Muscarinic Acetylcholine Receptor Agonists as Novel Treatments for Schizophrenia

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Schizophrenia remains a challenging disease to treat effectively with current antipsychotic medications due to their limited efficacy across the entire spectrum of core symptoms as well as their often burdensome side-effect profiles and poor tolerability. An unmet need remains for novel, mechanistically unique, and better tolerated therapeutic agents for treating schizophrenia, especially those that treat not only positive symptoms but also the negative and cognitive symptoms of the disease. Almost 25 years ago, the muscarinic acetylcholine receptor (mAChR) agonist xanomeline was reported to reduce psychotic symptoms and improve cognition in patients with Alzheimer's disease. The antipsychotic and procognitive properties of xanomeline were subsequently confirmed in a small study of acutely psychotic patients with chronic schizophrenia. These unexpected clinical findings have prompted considerable efforts across academia and industry to target mAChRs as a new approach to potentially treat schizophrenia and other psychotic disorders. The authors discuss recent advances in mAChR biology and pharmacology and the current understanding of the relative roles of the various mAChR subtypes, their downstream cellular effectors, and key neural circuits mediating the reduction in the core symptoms of schizophrenia in patients treated with xanomeline. They also provide an update on the status of novel mAChR agonists currently in development for potential treatment of schizophrenia and other neuropsychiatric disorders.

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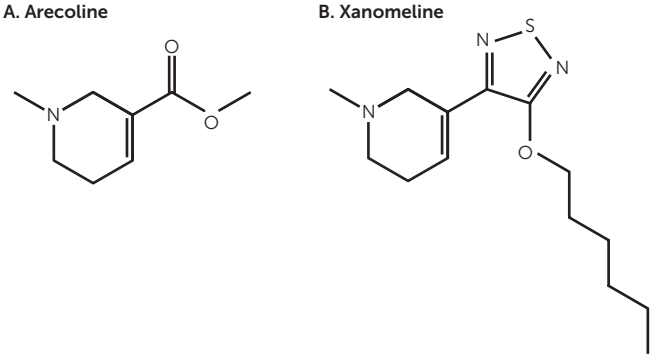
Schizophrenia is a common and complex psychiatric syndrome consisting of three primary symptom domains: positive symptoms (e.g., hallucinations, delusions, disordered thinking and speech), negative symptoms (e.g., lack of motivation, lack of emotional expression or flat affect, social withdrawal), and cognitive symptoms (e.g., impaired attention, concentration, memory, and executive functions) (1). Demographically, schizophrenia affects approximately 0.5%–1.0% of the population worldwide (2) and usually manifests by one's late teens or early adulthood. Although the natural progression of schizophrenia is highly variable, many patients remain disabled their entire lives (3). In fact, schizophrenia remains one of the top 10 causes of disability worldwide (3).

The very first commonly used antipsychotic drug, chlorpromazine, was discovered serendipitously by Laborit, Delay, and Deniker and was introduced in the United States in the 1950s (4). Many chemically unique first- and second-generation antipsychotic drugs have subsequently been developed; however, with the possible exception of clozapine, they all have relatively similar efficacy across the symptom domains of schizophrenia while differing in their side-effect profiles (5). This is not surprising, since all currently approved antipsychotics are either antagonists or partial agonists/antagonists of dopamine (DA) D_2 receptors (6). Whereas other neurotransmitter systems may contribute to their overall efficacy and/or side effects, DA D_2 receptors are believed to predominantly mediate their beneficial therapeutic effects by reducing positive symptoms of schizophrenia (7, 8).

Despite the effects of current antipsychotic medications on positive symptoms, many patients with schizophrenia continue to experience residual positive symptoms, and a significant percentage of patients remain treatment resistant (9). Moreover, there is currently no approved medication for the treatment of negative or cognitive symptoms (10) (the "dementia" of dementia praecox). Antipsychotic drugs are also associated with undesirable adverse events, including extrapyramidal side effects, akathisia, weight gain, metabolic disturbances, excessive sedation, hyperprolactinemia, and a risk of developing tardive dyskinesia (5, 11). More efficacious treatments based on new or unique mechanistic targets are desperately needed, especially if they are shown to be effective across all three symptom domains and/or possess better safety and tolerability profiles (12). Here, we review a considerable body of historical and recent preclinical and clinical evidence suggesting that muscarinic acetylcholine receptors, a small family of G protein–coupled receptors (GPCRs), may represent novel targets for treating the core symptom domains of schizophrenia.

FIGURE 1. The chemical structure of the natural pan-muscarinic acetylcholine receptor (mAChR) agonist alkaloid arecoline and the synthetic M_1/M_4-preferring mAChR agonist xanomeline



For more than six decades, muscarinic acetylcholine receptors (mAChRs) have been implicated in the pathophysiology of schizophrenia, based on the observation that brain-penetrant mAChR antagonists can either induce or exacerbate cognitive impairment (13, 14) as well as psychosis (15, 16). Almost 65 years ago, Pfeiffer and Jenney (17) reported that the natural mAChR agonist arecoline (Figure 1A) exhibited antipsychotic "activity" in a preclinical model of psychosis (i.e., conditioned avoidance responding) and produced what were described as "lucid intervals" in patients with schizophrenia. Although these lucid intervals were of relatively short duration, and despite the obvious limitations of this small, open-label, unblinded study, this was the first evidence that an mAChR agonist might reduce some of the symptoms of schizophrenia. Arecoline is also the most abundant psychoactive alkaloid in the betel nut, which is commonly chewed as a cultural practice in the south of Asia and Asia Pacific (18). Several small follow-on studies have again reported that betel nut chewing is associated with less severe positive and negative symptoms of schizophrenia (19, 20).

In the 1980s, the development of mAChR agonists as potential procognitive drugs became of interest to the pharmaceutical industry. Xanomeline, a potent synthetic mAChR agonist derivative of arecoline (21) (Figure 1B), was evaluated for its ability to improve cognition in a large placebo-controlled phase 2 study in patients with Alzheimer's disease. Bodick and colleagues (22) reported that xanomeline improved cognition in patients with Alzheimer's disease, but also reported the surprising and unexpected finding that xanomeline rapidly and dose-dependently reduced psychotic symptoms in patients manifesting these symptoms at baseline and prevented or delayed their onset compared with placebo during the 6-month trial. The antipsychotic properties of xanomeline in dementia-related psychosis were completely unanticipated and in many patients were quite dramatic.

Subsequently, Shekhar and colleagues (23) reported that xanomeline treatment led to rapid and robust antipsychotic and procognitive effects in a small double-blind placebo-controlled trial in inpatients with treatment-resistant schizophrenia. Treatment with xanomeline in both trials (22, 23), although not associated with the common side effects of first- and second-generation antipsychotics (e.g., weight gain, extrapyramidal side effects, and sedation), resulted in substantial peripheral "cholinergic" adverse events (e.g., nausea and vomiting) that precluded xanomeline's further development. Nonetheless, these clinical studies (22, 23) and related preclinical work (24, 25) with xanomeline strongly suggested that mAChR agonists can reduce psychotic symptoms without directly antagonizing DA D_2 receptors (26).

