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Positive allosteric modulation of M_1 and M_4 muscarinic receptors as potential therapeutic treatments for schizophrenia

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Abstract

Current antipsychotic drugs provide symptomatic relief for positive symptoms of schizophrenia, but do not offer symptom management for negative and cognitive symptoms. In addition, many patients discontinue treatment due to adverse side effects. Therefore, there is a critical need to develop more effective and safe treatment options. Although the etiology of schizophrenia is unclear, considerable data from post-mortem, neuroimaging and neuropharmacology studies support a role of the muscarinic acetylcholine (mAChRs) in the pathophysiology of schizophrenia. Substantial evidence suggests that activation of mAChRs has the potential to treat all symptom domains of schizophrenia. Despite encouraging results in demonstrating efficacy, clinical trials of nonselective mAChR agonists were limited in their clinical utility due to dose-limiting peripheral side effects. Accordingly, efforts have been made to specifically target centrally located M_1 and M_4 mAChR subtypes devoid of adverse-effect liability. To circumvent this limitation, there have been tremendous advances in the discovery of ligands that bind at allosteric sites, binding sites distinct from the orthosteric site, which are structurally less conserved and thereby afford high levels of receptor subtype selectivity. The discovery of subtype-specific allosteric modulators has greatly advanced our understanding of the physiological role of various muscarinic receptor subtypes in schizophrenia and the potential utility of M_1 and M_4 mAChR subtypes as targets for the development of novel treatments for schizophrenia and related disorders.

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PJC is an inventor on multiple composition of matter patents protecting allosteric modulators of GPCRs.

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1.1 Introduction

Schizophrenia is a complex heterogeneous disorder that affects approximately 1% of the population worldwide (Sullivan et al., 2000) and is characterized by three broad clusters of symptoms that are associated with significant psychological, social and occupational dysfunction. These symptom domains include positive symptoms (e.g., hallucinations, delusions), negative symptoms (e.g., anhedonia, social withdrawal, blunted affect) and cognitive dysfunction (e.g., impaired working memory, attention, cognitive flexibility; American Psychiatric Association, 2000). Schizophrenia is typically diagnosed at the first episode of psychosis that results in hospitalization (Strakowski et al., 1993) and is treated with broad spectrum G-protein coupled receptor (GPCR) antagonists that exert their therapeutic effects through inhibition of dopamine (DA) D_2 and serotonin (5-HT) 5-HT_{2A} receptors (Roth et al., 2004). While these agents are efficacious for treating positive symptoms, they offer little to no benefit for the negative or cognitive symptom domains (Green, 1996; Greenwood et al., 2005). In addition to partial responsiveness, other limitations for successful treatment include adverse side effects, such as development of movement disorders, abnormal weight gain and metabolic syndrome (Gerlach et al., 1975; Parsons et al., 2009). Therefore, there is a critical need to develop more effective and safe treatment options.

The etiological basis of schizophrenia is thought to arise from dysregulated DA neurotransmission in mesocortical and mesolimbic pathways (Guillin et al., 2007; Meisenzahl et al., 2007). However, the poorly elucidated pathogenesis and failure of current therapeutics to treat the negative and cognitive symptom domains has encouraged a reappraisal of the role of the DA system in schizophrenia. Newer approaches highlight several neurochemical abnormalities in schizophrenia and suggest a relationship between DA and other neurotransmitter systems, including 5-HT, Gamma-Aminobutyric acid (GABA), glutamate (GLU), and acetylcholine (ACh; Laruelle et al., 2003; Meisenzahl et al., 2007; Seo et al., 2008; Tandon et al., 1991). Although a significant role is still attributed to DA in the pathophysiology of schizophrenia, it is suggested that there is an imbalance between DA and one (or more) of these neurotransmitter systems that lead to symptom manifestation.

The ACh system is a regulator of neuronal activity throughout the peripheral (PNS) and central nervous systems (CNS; Picciotto et al., 2012), and is proposed to contribute to the pathophysiology of schizophrenia resulting from either hyperactivation of the pedunculopontine-lateral dorsal tegmental nuclei (Yeomans, 1995) or an imbalance between cholinergic and dopaminergic systems (Tandon and Greden, 1989). The importance of disturbances in the DA-ACh balance in schizophrenia is supported by the finding that psychotic symptoms are exacerbated by the psychostimulant methylphenidate and can be reversed by physostigmine, an anticholinesterase that enhances cholinergic transmission (Janowsky et al., 1973). ACh signals through two classes of receptors: ionotropic nicotinic receptors (nAChRs) and metabotropic muscarinic receptors (mAChRs). As discussed in more detail below, mAChRs are G protein-coupled and signaling through either $G_{\alpha q}$ (M_1, M_3, M_5 subtypes) or $G_{\alpha i}$ (M_2, M_4 subtypes). In contrast, nAChRs function as excitatory cation channels and occur as either homomeric or heteromeric assemblies of a large family

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of alpha- (α2-α7) or beta- (β2-β4) subunits. Cholinergic neurotransmission plays a critical role in a variety of functions, including sensory perception, attention, sleep, motivation, reward, mood, and cognitive processing; therefore, it is not surprising that abnormalities in the cholinergic system are known to contribute to a number of psychiatric and neurological diseases (Bohnen and Albin, 2011; Mufson et al., 2008; Scarr et al., 2013).

