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Muscarinic Receptors in Psychiatric Disorders – Can We Mimic 'Health'?

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Muscarinic receptors · Schizophrenia · Bipolar disorder · Depression · Signaling pathways

Abstract
The concept that acetylcholine is involved in the pathophysiologies of psychiatric disorders has existed since the 1950s. There is very strong evidence implicating a dysfunctional muscarinic system in schizophrenia, +with less information available for bipolar disorder and major depressive disorder. The translation of this evidence into clinically viable treatments has been disappointing; hampered by problems associated with developing drugs that target the requisite members of the muscarinic family, rather than all of the receptors, which results in unacceptable side-effect profiles. The discovery of additional binding sites, other than the one occupied by acetylcholine, has revitalised research into this aspect of psychopharmacology. New compounds are now being developed that have the potential to selectively target individual muscarinic receptors in the central nervous system. The question that remains to be answered is whether stimulating central muscarinic receptors will result in the re-establishment of normal central muscarinic activity? The purpose of this review is to (i) summarise the data supporting a role of the muscarinic system in schizophrenia, bipolar disorder and major depressive disorder, and (ii) give an overview of some of the new selective muscarinic ligands that are currently in development and try to address the issue of re-establishing appropriate central muscarinic function.

Acetylcholine is a phylogenically old chemical, present in both primitive plants and bacteria [for review, see 1]. Thus, it is not surprising that this neurotransmitter plays a major role in a number of functions mediated by the central nervous system (CNS), particularly information processing and the basic principles of cognition: learning and memory [2]. In the primate CNS, the cholinergic system consists of three major components: (i) the projections from the basal forebrain, in particular the medial septal nucleus, the nucleus basalis of Meynert, the vertical nucleus of the diagonal band and the horizontal limb of the diagonal band nucleus which innervate the hippocampus, most cortical regions and some subcortical nuclei [3]; (ii) the pedunculopontine-lateral dorsal tegmental projections, which innervate the thalamus as well as the midbrain and brainstem, and (iii) interneurons, particularly in the striatum but also those in the nucleus ac-

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cumbens [for review, see 4]. Acetylcholine mediates its effect through two families of receptors [5]: the ionotropic nicotinic receptors, which are heteromeric (α2–α6 and β2–β4 or α7 with α9 or α10) or homomeric (α7–α10) pentamers, for which 12 subunits have been identified [6], and the metabotropic muscarinic receptors (CHRMs). It is the latter family of receptors that this review will focus on, in particular their potential roles in the pathophysiology of psychiatric disorders and whether targeting these receptors, mimicking their downstream effects is an achievable pharmacological objective. However, it should be noted that this review is not intended to be taken in isolation; given the highly interactive nature of central nicotinic and muscarinic systems [7], it is extremely unlikely that only one arm of the cholinergic system is affected in psychiatric disorders.

Muscarinic Receptors

Muscarinic receptors are part of the superfamily of G protein-coupled receptors (GPCRs), more specifically, they are part of the family A typified by rhodopsin [8]. There are five CHRMs, M1–M5, products of different genes on different chromosomes, with distinct CNS distributions and functions [9]. The M1, M3 and M5 receptors preferentially couple to $G\alpha_{q/11}$ proteins, stimulating phospholipase C (PLC) activation and the subsequent mobilization of intracellular calcium. By contrast, M2 and M4 preferentially couple to $G\alpha_{i/o}$ proteins, inhibiting adenylyl cyclase and reducing intracellular levels of cAMP [10]. In addition, the M1, 3 and 5 receptors are generally considered to be postsynaptic in their localisation, whilst the M2 and M4 receptors can be both pre- and postsynaptic, depending on the brain region studied [for overview, see 11]. Despite being the product of distinct genes, there is a high degree of homology between the CHRMs, particularly in regions thought to contribute to the binding site of acetylcholine, the orthosteric site, hindering efforts to develop receptor-specific ligands [12]. Thus, much of our knowledge regarding the central effects of the CHRMs has come from the study of mice genetically modified to be null for a specific CHRM or combination of CHRMs rather than more traditional pharmacological studies [for review, see 13]. However, to date, there has been no distinction made between the functions of astrocytic [14, 15] and neuronal muscarinic receptors.