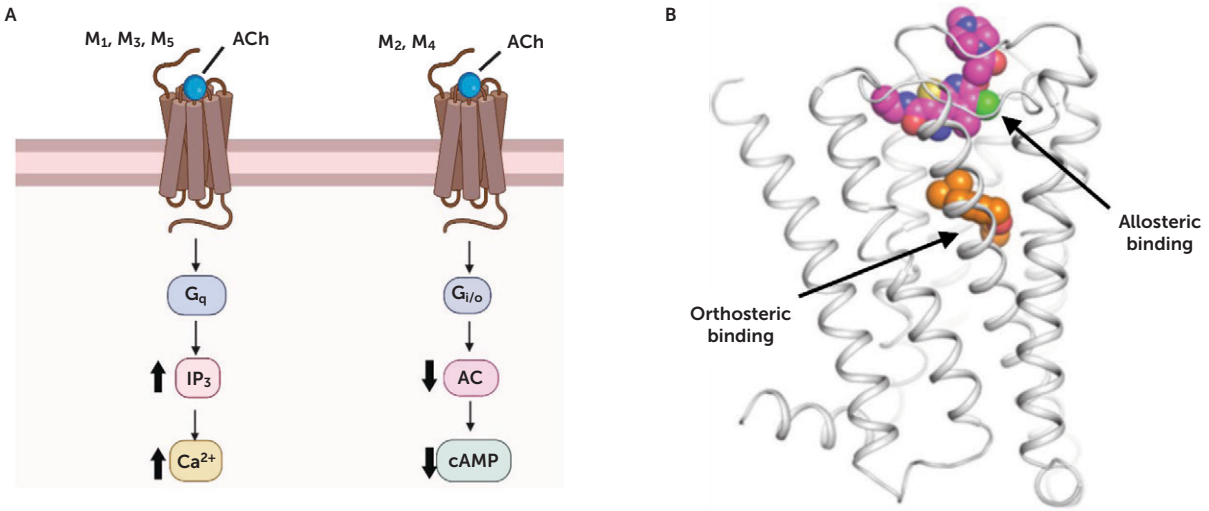
Over the ensuing two decades, much has been learned about the molecular, cellular, and neural circuit-based mechanisms of mAChR agonist–based antipsychotics. These findings and their application may lead to the discovery of potentially more effective treatments for managing the positive, negative, and cognitive symptoms of schizophrenia.

MUSCARINIC RECEPTOR PHARMACOLOGY

The discovery of mAChRs emerged from early Nobel Prize–winning discoveries on the regulation of frog heart contractility by Loewi and Dale and their seminal finding of the first neurotransmitter, acetylcholine (ACh), in 1921 (27). Cholinergic neurons are now known to form an intricate brain-wide network to modulate microcircuits through actions at both nicotine-sensitive (ionotropic) ion channels and muscarine-sensitive (metabotropic) mAChRs (28). The mAChR family is composed of five distinct subtypes (M_1–M_5) belonging to the superfamily of GPCRs, which, as transmembrane proteins, transmit external neurotransmitter signals via intracellular transducer proteins and second messengers (29). Although G protein–mediated signaling of GPCRs was the first signaling process associated with this superfamily of proteins, it is now well known that their signal transduction activities include complex combinations of G protein–dependent and –independent signaling pathways (see references 30, 31 for reviews). Historically, the mAChRs are subdivided into two distinct functional classes based on their propensity to couple to one of two primary G protein–dependent second messenger and signal transduction pathways (Figure 2A) (29, 32). The first class consists of the M_1, M_3, and M_5 receptor subtypes, which couple primarily through the G_q subtype of G protein to stimulate phospholipase C and the subsequent release of the intracellular second messenger, inositol 1,4,5-trisphosphate (IP_3), leading to increased intracellular calcium (Ca^{2+}) levels and facilitation of excitatory postsynaptic currents. The second class is composed of M_2 and M_4 receptors, which couple primarily through the $G_{i/o}$ subtype of G proteins that inhibit adenylate cyclase, leading to a reduction in the second messenger cAMP to generally suppress excitation.

All five mAChRs are broadly expressed throughout the body, supporting both peripheral autonomic functions and CNS control of arousal, attention, memory, and motivation (Figure 3A). The M_1 and M_4 receptors have their highest expression in the CNS, whereas the M_2 and M_3 receptors are

FIGURE 2. Signaling selectivity among muscarinic acetylcholine receptors (mAChRs)[a]



[a] As shown in panel A, the muscarinic receptor family consists of five members (M_1–M_5), which mediate the physiological effects of the neurotransmitter acetylcholine (ACh). The M_1, M_3, and M_5 receptors are stimulatory and couple primarily to the generation of intracellular G_q alpha subunit to stimulate phospholipase C (via inositol 1,4,5-trisphosphate [IP_3]), resulting in the mobilization of intracellular calcium (Ca^{2+}). The M_2 and M_4 receptors are inhibitory and negatively modulate adenylyl cyclase (AC) via $G_{i/o}$ alpha subunit to reduce cytoplasmic concentrations of cyclic adenosine monophosphate (cAMP). Panel B shows the crystal structure of the active state of the M_2 receptor with allosteric and orthosteric binding pockets simultaneously occupied by the mAChR orthosteric agonist iperoxo and the M_4/M_2 positive allosteric modulator LY2119620. Created with BioRender.com.

more highly expressed in the periphery. The M_5 receptor is relatively discretely expressed in DA-rich midbrain regions. The M_1 receptor is expressed predominantly postsynaptically, with high levels of expression in the cerebral cortex and hippocampus, and is primarily associated with modulating excitatory synapses (33). The M_2 receptor subtype primarily functions as a presynaptic neuromodulator, often acting as an autoreceptor on ACh-producing neurons (34) or as a heteroreceptor on neurons that release non-ACh neurotransmitters (e.g., glutamate, gamma-aminobutyric acid [GABA], and DA) to reduce their release (35). The M_2 receptor is also highly expressed in the brainstem and thalamus and is expressed at low levels in cortical regions (33). The M_3 receptor has a similar distribution pattern to the M_1 receptor's, but with a much lower level of expression in brain (33). The M_4 receptor is particularly abundant as either pre- or postsynaptic auto- or heteroreceptors in the basal ganglia, limbic system, hippocampus, and cortex (36, 37). In contrast to its other family members, the M_5 receptor has a more discrete localization in brain, primarily in midbrain DA neurons (38) (see references 39, 40 for mAChR distribution).

Early CNS drug development efforts resulted in agonists with only modest mAChR subtype selectivity and were associated with poor tolerability due to peripheral side effects that precluded clinical development (41). Further complicating the discovery of mAChR subtype–selective agonists was the experimentally observed difference in selectivity based on receptor binding potencies versus functional activity (42). Upon cloning and sequencing of the mAChRs (43), along with recent observations of their three-dimensional crystal and cryo-electron-microscopic structures (44–46), it became clear that the mAChR family is one of the most highly conserved with respect to protein sequence and structure, making it exceedingly challenging to develop subtype-selective orthosteric ligands (31). Lack of mAChR subtype–selective ligands has also confounded attempts to fully attribute the desired in vitro and in vivo pharmacology to one or more mAChR subtypes (47). In mice, this challenge has in part been addressed by the use of global tissue-specific and cell-type-specific transgenic mice, in which each of the mAChR subtypes has been genetically deleted to better define their physiological and behavioral roles as well as to characterize the pharmacology of mAChR ligands (48–50).