Numerous clinical and preclinical findings suggest that disruptions in central nicotinic cholinergic transmission may be associated with the symptom manifestation in schizophrenia. Presynaptic nACh receptors have been long implicated in the regulation of striatal DA release (see Jones et al., 2012; Picciotto et al., 2012 for review), and participate in the induction of striatal long term depression (Partridge et al., 2002), suggesting an important modulatory role of nAChRs on DA release and behaviors. Furthermore, data from autoradiographic studies indicate reduced binding at both heteromeric and homomeric nAChRs in the striatum, hippocampus and cortex in schizophrenic patients compared to healthy controls (Jones et al., 2012). Polymorphisms in the core promoter of the α_7 gene (CHRNA7) are indicative of abnormalities in sensory motor gating, sustained attention and cognition in schizophrenia (Kalkman and Feuerbach, 2016). Interestingly, two compounds that are currently in clinical use galantamine (anticholinesterase inhibitor) and topisetron ($5HT_3$ antagonist) possess efficacy at α_7 and are beneficial in patients with schizophrenia (see Olincy and Freedman, 2013 for review), suggesting that the development of selective nAChR activators may lead to important therapeutic interventions. However, a detailed discussion about nAChR in the neuropathology of schizophrenia is beyond the scope of this review (see Dineley et al. 2015; Jones et al., 2012; Martin and Freedman, 2007l; Ripoll et al., 2004 for detailed review).

Evidence for the involvement of mAChRs in schizophrenia is supported by data from post-mortem, neuroimaging and neuropharmacology studies (Dean et al., 2003; Scarr and Dean, 2009). Furthermore, recent studies using mAChR knockout mice have provided valuable insight into the potential role of mAChRs in the physiopathology of schizophrenia and cognitive deficits (Anagnostaras et al., 2003; Dencker et al., 2012; Gerber et al., 2001; Woolley et al., 2009). mAChRs are critical in modulating the activity of cholinergic projections from the midbrain, which innervate DA pathways implicated in psychotic symptoms of schizophrenia, as well as brain regions that are relevant to cognitive function, such as learning, memory and attention (Berman et al., 2007). Thus, these receptors have been proposed to contribute to the pathophysiology of schizophrenia as a result of an imbalance between central cholinergic and dopaminergic systems. In this review, we highlight the involvement of M_1 and M_4 muscarinic cholinergic receptors in schizophrenia and review data that suggest they may be a viable therapeutic target.

1.2 Muscarinic Receptors

As discussed in more detail below, the muscarinic cholinergic system has been implicated in the pathophysiology of schizophrenia (Raedler et al., 2007; Scarr and Dean, 2008, 2009) and such abnormalities may be significant to both the psychotic symptoms and cognitive deficits. mAChRs belong to the superfamily of GPCRs that either activate or inhibit signaling pathway systems through activation of intracellular second messengers such as cyclic

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adenosine monophosphate (cAMP) or inositol triphosphate (IP_3; Caulfield, 1993; Felder, 1995). Molecular cloning strategies have revealed five distinct muscarinic receptors (M_1-M_5) that can be subdivided into two functional classes based on G-protein coupling and location (Bonner et al., 1987; Bonner et al., 1988; Liao et al., 1989).

Individual mAChR subtypes are preferentially coupled to distinct heterotrimeric G-proteins that are capable of modulating a wide variety of ion channels and other signaling proteins. The M_1, M_3 and M_5 receptor subtypes couple to G_q/G_{11}, leading to activation of phospholipase C and formation of inositol phosphates and other second messengers, which promote closure of potassium (K+) channels in many neuronal populations, thus facilitating cell excitability. The M_2 and M_4 receptor subtypes, on the other hand, are coupled to G_i/G_o, resulting in an inhibition of adenylyl cyclase and reduction in cAMP, promoting inhibition of voltage-gated calcium (Ca^{2+}) channels, thus often diminishing cell excitability (Caulfield, 1993). In addition to their canonical signaling pathways, cell expression studies have revealed that mAChRs are capable of activating multiple signal transduction pathways, such as receptor tyrosine kinases (Kuhne et al., 2015; Ockenga et al., 2014).

Muscarinic receptors are widely expressed in both the CNS and PNS with distinct cellular and tissue localization of individual subtypes. As the predominant subtype in the CNS, M_1 receptors are highly expressed in the cortex, striatum, and hippocampus, where they are postsynaptically localized on hippocampal pyramidal neurons and dentate granule cells (Levey et al., 1991; Marino et al., 1998). Due to the location of M_1 receptors in the hippocampus and medial prefrontal cortex (mPFC), M_1 receptor signaling is thought to be important for cognitive function and neural circuits disrupted in schizophrenia. In support of this hypothesis, M_1 knockout mice show deficits in tasks that are dependent on hippocampal-cortical and mPFC function (Anagnostaras et al., 2003; Gould et al., 2015). In contrast to the widespread postsynaptic localization of M_1 on pyramidal cells, the M_4 subtype is presynaptically localized at glutamatergic synapses, cholinergic interneurons and DA D_1 receptor-expressing spiny projection neurons (D_1-SPNs) within the striatum (Ince et al., 1997; Levey et al., 1991; Santiago and Potter, 2001). Together with initial studies of whole body M_4 knockout mice (Gomeza et al., 2001; Tzavara et al., 2004; Zhang et al., 2002), mice that selectively lack M_4 receptors in D_1-expressing neurons (D_1-$M_4^{-/-}$ mice) have demonstrated the important modulatory role of M_4 in DA-dependent behaviors and neurotransmission (Dencker et al., 2012; Jeon et al., 2010).