Mice lacking the CHRM1 exhibit normal hippocampal-mediated learning and memory [16, 17]; however, they show deficits in paradigms that are thought to require interactions between the hippocampus and cortex [18], proposed to be analogous to working memory. In addition, $CHRM1^{-/-}$ animals have elevated striatal dopamine levels and show increased locomotor activity, both at baseline and in response to amphetamine [16, 19]. Likewise, $CHRM4^{-/-}$ animals have increased locomotor activity, both basally [20] and in response to dopaminetics [21], as well as increased basal levels of dopamine in the nucleus accumbens [22] and increased basal hippocampal acetylcholine levels [23]. Similarly, mice lacking CHRM2 have altered acetylcholine homeostasis in the hippocampus [23]. In addition, CHRM2 is involved in centrally mediated antinociception [21], whilst CHRM3 regulates food intake and appetite [24]. Finally, CHRM5 plays a significant role in the reward pathway underpinning addiction [25].

Acetylcholine and Psychiatric Disorders

The role of acetylcholine in psychiatric disorders has been theorized about for a number of decades. In schizophrenia, modulating the cholinergic system to induce coma using atropine [26] or seizures using acetylcholine [27] were among the early forms of pharmacological interventions, predating any neurochemical hypotheses for the pathophysiology of the illness. Later, it was theorized that the positive symptoms (hallucinations, delusions) of schizophrenia were associated with hypoactivity of cholinergic systems within the CNS, whilst negative symptoms (apathy, lack of emotion, social withdrawal) of the disorder were thought to be due to increased muscarinic activity [28]. More recently, it was proposed that CHRM1 agonists might prove to be beneficial in improving the cognitive deficits (disorganised thoughts, problems with attention and/or memory) seen in patients with schizophrenia. Furthermore, it was suggested that both CHRM1 and CHRM4 agonists might prove to have antipsychotic efficacy – providing a novel approach to managing the positive symptoms of the disorder [29].

The proposal of a role for the cholinergic system in affective disorders initially arose from the administration of a cholinesterase (the enzyme responsible for the catabolism of acetylcholine) inhibitor to patients with schizophrenia, manic depression (bipolar disorder, BD) or no psychiatric disorder. Patients with manic depression generally showed improvements in their mania but a worsening of their depressive symptoms. In addition, the non-psychiatric controls developed symptoms char-

acteristic of depression [30]. The worsening of depressive symptoms was supported by the observation that people exposed to insecticides containing organophosphates (which are also cholinesterase inhibitors) had an increased incidence of depressive symptoms, with the psychiatric effects persisting for up to 6 months after exposure ceased [31]. Similarly, the findings of improved mania was later supported by a small study showing the acetylcholinesterase inhibitor, physostigmine, diminished manic symptoms [32], with some of the patients becoming depressed after treatment. These data formed the basis of the cholinergic-adrenergic hypothesis of mania and depression, which proposes that affective disorders are due to an imbalance between central cholinergic and adrenergic systems. It is proposed that during depression the cholinergic system is dominant and the adrenergic system dominant during mania [33]. Further support for a role of muscarinic receptors in major depressive disorder (MDD) comes from a pilot study where 18 patients with either recurrent MDD or BD completed a double-blind, placebo-controlled crossover trial with scopolamine [34]. The design of the trial dictated that all of the patients received scopolamine at some stage, those who received placebo initially only showed improvements in their depression and anxiety ratings after the crossover to scopolamine. Patients who were in the scopolamine arm initially continued to have improved depression and anxiety ratings during the placebo arm. Whilst these results are encouraging, with 10 of the 18 patients reported as achieving remission (MADRS score $\leq$10), the number of patients is small and reports of adverse events were higher in the scopolamine arms than in placebo. However, the study highlights the potential for reducing depressive symptoms via modulation of the muscarinic system.

