revealed that residue D432 in the third extracellular loop (o3) of the receptor is involved in the potentiating effects of LY2033298. When co-administered with a low dose (0.1–0.3 mg kg⁻¹) of oxotremorine, LY2033298 attenuated conditioned avoidance responding (CAR) and reversed apomorphine-induced disruption of the prepulse inhibition (PPI) of the acoustic startle response. Potentiation of M₄ also augmented oxotremorine-stimulated dopamine (DA) release in dialysate from prefrontal cortex but not nucleus accumbens supporting a role for M₄ in modulating mesocortical but not mesolimbic DA levels. However, when compared to experiments performed using human M₄ AChRs (hM₄) membranes, the potency of LY2033298 for [³H]-oxotremorine-M potentiation in rat M₄ AChRs (rM₄) membranes was diminished 5- to 6-fold. This species difference was postulated to be responsible for LY2033298's inability to induce responses in rats when administered alone in CAR, PPI, and DA release assays.¹⁰¹ The efficacy of LY2033298 in rodent models and on DA release provided evidence that allosteric potentiation of M₄ is a viable approach for the development of muscarinic-based antipsychotic agents.

Our laboratory employed a chemoinformatic and medicinal chemistry-based approach to identify a new series of ligands that interacts with an allosteric site on M₄. Like LY2033298, compounds identified in these studies selectively potentiated M₄ responses to ACh.¹⁰⁸ Their binding profile supported an allosteric mode of action, as none of these ligands displaced [³H]-NMS in equilibrium binding assays. The lead compound in this series, VU100010 (Structure E; Fig. S2‡), possessed an EC₅₀ for potentiation of 400 nM and shifted the functional ACh response curve 47-fold to the left. As described in Shirey *et al.*,¹⁰⁸ binding studies indicated that VU100010 exerted its allosteric potentiation of M₄ by increasing the affinity of the receptor for ACh and by increasing downstream coupling of M₄ to G proteins. Electrophysiological studies revealed that VU100010 modulated hippocampal synaptic transmission at excitatory but not inhibitory CA1 synapses in acute slices.¹⁰⁸

Despite this notable advance in mAChR pharmacology, VU100010 suffered from poor physicochemical properties (log *P* ~ 4.5) and *in vivo* studies proved infeasible because

VU100010 could not be formulated into a homogeneous solution in any acceptable vehicle. For these reasons, subsequent efforts focused on the development of novel CNS-penetrant analogs of VU100010.¹⁰⁹ Of these, VU0152099 and VU0152100 (Structure F and G; Fig. S2‡) were identified as M₄ PAMS with EC₅₀ values for potentiation of ACh responses of approximately 400 nM. These novel modulators maintained a high degree of mAChR subtype selectivity when compared to VU100010 and also showed functional selectivity in a screen of 15 other GPCRs that are highly expressed in the CNS. Similar to the binding profile of VU100010, VU0152099 and VU0152100 did not compete for orthosteric [³H]-NMS binding but increased M₄ receptor affinity for ACh. The most exciting finding in this series of studies was that VU0152099 and VU0152100 exhibited improved physicochemical and pharmacokinetic properties as compared to VU100010; in contrast to the high log *P* of VU100010 (4.5), both VU0152099 and VU0152100 possessed log *P* values of 3.65 and 3.6, respectively, a full order of magnitude less lipophilic than VU100010. As a consequence, both VU0152099 and VU0152100 afforded homogeneous dosing solutions in multiple vehicles acceptable for *in vivo* studies. Furthermore, both compounds exhibited substantial systemic absorption and brain penetration after systemic dosing. Subsequent studies in an animal model used to predict antipsychotic efficacy showed that both novel M₄ PAMS reversed amphetamine-induced hyperlocomotion.¹⁰⁹ It will be of critical importance to further evaluate these compounds and their efficacy in other animal models of psychosis and cognition to yield a deeper understanding of the role of this receptor subtype in human diseases like schizophrenia and AD.

Lastly, it is important to note that, at present, the mechanisms underlying the selective nature of allosteric agents for M₁ and M₄ mAChRs are not fully established. As previously discussed, the initial rationale driving this approach was that allosteric ligands bind to evolutionarily less-conserved allosteric sites on the M₁ or M₄ receptors, as opposed to the highly conserved orthosteric (ACh) binding site and might, therefore, bind selectively to allosteric sites on individual mAChR subtypes. This hypothesis has been clearly established for metabotropic glutamate receptors where selective binding of allosteric ligands to individual subtypes has been established as the basis for the selectivity of some ligands.⁷³ However, this does not rule out the possibility that these compounds bind to allosteric sites on multiple mAChR subtypes with similar affinities but that selectivity is conferred by selective cooperativity with orthosteric-site agonists,^{73,93} as has been suggested for selective PAM activity of thiochrome at M₄.⁸¹ If this is the case, it is possible that selectivity of these compounds will vary depending on the system in which their effects are measured. Thus, it will be crucial to develop a clear understanding of the molecular basis for the observed selectivity in future studies. Optimally, this should be addressed with radioligands that act at the allosteric but not the orthosteric sites. However, studies involving detailed analysis of effects of allosteric compounds on binding of orthosteric-site ligands in addition to mutagenesis studies will also shed light on this important issue. Finally, it will be critical to understand whether selectivity of PAMs holds constant in the presence of multiple different classes of orthosteric agonists; examples of probe dependence of allosteric modulators are now surfacing.¹¹⁰ Preliminary findings by our group and others with the M₄ PAM LY2033298 and related analogs suggest that selectivity of these compounds across mAChR subtypes exists in the presence of ACh but not another orthosteric agonist, oxotremorine.