1.3 The muscarinic system and schizophrenia

Early insight into the role of the central muscarinic system in schizophrenia arose from investigations into polypharmacy. The administration of anticholinergic and antimuscarinic agents was once common practice in schizophrenics due to the capacity of these drugs to alleviate motor side effects (e.g. extrapyramidal symptoms) induced by typical antipsychotic medications (Tandon and Dequardo, 1995). However, it was also noted that treatment with anticholinergic drugs resulted in a worsening of neurocognitive impairment in schizophrenic patients (Johnstone et al., 1983; Singh et al., 1987). In addition to exacerbating existing symptoms in patients, antimuscarinic drugs also evoked a transient schizophrenia-like state (i.e., cognitive dysfunction and vivid multi-sensory hallucinations) in non-psychotic

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individuals (McEvoy, 1987; Perry and Perry, 1995; Potamianos and Kellett, 1982). Parallel to these findings in humans, administration of muscarinic antagonists, such as scopolamine, have been shown to induce psychomimetic-like effects in various animal models of schizophrenia, including reduced latent inhibition (LI), increased locomotor activity, and disruption of prepulse inhibition (PPI; Barak and Weiner, 2007, 2009; Furuie et al., 2013; Jones and Shannon, 2000). Postmortem, clinical imaging and genetic approaches have further implicated mAChR expression and function in the underlying pathophysiology of schizophrenia.

Several post-mortem radioligand-binding studies, using the mAChR antagonist [^{3}H]-pirenzepine, have demonstrated decreased M_1/M_4 expression in a number of cortical and subcortical regions in schizophrenic patients, including the hippocampus, PFC, striatum, and the anterior and posterior cingulate cortex (Crook et al., 2000, 2001; Dean et al., 2002; Dean et al., 2008). These findings are further supported by neuroimaging studies reporting reduced mAChR availability in unmediated schizophrenics (Raedler et al., 2003). Additionally, polymorphisms of the M_1 (CHRM1) and M_4 (CHRM4) receptor gene appear to be specific to schizophrenia (Scarr et al., 2013a; Scarr et al., 2013b), as patients with bipolar disorder and major depression have been reported to express normal levels of these receptors (Zavitsanou et al., 2004). It has been suggested that polymorphisms in CHRM1 and CHRM4 may represent distinguishable phenotypes within the syndrome of schizophrenia. For instance, compared to schizophrenic patients with heterozygous mutations, patients who have homozygous CHRM1 C267A nucleotide polymorphisms exhibit pronounced perseveration errors and responses on the Wisconsin Card Sorting Test, a neuropsychological measure of executive functioning and prefrontal lobe function (Cropley et al., 2015; Liao et al., 2003). It has been hypothesized that patients with decreased M_1 expression may exhibit more pronounced cognitive deficits compared to non-M_1-deficit patients (Scarr et al., 2009; Scarr et al., 2013a), however, no associations can be definitively made. Alterations in M_1 receptor binding and immunoreactivity have also been reported in autism (Perry et al., 2001a) and Alzheimer's disease (AD; Flynn et al., 1995; Shioaki et al., 2001), suggesting that this receptor plays an important role in cognitive function and actions at M_1 have been proposed to have cognition enhancing effects (Bymaster et al., 2003). Interestingly, polymorphisms in CHRM4 have not been reported in patients with cognitive disturbances (Yonan et al., 2013), further supporting the hypothesis that cognitive deficits are due to decreases CHRM1 in subjects with schizophrenia. Taken together these findings highlight the role of the central muscarinic system in the symptomology of schizophrenia.

1.4. Targeting Muscarinic Receptors for the Treatment of Schizophrenia

While atypical antipsychotics are the primary treatment for schizophrenia, they offer minimal benefit for cognitive dysfunction and negative symptoms. Therefore, novel therapeutic agents are necessary to adequately treat these other symptom domains. Acetylcholinesterase (AChE), which inhibits breakdown of acetylcholine, may be one such target. AChE inhibitors (AChEIs), such as tacrine, donepezil, physostigmine and galantamine, are currently used to treat AD, but some open-label studies suggest that these compounds may also ameliorate cognitive dysfunction, visual hallucinations and psychosis in schizophrenia (Ferreri et al., 2006; Friedman et al., 2002; Ribeiz et al., 2010; Rosse and

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Deutsch, 2002). Moreover, preclinical studies indicate that clinically used AChEIs have the ability to improve learning and memory in established rodent pharmacological models of pathological changes underlying schizophrenia (Kunitachi et al., 2009; Wang et al., 2007). However, the results from clinical trials with AChEIs in schizophrenic patients have been disappointing (Thakurathi et al., 2013), possibly owing to dose-limiting adverse effects caused by activation of peripheral receptors.