The first highly selective mAChR agonists were developed relatively recently by targeting ligand-binding pockets or sites outside of the ACh (orthosteric) binding pocket itself (51). Receptor subtype–specific amino acid residues residing in these unique allosteric binding pockets have enabled the discovery of allosteric ligands with highly receptor-selective modulatory properties (51) (Figure 2B). These modulatory properties can either enhance or reduce receptor activation by the endogenous neurotransmitter ACh and maintain the temporal and spatial signaling of natural cholinergic neurotransmission in the brain (52, 53).

Modulators are classified as either positive allosteric modulators (PAMs) or negative allosteric modulators (NAMs) (51). However, these designations should be interpreted with caution as they are highly contextual, being dependent on the exact receptor being studied, the choice of orthosteric ligand, and the signaling assay employed (31). For example, the M_1 receptor–selective modulator benzylquinolone carboxylic acid (BQCA) acts solely as a PAM when assayed in a cell line with low M_1 receptor expression (54) but acts as both a full allosteric agonist and as a PAM in a system

FIGURE 3. Quantification of muscarinic acetylcholine receptor (mAChR) mRNAs in central and peripheral human tissues using RNA sequencing[a]

[a] Relative expression of the five mAChR subtypes across key brain regions (panel A) and peripheral organs (panel B) associated with efficacy and tolerability of mAChR agonists. (Data used for the relative expression analysis described in this figure were obtained from the Genotype-Tissue Expression [GTEx] Portal on March 29, 2021. The GTEx Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health and by the National Cancer Institute, the National Human Genome Research Institute, the National Heart, Lung, and Blood Institute, the National Institute on Drug Abuse, the National Institute of Mental Health, and the National Institute of Neurological Disorders and Stroke.)

with high M_1 receptor reserve (55). Moreover, recent research on mAChRs and other GPCRs has revealed several additional and unexpected complexities, including receptor-receptor interactions (56, 57), the formation of homomeric versus heteromeric receptor complexes (58, 59), ligand-dependent biased signaling (47), and species specificity (60).

Notwithstanding these drug development challenges, both mAChR PAMs and NAMs remain a focus of investigation due to their potential to achieve receptor subtype selectivity and to maintain spatial and temporal signaling that are better aligned with natural cholinergic neurotransmission in the brain (52, 53). The ability of allosteric modulators to maintain temporal signaling compared to orthosteric agents could have potential advantages if persistent, full receptor activation is undesirable (i.e., due to potential side effects or receptor desensitization) (61).

However, the advantages ascribed to PAMs could, in some cases, be disadvantageous. For example, a PAM's reliance on cooperativity with the endogenous neurotransmitter, in this case ACh, could render such treatments less effective, especially in diseases where ACh levels decrease due to the progressive degeneration of cholinergic neurons, such as in Alzheimer's disease and Parkinson's disease (62, 63). Allosteric modulators are designed to regulate the affinity and efficacy of the natural ligand based on prevailing cholinergic tone, which may turn out to be therapeutic in one context but insufficient or even detrimental in another pathophysiologic state (64). The potential advantages or disadvantages of the selectivity afforded by allosteric mAChR agonists, however, will likely need to be determined in clinical studies to compare their relative efficacy and safety with direct-acting orthosteric agonists (see below).

Development of Muscarinic Receptor Agonists to Treat Schizophrenia

The initial development of mAChR-based therapies for CNS disorders focused primarily on treating Alzheimer's disease–associated cognitive impairment, which results, at least in part, from loss of cholinergic innervation from forebrain regions such as the nucleus basalis and longitudinal band projecting to the hippocampus, afferents important for cognition and memory (65, 66). This prompted drug developers to create direct-acting ACh-mimetic agonist drugs based on the structure of arecoline, a well-characterized ACh-mimetic agonist for mAChRs (41, 67, 68). The result was the development of a number of mAChR agonists, several of which were taken into clinical trials, such as RS-86, milameline, cevimeline, tazomeline, talsaclidine, alvameline, sabcomeline, and xanomeline, with the intent of stimulating postsynaptic M_1 receptors and thus circumventing the loss of ACh in Alzheimer's disease (41, 69). Although these agents were developed as M_1 receptor–targeted compounds, in practice they displayed only modest pharmacological selectivity across all five mAChRs (70).

Some of these M_1 receptor–targeted compounds demonstrated detectable, although modest, improvements in cognition; however, parasympathetically mediated adverse events, including nausea and vomiting, increased gastrointestinal motility, salivation, and sweating, prevented their further clinical development (41, 71). For example, cevimeline initially yielded positive results on both the Alzheimer's Disease Assessment Scale–Cognitive Subscale (ADAS-Cog) and caregiver impression scales (72). However, in a larger follow-up study, cevimeline subsequently failed to improve these same prespecified primary endpoints (73). Similarly, initial results from a clinical trial of sabcomeline in patients with Alzheimer's disease indicated that it, too, improved ADAS-Cog scores (74), but it failed to meet its primary cognitive endpoint in a phase 3 trial (75). In a phase 2 study in patients with schizophrenia, sabcomeline was also reported to have no effect on Positive and Negative Syndrome Scale (PANSS) scores compared with placebo; however, trends in improved cognitive function were reported (76).

Overall, the often-cited reasons for discontinuation of these first-generation full or partial M_1 receptor–targeted orthosteric agonists were the lack of efficacy and/or poor tolerability. The trials did not include any CNS assessment of activity, such as CNS muscarinic receptor occupancy, which is not surprising given the lack of reliable positron emission tomography ligands when these trials were conducted. Thus, it is difficult to conclude whether these trials failed owing to suboptimal dosing caused by dose-limiting peripheral mAChR adverse events or a lack of therapeutic activity for any given compound. At the time, the poor tolerability of these compounds was believed to be primarily due to their M_2 and M_3 receptor activity (48), but further work (see below) with mAChR subtype–selective ligands suggested that M_1 and M_4 receptors also likely played a role in the peripherally mediated adverse events observed with this first generation of mAChR-targeted drug candidates.

Although initially developed to specifically treat the cognitive symptoms of Alzheimer's disease, the mAChR agonist xanomeline, as briefly discussed above, significantly reduced and prevented the emergence of behavioral, including psychotic, disturbances in patients with Alzheimer's disease and resulted in modestly improved cognitive impairment as measured by the ADAS-Cog (22). These promising clinical data in patients with Alzheimer's disease encouraged a considerable number of preclinical studies across academia and industry (discussed in detail below) to substantiate the neurobiological underpinnings of these surprising clinical results. Moreover, these findings also prompted a small (N=20), proof-of-concept, phase 2 follow-up trial in acutely psychotic patients with chronic schizophrenia (23). In that trial, when compared with placebo, treatment with xanomeline resulted in significant improvements in total Brief Psychiatric Rating Scale and PANSS scores as well as improvement in several cognitive domains, specifically in measures of verbal learning and short-term memory function. These rather compelling results obtained in patients with either Alzheimer's disease (22) or schizophrenia (23) stimulated further research on the potential utility of mAChR agonists for treating a variety of neuropsychiatric disorders. Also evident from these early studies, however, was the need for improved tolerability, primarily with respect to limiting peripheral mAChR stimulation.