Considering that co-administration of low doses of systemically active muscarinic agonists may be required to see efficacy of PAMs in different behavioral models, it will be critical to explore selectivity profiles of allosteric modulators not only in careful radioligand binding experiments but also in multiple functional assays *in vitro* with different classes of orthosteric agonists.

Conclusions

Exciting new data from pre-clinical and clinical studies suggest that selective M₁ or M₄ mAChR activation might provide a novel approach to the treatment of multiple CNS disorders. This includes compelling evidence that selective increases in the activity of M₁ and M₄ could provide a novel approach to the treatment of AD and schizophrenia and perhaps have efficacy in treating psychosis and cognitive impairment in other neurodegenerative diseases. Major advances have been made in developing selective allosteric agonists and PAMs of both M₁ and M₄ as an alternative approach for the development of selective receptor activators. These molecules have now been optimized for use in animal models, and early studies indicate that they have effects in animal models that predict efficacy in the treatment of these disorders. In addition to potential clinical utility of selective mAChR activators, abundant clinical and animal studies indicate that highly selective antagonists of specific mAChR subtypes might have utility in the treatment of other CNS disorders including dystonia, Parkinson's disease, epilepsy and others.^{24,42,47,111–115} In each case, subtype selectivity will be a key to achieving clinical efficacy in the absence of adverse effects. For other GPCRs, it has been possible to discover allosteric modulators that serve as potentiators or inhibitors (*i.e.* NAMs) of receptor function.^{116–118} Thus, it might be possible to discover selective allosteric antagonists of mAChR subtypes. Indeed, a recent high-throughput screening campaign that focused on M₁ PAMs was successful in identifying NAMs of mAChR subtypes that are still under development.¹⁰² These exciting advances are providing a fundamental advance in our approach to modulating mAChRs as drug targets and may provide a novel approach to treatment of AD and schizophrenia in addition to other CNS disorders.

Supplementary Material

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Biography

Gregory J. Digby completed his PhD in 2008 in the Department of Pharmacology, Medical College of Georgia, supervised by Dr Nevin Lambert. He is currently a postdoctoral research fellow at Vanderbilt University in Dr Jeff Conn's laboratory.

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Dr Jeff Conn is the Lee E. Limbird Professor of Pharmacology at Vanderbilt University. He is also the director of the Vanderbilt Program in Drug Discovery. Dr Conn's research involves developing a detailed understanding of the cellular and molecular mechanisms involved in regulating chemical and electrical signaling in the central nervous system. He is especially interested in understanding how signaling is regulated in identified neuronal circuits that are important for human neurological and psychiatric disorders such as Alzheimer's disease and schizophrenia. His work employs a broad range of techniques including electrophysiology, biochemistry, behavior, high throughput screening (HTS), and medicinal chemistry of novel small molecule therapeutics to aid in validating novel therapeutic approaches and in understanding the biology of signaling in the central nervous system.

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Fig. 1.

Muscarinic receptor structure and G protein coupling. M₁, M₃, and M₅ AChRs couple to Gα_q family G proteins which modulate effectors including PLC whereas M₂ and M₄ mAChRs couple to Gα_{i/o} family G proteins which modulate effectors such as adenylyl cyclase. It is also important to note that several other effectors can be modulated by active mAChRs (*e.g.* ion channels and other protein kinases). The amino acids that contribute to the orthosteric binding site of mACh receptors are located in the transmembrane (TM) domain. In contrast, “ectopically” located amino acids contribute to the putative allosteric binding sites on mAChRs.

Fig. 2.

Schematic representation of concentration-response curves (CRC) of allosteric potentiators when added alone (A) or in the presence of an orthosteric agonist ACh (B). In (A), increasing concentrations of positive allosteric modulator (PAM) or orthosteric agonist are added and a functional response is measured. When added alone, the PAM has no effect whereas increasing concentrations of the orthosteric agonist induce a dose-dependent response. In (B), a single concentration of PAM or vehicle is added in the presence of increasing amounts of the orthosteric agonist ACh. A PAM shifts the agonist CRC to the left, resulting in an increase in ACh potency as depicted by the intersection of the descending lines to the x-axis.