Xanomeline, an M_1/M_4 preferring agonist, has been shown to have positive effects on cognitive and psychotic-like symptoms (e.g., hallucinations, delusions) in AD (Bodick et al., 1997a; Bodick et al., 1997b; Veroff et al., 1998) and may hold therapeutic efficacy for the treatment of schizophrenia. In support of this notion, xanomeline displays robust antipsychotic-like efficacy in several rodent models that have been used to predict antipsychotic efficacy (Jones et al., 2005; Mirza et al., 2003; Perry et al., 2001b; Shannon et al., 2000; Stanhope et al., 2001). Schizophrenia patients show impaired PPI, which is mimicked in rodents following administration of the non-selective DA antagonist, apomorphine. In this pharmacological model of psychosis, administration of xanomeline reversed apomorphine-induced deficits in PPI similar to other clinically effective antipsychotics (Jones et al., 2005; Stanhope et al., 2001). Xanomeline also demonstrated antipsychotic-like efficacy in other preclinical assays including conditioned avoidance responding (CAR), amphetamine-induced hyperlocomotion (AHL), apomorphine-induced climbing, and amphetamine-induced deficits in LI (Jones et al., 2005; Shannon et al., 1999). In addition, xanomeline significantly attenuated deficits in models of cortical pathology associated with schizophrenia, such as novel object recognition (NOR) and contextual fear conditioning (Brown et al., 2014) and attenuated MK-801-induced disruptions in LI (Barak and Weiner, 2011), a model of cognitive and negative symptoms of schizophrenia. Taken together these studies suggest that M_1/M_4 selective agents may have clinical utility in positive, negative and cognition symptom domains of schizophrenia and thus warrant further investigation.

In the only proof of concept human clinical trial, xanomeline has been shown to improve positive, negative and cognitive symptom domains in schizophrenia patients (Shekhar et al., 2008). Interestingly, xanomeline treatment was superior to that of traditional antipsychotic agents (e.g., the DA D_2 antagonist haloperidol), and significant antipsychotic effects were documented within the first week. This clinical trial highlights not only xanomeline's potential, but that of M_1/M_4 receptor agonists to serve as an alternative treatment in schizophrenia. Although xanomeline displayed efficacy in improving cognition and reducing negative and psychotic symptoms of schizophrenia, its clinical utility was limited due to adverse side effects elicited by its agonism of peripheral M_2 and M_3 receptors (Bymaster et al., 2003).

1.5 Allosteric Modulators of Muscarinic Receptors

Accordingly, efforts have been made to specifically target M_1 or M_4 receptors to retain therapeutic efficacy while minimizing the adverse side effects (Foster et al., 2014; Foster et al., 2012; Jones et al., 2012). mAChRs have an orthosteric binding site for natural or exogenous agonists that is highly conserved among individual receptor subtypes, making it

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difficult to develop subtype-selective ACh site ligands. To circumvent this problem, an approach of targeting allosteric binding sites that are topographically distinct from the orthosteric site and less conserved across receptor subtypes has been developed (Bridges et al., 2010; Christopoulos, 2002; Conn et al., 2009a; Digby et al., 2010; Foster and Conn, 2017; Nickols and Conn, 2014; Wenthur et al., 2014). Allosteric modulators possess high subtype selectivity and can either activate the receptor by themselves or modulate receptor activation by ACh. Allosteric activators can include allosteric agonists, which act at a site removed from the orthosteric site to directly activate the receptor in the absence of ACh, or positive allosteric modulators (PAMs), which do not activate the receptor directly but potentiate activation of the receptor by the endogenous orthosteric agonist ACh (Conn et al., 2009a; Conn et al., 2009b; Marlo et al., 2009). It is also possible for a single molecule to have both allosteric potentiator and allosteric agonist activity. Since allosteric mechanisms are governed by both affinity and cooperativity factors, it presents practical implications and challenges for drug discovery (Conn et al., 2009).

Compounds that possess an allosteric mode of action can display a number of advantages over orthosteric ligands as potential therapeutic agents. For example, allosteric modulators that do not display any agonism are quiescent in the absence of endogenous orthosteric activity and only exert their effect in the presence of the released orthosteric agonist (Conn et al., 2009a; Conn et al., 2014; Foster and Conn, 2017; Lindsley et al., 2016; Lutjens and Rocher, 2017). A key advantage of allosteric modulators is that their modulation is in concert with the temporal and spatial organization of physiological receptor activation (Conn et al., 2009a). Another advantage stemming from allosteric modulators is that their effect is given by the factor of cooperativity with orthosteric ligands that dictates a maximal degree of interaction of binding both agents, thus imposing a “ceiling” on the magnitude of allosteric effect (May et al., 2007). Together, these properties may reduce the side effect potential relative to orthosteric agonists, which stimulate a given receptor independently of its physiological state. The possibility of limiting side effects in the treatment of schizophrenia is obviously of significant importance given the current adverse side effect profile of current therapies (Ucok and Gaebel, 2008). As discussed below, the discovery of subtype-specific allosteric modulators has greatly advanced our understanding of the physiological role of various muscarinic receptor subtypes in brain regions important for schizophrenia and have emphasized the potential utility of M_1 and M_4 mAChR subtypes as targets for the development of novel treatments for this disorder.