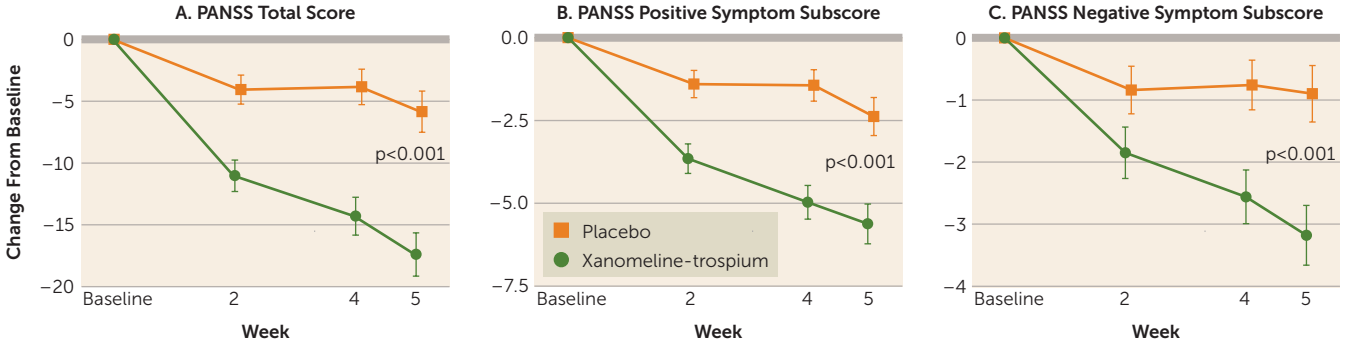
Following these early encouraging clinical data with xanomeline, the field set out to develop "mAChR-targeted" agents that would maintain the clinical benefits associated with xanomeline while reducing the associated mAChR-mediated adverse events. To accomplish this, two different approaches have been pursued. The predominant approach has been to develop compounds that more selectively target M_1 and/or M_4 receptors, the two mAChRs believed to be most associated with the benefits of xanomeline, via their allosteric (63) rather than orthosteric binding sites.

The other approach is a strategy recently adopted for reducing the peripheral side effects of xanomeline while maintaining its therapeutic benefits in the CNS: coadministration of xanomeline with a peripherally restricted mAChR antagonist (77). By blocking peripheral mAChRs with trospium (a U.S. Food and Drug Administration–approved, non–brain-penetrant, pan-mAChR antagonist) (78) while simultaneously activating central mAChRs with xanomeline, tolerability was markedly improved while maintaining xanomeline's centrally mediated therapeutic benefits. The high brain-to-plasma ratio (>10:1) of xanomeline also likely favors CNS-mediated therapeutic activity observed across these clinical trials (79–81).

The results from a recently completed placebo-controlled phase 2 trial in patients with schizophrenia experiencing acute psychotic symptoms (82) demonstrated that treatment with the investigational drug combination of xanomeline and trospium (KarXT) resulted in a significant reduction in PANSS total score as well as a number of secondary endpoints (e.g., PANSS positive subscale, PANSS negative subscale, and Clinical Global Impressions severity scale) compared with placebo (Figure 4). Additionally, an exploratory post hoc analysis (83) suggests that KarXT treatment was also associated with greater improvement in cognitive performance compared with placebo in patients who were cognitively impaired at baseline. However, additional prospective studies will be needed to confirm the potential procognitive benefits of KarXT. KarXT was associated with mild to moderate cholinergic and anticholinergic adverse events that were generally transient and did not lead to discontinuation from the trial. Treatment with KarXT was not associated with extrapyramidal side effects, weight gain, or sedation (82). Although these phase 2 data are encouraging, phase 3 studies will be required to confirm the efficacy and safety profile observed in phase 2. Several phase 3 studies are currently under way (NCT04659161, NCT04738123, NCT04659174, NCT04820309, NCT05145413).

These results, which replicate the earlier phase 2 trials with xanomeline (22, 23), strongly suggest that mAChRs are compelling drug targets to potentially modulate neural

FIGURE 4. Treatment with the muscarinic acetylcholine receptor (mAChR) M_1/M_4-preferring agonist xanomeline in combination with the peripherally restricted mAChR antagonist trospium[a]



[a] Treatment with xanomeline-trospium was superior to placebo on symptom improvement as assessed by the Positive and Negative Syndrome Scale (PANSS) at week 5 (panel A). Secondary efficacy endpoints, the PANSS positive symptom subscore (panel B) and negative symptom subscore (panel C), were also statistically significant compared with placebo at week 5. (From Brannan SK et al., Muscarinic cholinergic receptor agonist and peripheral antagonist for schizophrenia, New England Journal of Medicine, vol. 384, pp. 717–726. Copyright © 2021 Massachusetts Medical Society. Reprinted with permission.)

circuits disrupted in patients with schizophrenia. Still to be explained, however, is whether one or more mAChR subtypes contribute to xanomeline's antipsychotic and procognitive activity and how mAChR agonists may impact neural circuits likely responsible for their potential benefits in treating the core symptoms of schizophrenia.

ROLE OF SPECIFIC MUSCARINIC RECEPTOR SUBTYPES

Xanomeline has been reported to bind with relatively equal affinity to all five mAChRs, as determined by the displacement of the nonselective radiolabeled mAChR antagonist [^{3}H]-*N*-methyl scopolamine (84, 85), and has been reported in some (86) but not all (26, 85) studies to bind to several serotonin (5-HT) receptors with low to high nanomolar affinity. Historically, radioligand binding assays with receptor-containing isolated whole-cell or synaptic membranes were used to rapidly screen compounds that target GPCRs, but these simple binding assays do not adequately establish whether a given compound of interest is an agonist or an antagonist or, importantly, whether it has functional activity as either a full or partial agonist or antagonist (42). Efforts have been made to develop signaling-dependent cell-based functional assays to provide more accurate and comprehensive data sets of compounds targeting GPCRs. Based on several cellular and in vivo functional assays, xanomeline appears to be selective for M_1 and M_4 receptors, and at pharmacologically relevant concentrations, it does not have significant functional activity at other GPCRs (85, 87, 88), including DA or 5-HT receptors.