Species differences among receptors can cause variability in the response between an orthosteric and an allosteric ligand (Wootten et al. 2013), presenting a challenge for drug discovery. Allosteric sites are less evolutionarily conserved across receptor subtypes (Conn et al., 2009) and amino acid residues in allosteric sites may be subject to change the allosteric behavior of enzymes in different species (Hines et al. 2007). Therefore, it is possible that compounds that are identified using human GPCR cell lines do not produce desired effects when tested *in vivo* in animal models, which may be due to lack of cooperativity with the endogenous agonist or pharmacokinetic limitations. Recently, Suratman and colleagues (2011) discovered that the M_4 PAM LY2033298 (discussed in more detail below) displayed differences at rodent and human M_4 receptors due to cooperativity factors and probe-dependence (the need to co-administer an orthosteric agonist

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with an allosteric modulator to provide sufficient tone). When possible, it is advantageous to select for allosteric ligands that do not display pronounced species bias (Conn et al., 2014; Lindsley et al., 2016).

1.6 Animal Models of Schizophrenia

While this review focuses on allosteric modulators of M_1 and M_4 for symptom management in schizophrenia, it is important to keep in mind that current pharmacological animal models of schizophrenia are not intended to serve as the complete equivalent of the human disorder, but rather are designed to test specific causative or mechanistic hypothesis (Jones et al., 2011; Marcotte et al., 2001). A common approach for developing animal models has been to use drug-induced states that produce schizophrenic-like symptoms in nonschizophrenic individuals. In rodents, administration of dopaminergic stimulants (i.e, amphetamine) elevate locomotor activity and impair PPI, which is thought to mimic the hyperdopaminergic tone observed in schizophrenic patients. Measures of locomotor hyperactivity are useful for providing a functional measure of the antidopaminergic activity of neuroleptics. Although dopaminergic psychostimulants provide a model of psychosis, it does not accurately mimic the cognitive or negative symptom domains (Pratt et al., 2012). In contrast, N-methyl-D-aspartate (NMDA) receptor antagonists generate a more complete model of schizophrenia, including aspects of the positive, negative and cognitive symptoms (Marcotte et al., 2001; Nabeshima et al., 2006). While pharmacological models may never be able to accurately mimic symptom domains observed in schizophrenic patients, they still provide valuable insight into the neurobiological mechanisms (Steeds et al., 2015) and to facilitate the development of improved therapeutics. In addition to pharmacological models, genetic models of schizophrenia based on human mutations have been established (Nestler and Hyman, 2010). Therefore, novel therapeutics should be assessed in translational assays with high construct validity and genetic models.

1.7 M_1 Positive Allosteric Modulators (PAMs)

Of the five mAChR subtypes, the M_1 receptor is viewed as the most important for memory and attention mechanisms. Due to the postsynaptic localization of M_1 on cholinergic projections to the PFC and colocalization with NMDA receptors in the hippocampus (Levey et al., 1991; Marino et al., 1998), these receptors have long been a target for the treatment of cognitive deficits in schizophrenia. In rodents, activation of M_1 has been shown to increases synaptic excitation of pyramidal cells in the mPFC (Shirey et al., 2009) and potentiate CA1 hippocampal pyramidal cell firing (Buchanan et al., 2010), a physiological response associated with learning and memory. In support of this hypothesis, M_1 knockout mice show deficits in tasks that require mPFC function (e.g., non-match to sample working memory and consolidation; Anagnostaras et al., 2003), lack the ability of the cholinomimetic carbachol to induce long-term potentiation (LTP) in the hippocampus (Buchanan et al., 2010; Hamilton and Nathanson, 2001), and have reduced expression of extracellular signal-regulated kinase (ERK 1 and 2) in the hippocampus, a protein involved in synaptic plasticity (Berkeley et al., 2001).

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In addition to regulating cognition, M_1 has been shown to modulate DA signaling. M_1 knockout mice have elevated levels of extracellular DA in the striatum and increased basal and amphetamine-induced locomotion (Gerber et al., 2001), indicating an inhibitory role for M_1 control of subcortical dopaminergic transmission. M_1 receptors are highly expressed in both striatonigral and striatopallidal medium spiny neurons (MSNs; Yan et al., 2001). Through coordinated modulation of potassium and calcium channels (Ben-Ari et al., 1992; Perez-Burgos et al., 2010), M_1 can shape the synaptic integration and spiking activity in MSNs. Correspondingly, muscarinic agonists, particularly those with M_1-preferring activity (e.g., AC260584, sabcomeline, xanomeline) have been shown to acutely stimulate DA efflux within the PFC and striatum (Li et al., 2007; Li et al., 2008). Data from neuroimaging studies have demonstrated that frontal and striatal DA release is critical for working memory representations and behavioral flexibility, respectively (Cools and D'Esposito, 2011; Frank et al., 2001). In schizophrenia it is hypothesized that DA hypofunction may contribute to cognitive and negative symptom manifestation, thus agents that enhance DA transmission in mesocortical pathways, such as M_1, may possess antipsychotic efficacy.

Remarkable progress has been achieved in the discovery of highly selective M_1 PAMs that provide tools to further understand the contributions of M_1 to the preclinical and clinical efficacy of xanomeline. BQCA (benzyl quinolone carboxylic acid), a second generation M_1 PAM which potentiates responses to ACh in CHO cell lines expressing rhesus, dog, rat and mouse M_1 (Ma et al., 2009), was found to express antipsychotic drug-like qualities and produce pro-cognitive responses, including enhancing memory function and increasing spontaneous prefrontal brain activity in rodent models (Chambon et al., 2012; Gould et al., 2015; Ma et al., 2009; Shirey et al., 2009). The dissociative anesthetic phencyclidine (PCP) and MK-801 are noncompetitive NMDA receptor antagonists suggested to be validated pharmacological model of all symptom domains of schizophrenia (Steeds et al., 2015). Recently, it was found that BQCA can attenuate deficits induced by MK-801 and potentiate the effects of atypical, but not typical, antipsychotics in a Y-maze test, a short-term spatial memory paradigm for assessing hippocampal-dependent memory function (Choy et al., 2016). An analog of BQCA, PQCA (1-((4-cyano-4-(pyridine-2-yl) piperidin-1-yl) methyl-4-oxo-4 H-quinolizine-3-carboxylic acid) demonstrated robust efficacy in rodent and non-human primate cognition assays (Lange et al., 2015). Taken together these results provide preclinical insights into M_1 PAMs' procognitive effects as well as synergic effects with atypical antipsychotics.

Further behavioral effects of M_1 receptors have been characterized through use of second generation M_1 PAMs, such as VU0453595, PF-06767832 and VU6004256. Behavioral deficits following administration of PCP to mice tested in social interaction and NOR were reversed following acute treatment with VU0453595 (Ghoshal et al., 2016). Recent findings indicate that M_1-mediated plasticity in the PFC is highly dysregulated following acute or chronic NMDA receptor blockade (Ghoshal and Conn, 2015; Thomases et al., 2014). Namely, acute administration of NMDA antagonists lead to a tonic excitation of PFC neurons (Homayoun and Moghaddam, 2007; Ninan and Wang, 2003), and this aberrant plasticity occurs in conjunction with cognitive deficits in animal models (Blot et al., 2013). The M_1 PAM VU0453595 was found to restore muscarinic LTD (mLTD), thus providing evidence that loss of M_1-mediated mLTD at the hippocampal-PFC synapse contributes to the

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increased activation of the PFC and cognitive impairments (Ghoshal et al., 2016). Additional evidence suggests that activation of M_1 triggers release of an endocannabinoid leading to CB_1 mediated depression of GLU transmission (Martin et al., 2016); however, how this form of LTD correlated with mPFC dependent learning and function is still speculative. At present, very few studies have focused on understanding the cellular mechanisms underlying mLTD in the PFC (Caruana et al., 2011; Ghoshal et al., 2016; Scheiderer et al., 2008). It has also been suggested that the loss of mLTD could be related to a dysfunction in the muscarinic regulation of GABAergic neurotransmission in the PFC (Yi et al., 2014); however, future studies need to conducted to test this possibility.

In addition, PF-0676832 was found to attenuate learning and memory deficits induced by the anticholinergic scopolamine in the Morris water maze (MWM) assay (Davoren et al., 2016) and VU6004256 was shown to ameliorate cognitive abnormalities in a genetic mouse model of a global reduction in the NR1 subunit of the NMDA receptor (Grannan et al., 2016). These studies support the idea that M_1 activation may have a critical role in mPFC-dependent cognitive functions and suggest that M_1 allosteric activators may serve as a novel approach for the treatment of PFC deficits observed in schizophrenic patients.

More recently studies are investigating whether M_1 activation could potentially provide antipsychotic effects. Davoren and colleagues (2016) tested PF-06767832 in amphetamine-induced disruptions in PPI of acoustic startle, a preclinical model of sensorimotor gating deficits observed in schizophrenia. Interestingly, administration of PF-06767832 was found to significantly block amphetamine-induced deficits in PPI. Although well tolerated in rodents, PF-06767832 was poorly tolerated in dogs as evidenced by dose-dependent cholinergic signs, such as salivation, watery stool, ataxia and convulsions (Davoren et al., 2016), which may limit clinical utility. M_1 activation is generally not thought to be associated with GI adverse effects; however, these results are consistent with toxicology data on BQCA, PQCA, and a related analog (Alt et al., 2016). The M_1 mechanism is associated with convulsions (Cruickshank et al., 1994), most likely due to unbound plasma values and agonist activity (Davoren et al., 2016; Rook et al., 2017). To compliment these findings, the highly potent M_1 PAM VU6004256 was found to attenuate spontaneous hyperlocomotion in a genetic model of NMDA hypofunction (Grannan et al., 2016), suggesting that M_1 modulation in cortical and limbic regions may contribute to antipsychotic-like effects of xanomeline.