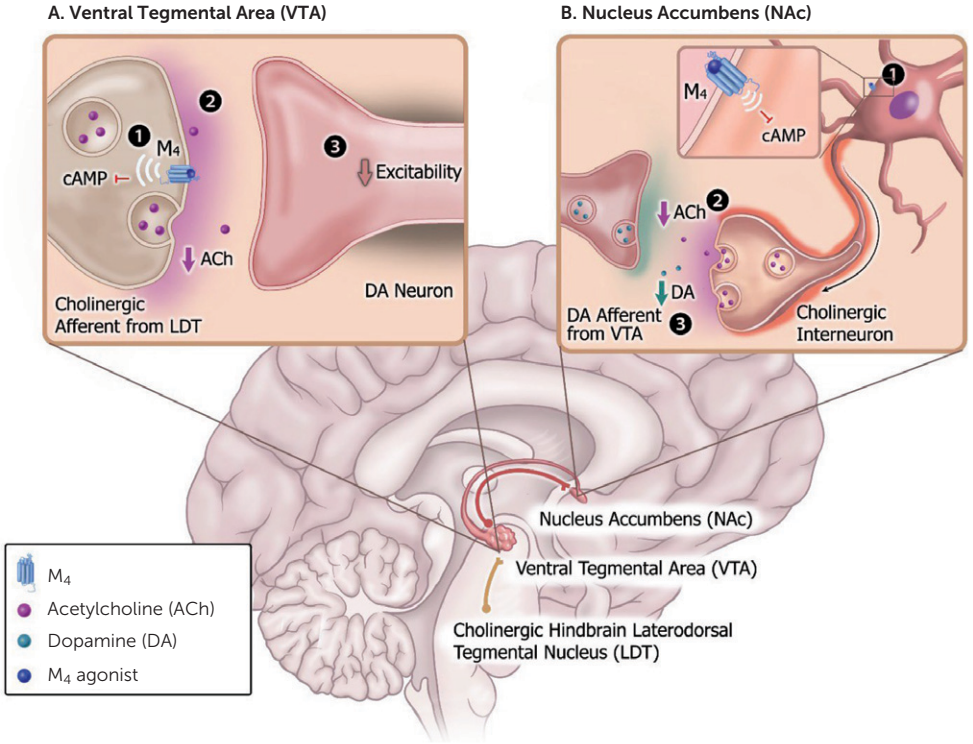
Recent studies using X-ray crystal or cryo-electron-microscopy–solved structures of mAChRs at the <3.0-angstrom level, along with in silico molecular dynamic simulations, have allowed more detailed descriptions of both the static and kinetic interactions of mAChR ligands, such as xanomeline, at each of the mAChR subtypes (89). Based on these studies, xanomeline has been found to have unique and unexpected binding properties and pharmacology, including functional selectivity for M_1 and M_4 receptors (24, 90) and some level of persistent or "wash-resistant" binding that suggests pseudo-irreversible binding properties (91–93), which may contribute to its potentially unique functional pharmacology. Data generated from pharmacological models (94), paired with genetic deletion of mAChRs in mice (49), have provided considerable insight into which receptor subtypes are responsible for xanomeline's behavioral pharmacology.

Xanomeline exhibits robust "antipsychotic drug–like" activity in animals treated with psychostimulant drugs that increase DA neurotransmission (e.g., amphetamine or apomorphine) (25, 26, 90, 95–97) or that block *N*-methyl-D-aspartate (NMDA) receptors (e.g., phencyclidine [PCP], dizocilpine [MK-801], or ketamine) (98, 99). The ability of xanomeline to decrease the functional effects of dopaminergic and glutamatergic psychostimulants is consistent with the putative antipsychotic actions of this compound, findings that have been replicated across numerous preclinical behavioral models of "psychosis" (24, 25, 90, 95, 97, 98, 100) and in recent pharmacological MRI studies (101, 102). Moreover, xanomeline's antipsychotic activity is fully blocked by a centrally penetrant but not peripherally restricted pan-muscarinic-subtype antagonist, indicating that activation of one or more central mAChRs is sufficient to elicit antipsychotic-like activity (103) and likely to regulate neurotransmitter systems and neural circuits implicated in the pathophysiology of schizophrenia. Evidence for which mAChR subtypes are involved in the behavioral actions of xanomeline has only recently been made possible with the development of mice bearing genetic deletions of one or more mAChR subtypes as well as by using mAChR subtype–selective agonists.

Role of M_4 Receptors

The onset of psychotic (i.e., positive) symptoms is believed to be associated with neural network dysfunction that includes a variety of brain regions and neurotransmitters, including increased activity of ventral tegmental area (VTA) DA neurons and heightened terminal DA release (104). Although the exact mechanism of how antipsychotic drugs achieve their therapeutic activity is still not completely understood, it has been postulated, with considerable experimental support, that most, if not all, antipsychotic drugs induce "depolarization block" or inactivation of DA neurons in both the VTA ("mesolimbic DA circuit") and the substantia nigra (SN; "motor DA circuit"); this phenomenon takes place over a period of time that parallels the delay in the onset of clinical improvement (105). Based on the "depolarization block" hypothesis, antipsychotic drugs with more selective actions on VTA DA neurons consistently show a lower motor symptom liability (i.e., low extrapyramidal side effects), whereas high liability to extrapyramidal side effects is associated with antipsychotic drugs that more markedly reduce the activity of DA neurons in the SN, which is involved in the control of the extrapyramidal motor control system (106, 107). Importantly and often overlooked is that the activity of midbrain VTA DA neurons is modulated by cholinergic input from hindbrain structures (108), particularly the laterodorsal tegmental nucleus (LDT), where M_4 receptors are abundantly expressed and function as autoreceptors (49, 109). M_4 receptors play a key role in blunting the stimulatory effects of ACh on DA neurons, making them less active and thereby reducing downstream DA release in brain regions implicated in psychosis, such as the nucleus accumbens and ventral striatum (Figure 5A) (49, 109).

FIGURE 5. Activation of M_4 receptors modulates cholinergic tone within midbrain dopamine (DA) centers to regulate terminal DA release[a]



[a] The M_4 receptor is an autoreceptor on cholinergic afferents (point 1 in panel A) that project into the ventral tegmental area (VTA). Upon activation, M_4 receptors reduce acetylcholine (ACh) release onto VTA DA neurons (point 2 in panel A) and subsequently decrease neuron firing activity due to reduced activation of ACh receptors located on DA cell bodies (point 3 in panel A). This leads to a downstream reduction of DA release within the nucleus accumbens (NAc). Locally, within the NAc, M_4 receptors are located on cholinergic interneurons (ChIs) (point 1 in panel B). Upon activation, M_4 receptors decrease ChI spontaneous activity, and thus decrease cholinergic release from these neurons (point 2 in panel B). Reduced local cholinergic tone will also decrease stimulation (i.e., activation) of DA terminals (point 3 in panel B).

Interestingly, xanomeline has been shown to selectively and rapidly reduce VTA DA neuron firing rates but not SN DA neuron activity (24), an effect now believed to be mediated by selective activation of LDT afferents (110, 111). This very rapid reduction in VTA DA neuron activity observed following acute administration of xanomeline (24) contrasts with the slow, time-dependent depolarization block of these same DA neurons that occurs following treatment with second-generation antipsychotics (105, 112, 113). The DA D_2 receptor–independent modulation of DA microcircuits may also explain the relatively rapid antipsychotic effects of xanomeline reported in patients with Alzheimer's disease (22) or schizophrenia (23). Also consistent with xanomeline's selective action on mesolimbic VTA DA neurons is the lack of overt motor effects (e.g., catalepsy) observed in preclinical models (24, 90) or its lack of effect in inducing immediate early gene expression (e.g., cFos) in brain regions associated with extrapyramidal side effects (114). This highly selective regulation of DA-containing neural circuits seen with xanomeline may therefore account for the lack of observed extrapyramidal side effects in multiple clinical trials (22, 23, 82) and a much lower, if not absent, risk of developing tardive dyskinesia.

In addition to their ability to regulate midbrain VTA DA neuron activity, M_4 receptors are expressed in cholinergic interneurons that reside locally in the nucleus accumbens and modulate terminal DA release (115, 116). Within the nucleus accumbens, activation of M_4 autoreceptors on cholinergic

interneurons decreases spontaneous activity, which reduces ACh release to dampen nicotinic receptor feed-forward stimulation on DA terminals (Figure 5B) (116, 117). It should be noted, however, that there are other postulated mechanisms by which M_4 receptor–mediated inhibition of local DA release may occur, such as activation of M_4 receptors coexpressed in D_1 receptor–expressing medium spiny neurons (MSNs) of the dorsolateral striatum (118, 119). In support of this hypothesis, selective deletion of M_4 receptors from D_1 MSNs increases DA-dependent behavioral phenotypes (119) and blunts M_4 receptor–mediated inhibition of DA release in preclinical models (120), an effect likely mediated through competition of convergent second messenger systems (56), recruitment of endocannabinoids (120), interactions with G_q-coupled receptors (121), or direct actions on enhanced Ca^{2+} currents via Ca_v1 channels (122). Genetically eliminating M_4 receptors from D_1 MSNs also markedly reduces the inhibitory effects of xanomeline on amphetamine-stimulated locomotor activity (118).

More recent studies using highly selective M_4 receptor PAMs support the important role that these receptors play in mediating antipsychotic drug–like behavioral activity, as numerous chemical scaffolds that modulate M_4 receptors display antipsychotic activity (60, 120, 123–125). In addition to modulating classical neural circuits implicated in psychosis, M_4 receptor PAMs have been shown to enhance attentional and memory network function in preclinical rodent models (126). M_4 receptor agonists can reduce elevated CA1 pyramidal neuron activity, which may partially contribute to their procognitive effects (85), as changes in CA1 excitability have been postulated to contribute to the cognitive deficits in schizophrenia (27). However, additional detailed studies are needed to fully understand the multinodal mechanisms by which M_4 receptors impact local subcortical microcircuits within the dorsal (i.e., associative striatum) and ventral striatum to inhibit DA neurotransmission (for a review, see reference 9).

Although M_4 receptor regulation of DA microcircuits appears to be important in the central mechanism of action of xanomeline, glutamatergic microcircuits may also contribute to its antipsychotic activity. In rodents, acute administration of NMDA receptor antagonists, such as PCP or MK-801, produces behavioral hyperactivity and cognitive deficits that correlate with a disinhibition of pyramidal cell firing in the prefrontal cortex and increased DA and glutamate levels (127, 128). Administration of xanomeline or a selective M_4 receptor PAM can attenuate PCP- or MK-801-evoked locomotor activity, an effect that is absent in global M_4 receptor knockout mice (123). M_4 receptor–mediated regulation of MK-801-induced hyperactivity may involve actions at corticostriatal terminals to normalize the function of overactive excitatory glutamatergic projections to the striatum (129) or via a dampening of thalamocortical synapses (130). This "top-down" control of cortical glutamatergic projections has important implications, as these projections indirectly modulate phasic DA release (118). In addition, activation of M_4 receptors attenuates MK-801-induced disruptions in learning and memory (123) and elevations of high-frequency gamma power as well as state-dependent alterations in sleep architecture and arousal similar to the effects observed with atypical antipsychotics in preclinical models (131). Taken together, these findings suggest an important role for M_4 receptors in modulating neural circuits involved in the psychotic, motivational, cognitive, and executive functions disrupted in schizophrenia via glutamatergic microcircuits.

These behavioral studies describing an important role for M_4 receptors in animal models of psychosis have led to further studies exploring the potential role of M_4 receptors in the pathophysiology of schizophrenia. In postmortem studies, M_4 receptor expression has been reported to be decreased in striatal and hippocampal brain regions of patients with schizophrenia compared with those of healthy control subjects (132). Other studies using modestly selective mAChR antagonist radioligands also suggest that M_4 receptors are decreased in the frontal cortex, hippocampus, and striatum of patients with schizophrenia compared with those of control subjects (133, 134). In tandem with the apparent decreases in M_4 receptor expression, these morphometric changes could also contribute to decreased regional brain volumes and cortical thickness. Genetic markers (single-nucleotide polymorphisms) in a region on chromosome 11 have been reported to be associated with schizophrenia (135), and this region contains several candidate genes, including *CHRM4*, the gene encoding the M_4 receptor. Two genomic variants of the M_4 receptor, rs2067482 and rs72910092, have also been reported to be associated with an increased risk of schizophrenia (136). It should be noted, however, that these allelic associations have not been consistently replicated across studies (137), which may be due to differences in the patient populations sampled and diagnostic subtyping.

Importantly, recent clinical results were released from a phase 1b placebo-controlled trial of emraclidine (CVL-231), an M_4 receptor PAM, in patients with schizophrenia (138). In an exploratory analysis, treatment with CVL-231 was associated with reduced psychotic symptoms compared with placebo after 6 weeks of treatment (138). Rates of gastrointestinal adverse events were minimal compared to those in historical mAChR agonist trials (41, 69). These data provide additional evidence that M_4 receptors play key roles in mediating the antipsychotic properties of mAChR agonists (138) and preliminary clinical validation for drugs that target allosteric binding pockets of mAChR receptors (in this case M_4 receptors). Although encouraging, these data have yet to be peer reviewed, and further placebo-controlled trials of CVL-231 will be necessary to establish its efficacy and safety profile in patients with schizophrenia.

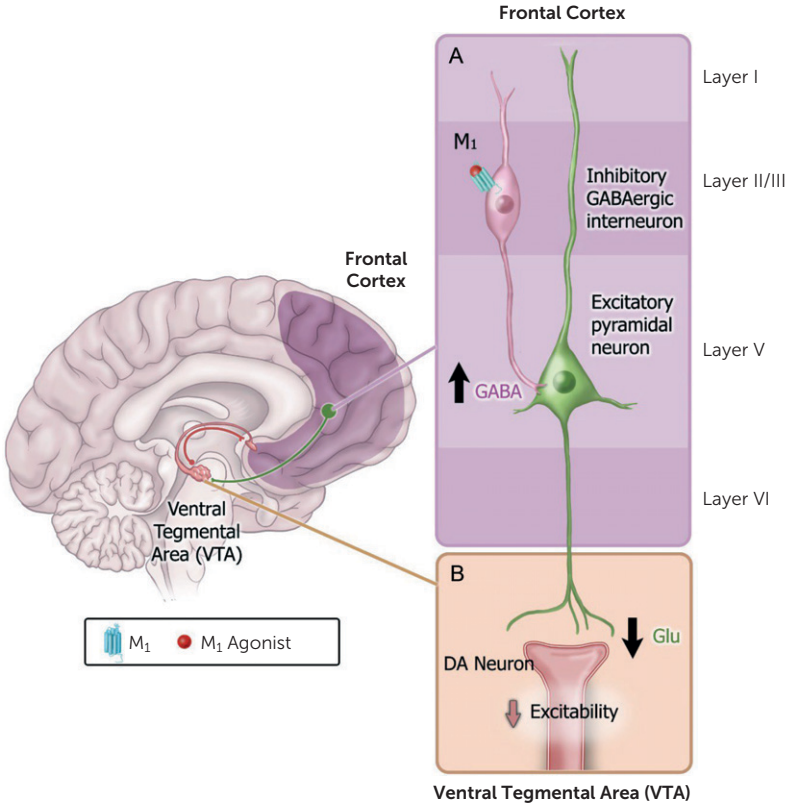
Role of M_1 Receptors

Although an important role for M_4 receptors in mediating the antipsychotic effects of xanomeline is quite likely (see above) there is also substantial evidence that M_1 receptors regulate

neural circuits underlying psychosis as well as learning and memory (especially working memory). Xanomeline's "antipsychotic" activity as measured by amphetamine-induced hyperactivity was abolished in mice lacking M_4 receptors but also was partially attenuated in mice lacking M_1 receptors, suggesting that M_4 receptors and, to a certain degree, M_1 receptors may both contribute to xanomeline's efficacy in treating the positive symptoms of schizophrenia (97). As xanomeline is a "dual" M_1 and M_4 receptor agonist, M_1 receptors may also contribute to its reported antipsychotic and procognitive activity.