The dopaminergic system has been shown to have an excitatory influence on the hippocampal-PFC pathway (Bernardi et al., 1982; Li et al. 2015). Activation of the mesocortical DA system at a frequency that leads to DA overflow causes a long-lasting enhancement in the magnitude of hippocampal-PFC tetanic LTP *in vivo* (Jay et al., 1995) and depletion of DA in the PFC has the opposite effect (Gurden et al., 1999). The exact physiological roles of M_1 on DA release still remains to be illuminated. Therefore, future studies should determine the modulatory role of M_1 dependent DA release and behaviors through optogenetic techniques. Together with multiple studies demonstrating robust effects of M_1 PAMs on cognitive function, these studies support the exciting possibility that highly selective M_1 PAMs may provide a novel approach for reducing symptomology associated with changes in cortical plasticity in schizophrenia patients. Thus, it will be critical to

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advance M_1 PAMs into clinical development that has robust actions on M_1-mediated responses in PFC neurons and in MSNs.

1.8 M_4 Positive Allosteric Modulators (PAMs)

The M_4 receptor is believed to play a crucial role in the antipsychotic properties of the muscarinic agonist xanomeline (Bymaster et al., 2003; Woolley et al., 2009). M_4 receptors are abundantly expressed in the striatum (Hersch et al., 1994), and are co-expressed with DA D_1 receptors on SPNs (Ince et al., 1997; Santiago and Potter, 2001), suggesting that M_4 is ideally located to modulate dopaminergic signaling. All currently approved antipsychotics that are efficacious for the management of positive symptoms act to reduce DA transmission (Howes et al., 2009). This is significant as there is evidence that links M_4 receptor activation with dopaminergic regulation, and that atypical antipsychotic medications may act was M_4 receptor agonists (Brady et al., 2008; Jeon et al., 2010; Tzavara et al., 2004; Wess, 2004). In preclinical studies, mAChR agonists with partial M_4 selectivity exert antipsychotic-like efficacy in animal models of psychosis (Bymaster et al., 1998; Thomsen et al., 2010; Watt et al., 2013) and these behavioral effects are absent in M_4 knockout mice (Dencker et al., 2011). Additional evidence for the involvement of M_4 in modulating the activity of the central dopaminergic comes from whole body M_4 knockout mice, that display enhanced hyperlocomotor activity and increased behavioral sensitization following treatment with psychostimulants (Gomeza et al., 2001; Koshimizu et al., 2012; Tzavara et al., 2004; Zhang et al., 2002). Moreover, the antipsychotic-like effects of xanomeline are absent in mice lacking the M_4 mAChR in D_1 DA receptor expressing cells (D_1-M_4 knockout mice; Jeon et al., 2010). Taken together, these findings support the hypothesis that M_4 mAChRs represent a viable drug target for the treatment of schizophrenia.

An important breakthrough for M_4-selective compounds occurred with the discovery of the first generation allosteric agents, VU0010010 and LY2033298 (Chan et al., 2008; Shirey et al., 2008). These agents do not directly activate M_4, rather they serve as allosteric potentiators that increases responses of the receptor to ACh. In brain slices, VU0010010 selectively potentiated mAChR-mediated reductions in excitatory, but not inhibitory, synapses in hippocampal neurons, indicating a key role for M_4 in regulating hippocampal function. These findings validated the functional activity of M_4 PAMs *ex vivo* and lead to the chemical optimization of future compounds (Shirey et al., 2008). Unlike VU0010010, LY2033298 possessed physiochemical properties suitable for *in vivo* dosing and was found to potentiate the behavioral effects of the nonselective mAChR agonist oxotremorine in animal models of psychosis, such as CAR and PPI, and modulate DA release in the PFC. Additionally, the effects of LY2033298 were significantly attenuated in M_4 knockout, indicating the critical role of the M_4 receptor in governing antipsychotic-like effects (Chan et al., 2008; Leach et al., 2010). However, LY2033298 does not provide an optimal tool compound for rodent studies in that it has relatively low potency at the rat M_4 mAChR (Chan et al., 2008; Leach et al., 2010) and displays only weak cooperativity with ACh, the endogenous agonist of M_4 (Suratman et al., 2011).

The scaffolds of VU0010010 and LY2033298 have led to the advancement of M_4 PAMs with central penetration and suitable pharmacokinetic properties for preclinical studies. New

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generations of M_4–selective PAMs, such as VU1052100 and VU0467154, have demonstrated robust effects, similar to those seen with xanomeline and the atypical antipsychotic clozapine, in multiple animal models of psychosis (Brady et al., 2008; Bubser et al., 2014; Byun et al., 2014; Chan et al., 2008; Foster et al., 2016). VU0467154 is a highly valuable rodent *in vivo* tool compound with excellent physiochemical properties (reviewed in Wood et al., 2016). M_4 PAMs have been shown to attenuate amphetamine-induced increases in extracellular DA in the striatum and nucleus accumbens (Byun et al., 2014). Taken together with data from D_1-$M_4^{-/-}$ mice depicting increased DA efflux in response to psychotomimetics (Jeon et al., 2010), it is hypothesized that activation of M_4 on D_1 SPNs may provide feedback control on basal and evoked DA release in the striatum.