Several hypotheses have emerged regarding how M_1 receptors may modulate neural circuits implicated in psychosis, including regulation of top-down circuits that synapse onto VTA DA neurons (Figure 6) (139, 140). For example, activation of M_1 receptors facilitates excitability of cortical GABAergic interneurons that synapse onto pyramidal neurons (139), causing a decrease in excitability of principal cortical output neurons. However, there are additional hypotheses regarding how M_1 receptor activators may exhibit antipsychotic activity, including augmentation of corticostriatal plasticity (141), modulation of MSN excitability (142), and enhanced communication between MSNs (e.g., via nucleus accumbens output neurons) (143). Additional studies have demonstrated a "psychosis-like" phenotype in global M_1 receptor knockout mice (144) as well as antipsychotic drug–like activities in various preclinical models following administration of selective M_1 receptor PAMs (145, 146). M_1 receptor PAMs have also been reported to reverse excessive spontaneous locomotor activity in NMDA receptor NR1-subunit knockdown mice that display an NMDA receptor–mediated hypofunction phenotype (147).

FIGURE 6. Activation of M_1 receptors in the frontal cortex exerts top-down control onto midbrain dopamine (DA) circuits[a]



[a] As illustrated in panel A, activation of M_1 receptors expressed on layer II/III inhibitory GABAergic interneurons (pink) facilitates inhibitory drive onto excitatory output neurons (i.e., pyramidal neurons; green). In panel B, enhanced inhibitory drive onto pyramidal neurons decreases glutamatergic input to the ventral tegmental area (VTA). A reduction of excitatory input leads to a decrease in VTA dopamine (DA; red) neuron activity and reduced terminal DA release.

As summarized above, significant effort has been made to develop M_1 receptor agonists to treat the cognitive impairment associated with various neuropsychiatric and neurodegenerative disorders (64, 70). In memory circuits, M_1 receptor activators have been shown to enhance synaptic plasticity (85, 148), increase neuronal excitability (149), and facilitate learning and improve cognition in aged animals (150) and in a variety of NMDA receptor hypofunction models of impaired learning and memory (147, 149, 151, 152). Previous studies have shown that M_1 receptors are physically and functionally coupled to NMDA receptors and that activation of M_1 receptors potentiates NMDA receptor currents in cortical and hippocampal pyramidal neurons (54, 153, 154). Conversely, global M_1 receptor knockout mice have impaired performance in prefrontal cortex–dependent cognitive tasks (155, 156) and reduced hippocampal long-term potentiation (157). Administration of an M_1 receptor potentiator in genetic models of NMDA receptor hypofunction restored plasticity deficits and improved impaired learning and memory in these mice (147). Moreover, M_1 receptor PAMs have been shown to modulate sleep-wake architecture in rodents and nonhuman primates (150), suggesting that activation of M_1 receptors may participate in restorative sleep–mediated plasticity, which has been postulated to be dysregulated in patients with schizophrenia (158). Beyond cognition, M_1 receptor PAMs have been reported to reverse behavioral and electrophysiological deficits in chronic PCP rodent models, which are thought to recapitulate the "deficit state" or negative symptoms of schizophrenia (149). Recently it was shown that M_1 receptor PAMs can reverse PCP-induced disruption of mAChR-stimulated long-term depression (149), a plasticity measure that is important for adapting neural networks to physiological activity. These studies support the idea that M_1 receptor activation may, by itself, contribute to improvements in the cognitive, negative, and even positive symptom domains of schizophrenia, and thus a dual M_1/M_4-preferring mAChR agonist may be particularly effective in treating schizophrenia.

As mentioned earlier, clozapine appears to be unique among the drugs used to treat schizophrenia, as it appears to be more effective in treating positive and negative symptoms in patients with treatment-resistant illness (159). Moreover, some (160) but not all (161) studies have reported that clozapine can improve cognition and especially working memory performance in patients with schizophrenia. Interestingly, although clozapine itself is a potent mAChR antagonist (162), its major metabolite, *N*-desmethylclozapine (NDMC), is a potent partial M_1 receptor agonist (163). Recently, it has been reported by several groups that lower clozapine-to-NDMC ratios are associated with improvements in working memory and executive function, whereas higher ratios are associated with cognitive deficits (164–167). These findings raise the intriguing possibility that the M_1 receptor activity of NDMC contributes to clozapine's unique clinical profile, including its reported procognitive benefits (160). Several lines of evidence suggest that M_1 receptors may also play a key role in the ability of clozapine to modulate schizophrenia-related circuity in rodents (168–170). Subefficacious doses of clozapine can reverse MK-801-induced deficits in sensorimotor gating, and this is potentiated by coadministration of an M_1 receptor PAM (151). From a circuit-level perspective, local administration of NDMC can alter DA release in brain regions implicated in psychosis, an effect that is opposite to that of clozapine (171).

However, in preclinical models (172), and in marked contrast to clozapine, NDMC did not display antipsychotic activity, and a subsequent phase 2 clinical trial in patients with schizophrenia confirmed its lack of efficacy in treating positive symptoms (173). Although NDMC shares many of clozapine's pharmacological properties beyond mAChR activity, it does not occupy or block DA D_2 receptors (174); this may account for its lack of efficacy in treating positive symptoms. However, it is quite possible that NDMC accounts for the procognitive effects of clozapine and possibly its beneficial effects on negative symptoms. NDMC could also be responsible for some of the peripheral adverse effects observed with clozapine (175), such as hypersalivation. Moreover, the peripheral gastrointestinal adverse events reported for xanomeline are almost certainly due to stimulation of peripheral mAChRs. Nonetheless, these data fit with the hypothesis that M_1 receptor activation could be a key mechanism through which clozapine exerts its unique clinical profile (175).