Studies using fast scan cyclic voltammetry (FSCV) have demonstrated that D_1-$M_4^{-/-}$ mice lack sustained reductions in striatal DA release seen in littermate controls and antipsychotic-like effects following administration of M_4 PAMs and xanomeline (Dencker et al., 2011; Foster et al., 2016), suggesting that M_4 expressed on D_1-containing neurons mediate these effects. One proposed mechanism for these effects is that activation of M_4 on D_1-containing MSNs leads to decreased GABA release from nerve terminals via a multisynaptic mechanism. This inhibition is thought to underlie the antipsychotic-like profile of M_4 PAMs as well as atypical antipsychotics with M_4 selectivity (Mirza et al., 2003; Olianas et al., 1999; Stanhope et al., 2001), however, this model remains to be rigorously tested. Another possible mechanism supported by Foster et al. (2016) posits that M_4 activation induces release of a local messenger that acts on neighboring DA terminals to inhibit DA release. In support of this notion, M_4-mediated effects on DA release are blocked by a CB_2 endocannabinoid (eCB) receptor antagonist, absent in CB_2 knock out mice, and are occluded by inhibition of the eCB synthetic enzyme diacylglycerol lipase (Foster et al., 2016). Taken together, these data suggest that the effects of M_4 PAMs on DA release in the striatum are mediated, at least in part, by activation of CB_2 receptors, possibly expressed on neighboring DA terminals.

In addition to displaying antipsychotic-like properties, M_4 PAMs have been reported to display cognition enhancing properties in associative learning paradigms. Administration of VU0467154 improves the acquisition of both contextual and cue-mediated fear conditioning and reverses stimulant-induced deficits in learning and memory (Bubser et al., 2014). Interestingly, it has recently been reported that M_4 PAMs improve memory of rodents that perform poorly at baseline, more so than an M_1 allosteric agonist BQCA (Galloway et al., 2014), suggesting that M_4 PAMs may offer improvement to the cognitive symptoms of schizophrenia. Recent physiology studies have revealed that activation of presynaptic M_4 receptors decreases GLU release from excitatory terminals in the hippocampus (Shirey et al. 2008) and from corticostriatal terminals (Pancani et al., 2015), suggesting that M_4 is the primary mAChR mediating cholinergic inhibition of excitatory transmission in these brain regions. Psychotomimetic agents like the MK-801 enhance spontaneous firing at glutamatergic synapses within the mPFC and induce cognitive impairments (Blot et al. 2015; Wang and Gao, 2012). These data raise the possibility that M_4-PAMs could reverse MK-801-induced deficits in cognition that may involve actions at excitatory synapses, including corticostriatal terminals to normalize the function of overactive excitatory

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projections from layer V pyramidal cells of the mPFC to the striatum; however, additional studies are needed to confirm the role of M_4 in modulating cognitive function.

While these results are exciting, the potential of M_4 PAMs as clinical candidates has been hindered by a significant species disconnect (35x less potent at human M_4; Wood et al., 2017). Due to the disconnect between rodent and human M_4 receptors, it lead to the discovery of a potent, selective, and orally bioavailable M_4 PAM (VU0467485) that displayed robust efficacy in hyperdopaminergic states and NMDA hypofunction (Wood et al., 2016). Excitingly, VU0467485 is the first potent M_4 PAM to overcome major species differences in potency while maintaining high selectivity (Wood et al., 2016), however, further advancement was halted due to solubility issues.

1.9 Conclusions

Significant progress has been made in terms of our scientific understanding of the neurochemical origins of the symptoms of schizophrenia. From the evidence reviewed here, it is apparent that an abnormal central muscarinic system contributes to positive, negative and cognitive symptom domains of schizophrenia. These observations combined with the positive clinical data observed with xanomeline suggest that M_1/M_4 activity warrant further investigation as potential therapeutic options for schizophrenia. Major attention has been focused on developing highly selective allosteric modulators to use as research tools to achieve a better understanding of the exact role of these receptor subtypes in schizophrenia. Excitingly, new generations of M_1 and M_4 PAMs have demonstrated efficacy in preclinical assays that predict antipsychotic-like and cognition enhancing effects, suggesting these compounds may be beneficial for symptom management in schizophrenic patients. However, additional studies are needed to further understand the effects of these compounds in preclinical models of negative symptoms.

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List of Abbreviations

GPCR	G-protein coupled receptor
DA	dopamine
5-HT	serotonin
GABA	γ-amino butyric acid
Acetylcholine	ACh
mAChRs	muscarinic acetylcholine receptors

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CNS	central peripheral nervous system
MSNs	medium spiny neurons
PNS	peripheral nervous system
PFC	prefrontal cortex
mPFC	medial prefrontal cortex
D_1-SPNs	D_1 spiny projection neurons
LI	latent inhibition
PPI	prepulse inhibition
AChEIs	Acetylcholinesterase inhibitors
CAR	conditioned avoidance responding
AHL	amphetamine-induced hyperlocomotion
NOR	novel object recognition
PAMs	positive allosteric modulators
NMDA	N-methyl-D-aspartate
LTP	long-term potentiation
ERK1/2	extracellular signal-regulated kinase
PCP	phencyclidine
mLTD	muscarinic long term depression
MWM	Morris water maze
FSCV	fast scan cyclic voltammetry
eCB	endocannabinoid

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Highlights

- Allosteric modulators demonstrate unique mechanisms of action and high subtype selectivity.
- M_1 PAMs have procognitive effects and have efficacy in some models of negative symptoms.
- M_4 PAMs exhibit antipsychotic efficacy via influence on dopaminergic signaling.
- M_1 and M_4 activators may provide novel therapeutic approaches with minimal adverse side effects.