Previous studies have also suggested a role for M_1 receptors in the pathophysiology of schizophrenia. For instance, using [^{123}I]quinuclidinyl benzilate, mAChR availability was found to be reduced in unmedicated patients with schizophrenia (21), a finding similar to that in the postmortem studies. Several postmortem studies using the mAChR antagonist [^{3}H]-pirenzepine have demonstrated decreased M_1 receptor expression in cortical and subcortical regions in patients with schizophrenia (62, 176, 177). The authors refer to these patients as having "mAChR deficit" schizophrenia. Interestingly, whereas there is a pronounced loss of M_1 receptors in the mAChR deficit schizophrenia subtype, the residual M_1 population has increased receptor–G protein coupling efficiency, suggesting an adaptive change to compensate for reduced receptor expression (178). Importantly, these changes in M_1 receptor expression appear to be specific to schizophrenia and may represent a distinguishable endophenotype (177). Additional evidence that M_1 receptors may contribute to the pathophysiology of schizophrenia include recent reports that homozygous carriers of *CHRM1* C267A nucleotide polymorphisms exhibit pronounced perseveration errors and poor performance on tests of executive functioning (179, 180). More recently, elevated serum titers of anti–M_1 receptor antibodies have been reported in up to one-third of people diagnosed with schizophrenia (181, 182), and their presence was correlated with the severity of negative symptoms (181).

In actual practice, it is likely that many patients with schizophrenia will be treated simultaneously with conventional antipsychotic drugs and novel therapies to improve overall efficacy before they are switched to monotherapy with a novel drug (183). This concept is supported by preclinical data showing that treatment with the M_1 PAM BQCA in combination with atypical antipsychotics (i.e., aripiprazole and clozapine) provided synergistic procognitive activity in deficit states induced by the NMDA receptor antagonist MK-801 (151). Although additional studies are needed to fully understand the underlying neural circuits involved in mediating the procognitive effects of BQCA, they likely involve modulation of hippocampal synaptic plasticity (184, 185).

In the context of psychosis, subeffective doses of the M_4 PAM PGM039678 in combination with subeffective doses of the atypical antipsychotics olanzapine or risperidone significantly augmented conditioned avoidance responding in rats (186). Recently, it was also reported that subeffective doses of xanomeline augmented the activity of aripiprazole and risperidone in the conditioned avoidance response assay in mice (103). In the same study, subthreshold doses of xanomeline and risperidone administered together significantly attenuated MK-801-induced hyperactivity (103). These results raise the intriguing possibility that M_1 receptor, M_4 receptor, or dual M_1/M_4 receptor agonists may represent adjunctive treatments, when used together with currently prescribed first- or second-generation antipsychotics, to improve the core symptoms of schizophrenia, especially in patients with treatment-resistant illness.

Role of M_2, M_3, and M_5 Receptors

Pharmacological studies using M_2 receptor–preferring antagonists have produced contradictory results regarding their potential role in cognition. Whereas some studies suggest that blockade of central M_2 receptors enhances learning and memory in various experimental settings (187), other studies arrived at the opposite conclusion (188). M_2 receptor global knockout mice display deficits in behavioral flexibility, working memory, and passive avoidance learning (189). However, M_2 receptors are apparently not required for

stimulus-reward learning. Reduced expression of subcortical (190, 191) but not cortical (192) M_2 receptors has also been found in patients with schizophrenia. In preclinical models, the antipsychotic-like activity of BuTAC, an M_2 receptor–preferring orthosteric agonist and a partial M_1/M_4 receptor agonist, is lost in global M_2/M_4 double-knockout mice (193). From a microcircuit perspective, there appears to be a functional interplay between M_2 and M_4 receptors to regulate DA release in striatal regions (116), suggesting that M_2 receptor activation may play an important role in M_4-mediated suppression of DA release. Additional studies are needed to understand the therapeutic potential of dual M_2-M_4 receptor agonists or whether M_2-M_4 heterodimers play a role in psychosis.

The M_3 receptor is expressed widely in the CNS, including in the hippocampus (194), and may play an important role in learning and memory. M_3 receptor knockout mice have severe deficits in hippocampus-dependent memory, suggesting that selective M_3 receptor activators may be beneficial for cognition (195). Recently, it has been reported that M_3 receptors regulate feed-forward inhibition, which may facilitate memory consolidation by reducing interference signals (196). Detailed biochemical studies support an important role for M_3 receptor signaling through phosphorylation events as contributing to these procognitive effects (195) and raise the possibility that M_3 receptor–biased ligands that increase β-arrestin-dependent (non–G protein) signaling might promote cholinergic-mediated learning and memory (197).

Finally, M_5 receptors may also be a promising drug target for schizophrenia given their expression in and control of midbrain DA neurons (198). M_5 receptor global knockout mice display reduced striatal DA release and blunted responses in preclinical models of psychosis (199). Activation of M_5 receptors increases the activity of midbrain DA neurons (200) to facilitate terminal DA release (201), suggesting that therapeutic agents that inhibit the M_5 receptor will decrease elevated DA levels reported in various forebrain regions in schizophrenia. More recent preclinical evidence suggests that M_5 receptor agonists or PAMs may also be effective for treating negative symptoms (202) and cognitive deficits (203). Continued efforts are needed to develop CNS-penetrant compounds with appreciable M_5 receptor agonist or antagonist selectivity to further elucidate their potential benefits (204, 205). In this regard, xanomeline has been shown to be an M_5 receptor partial agonist (206) and thus is likely to have a predominant M_5 receptor antagonist pharmacological profile (206). The contribution, if any, of xanomeline's M_5 receptor activity, along with its M_1 and M_4 receptor pharmacology, to its reported clinical (antipsychotic and procognitive) activity in patients with schizophrenia is unclear but cannot be excluded.

SUMMARY AND CONCLUSIONS

Current treatments for schizophrenia include a wide array of antipsychotic drugs that are chemically distinct, but all target the DA D_2 receptor, just like the very first antipsychotic drug, chlorpromazine, which was introduced in the United States 70 years ago (4, 207). With the exception of clozapine, all current antipsychotics have relatively similar efficacy profiles. These drugs work primarily to reduce the positive symptoms of schizophrenia, with little beneficial impact on either negative or cognitive symptoms (9). Currently approved antipsychotic drugs are all believed to work by initially binding to (and occupying) DA D_2 receptors, where they behave as either antagonists or partial agonists/antagonists, leading to downstream adaptive neurochemical and neurophysiological changes in neural circuits underlying psychotic symptoms, which may also account for the time-dependent lag of several weeks for a maximal antipsychotic response (208). Due to their conserved pharmacology, these drugs all share many of the undesirable side effects and longer-term adverse events associated with their use, including extrapyramidal side effects, akathisia, sedation, weight gain, hyperprolactinemia, and a risk of developing tardive dyskinesia (5, 209), among others.

The need for new and mechanistically unique antipsychotic drugs that can treat not only the positive but also negative and cognitive symptoms of schizophrenia cannot be overstated (9). Based on the clinical observations of the antipsychotic properties of the M_1/M_4-preferring mAChR agonist xanomeline (22) and other mAChR agonists, it appears that mAChRs may represent viable non-DA D_2 receptor drug targets for discovering mechanistically unique treatments for the core symptoms of schizophrenia. Preclinical and clinical data support the role of both the M_1 and M_4 mAChRs (as well as potentially other mAChRs) in mediating the antipsychotic and procognitive effects of xanomeline, as well as potentially other orthosteric and allosteric mAChR modulators. The evidence suggests that these drugs can rather rapidly and selectively impact the cellular and neural circuits that may underlie their novel antipsychotic and procognitive properties. mAChR agonists represent a promising new class of medication with the potential to treat the core symptoms of schizophrenia, including positive, negative, and cognitive symptoms, while not being associated with the long-term side effects of DA-based antipsychotics.

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