Muscarinic Receptors and Psychiatric Disorders

Most of the direct data supporting a pathophysiological role of muscarinic receptors in psychiatric disorders has come from studies of the human CNS, in the form of neuroimaging or post-mortem studies. Although these research efforts have been hampered by the lack of specific ligands for the CHRMs, there is a significant body of evidence to suggest that CHRMs are involved in the pathophysiology of schizophrenia and some evidence suggesting they play a role in the pathophysiology of MDD and BD.

Muscarinic Receptors in Schizophrenia

The initial post-mortem investigations into the involvement of muscarinic receptors in schizophrenia were conducted as membrane-binding studies using the ligand [^{3}H]quinuclidinyl benzilate (QNB), which binds to all 5 muscarinic receptors [35]. The outcomes of these studies were disparate, with an initial report of no change in muscarinic receptor density in schizophrenia in one cohort but a decreased density of binding in a smaller, follow-up cohort [36]. Later studies, on subjects with schizophrenia who were not on medication at the time of death, reported increases in binding density in the caudate, but not the putamen [37], as well as the caudate and orbito-frontal but not medial frontal cortices [38]. The latter study also reported a decreased affinity for the radioligand in tissue from subjects with schizophrenia.

Whilst the different outcomes of these early studies were not encouraging, the development of alternative techniques and more selective ligands for the muscarinic receptors revitalised research in this field. This work has been conducted by a number of research groups, with tissue samples sourced from America, England and two separate sites in Australia. Much of this work has been carried out using the ligand [^{3}H]pirenzepine which binds to both CHRM1 and CHRM4 [39]. We and others have shown that there are widespread, significant decreases in the density of [^{3}H]pirenzepine binding in tissue obtained from subjects with schizophrenia compared to the levels seen in tissue from control subjects. A lot of work has focussed on the cortex, in part because cortical regions are strongly implicated in the pathophysiology of the disorder [40]. Thus, [^{3}H]pirenzepine-binding density has been shown to be decreased in the frontal cortex [41, 42], dorsolateral prefrontal cortex [42, 43], the anterior cingulate cortex [44], superior temporal cortex [45], and the hippocampus [46, 47]. Studies in subcortical areas have also reported decreased [^{3}H]pirenzepine-binding density [48, 49]. To date, the only region where [^{3}H]pirenzepine-binding density is unchanged in schizophrenia is the parietal cortex [43].

Given this systematic decrease in [^{3}H]pirenzepine-binding density, it is notable that similar patterns are not seen with ligands selective for other CHRMs. The binding density of [^{3}H]AF-DX 384, which binds to both CHRM2 and CHRM4, is decreased in the caudate putamen [50] but unchanged in cortical regions [45, 51, 52]. The only study that looked at [^{3}H]4-DAMP, which binds to CHRM1 and CHRM3, reported no change in the frontal cortex [41]. This pervasive decrease in the density of muscarinic receptors reported from post-mortem studies

has been substantiated by single photon emission computed tomography (SPECT) imaging study in patients with schizophrenia who were off antipsychotic medication at the time of the study, using [^{123}I]QNB [53]. The SPECT study showed decreased muscarinic receptor availability in all brain regions, except the pons and the investigators made particular comment on the extensive nature of this decrease.

Is it possible to ascribe these decreases to specific CHRMs despite the non-selective nature of the ligands used in these studies? In our hands, we consider cortical [^{3}H]pirenzepine binding to be a surrogate measure of CHRM1s for the following reasons: (1) [^{3}H]pirenzepine has a higher on-rate for CHRM1 compared to CHRM4, thus with short incubation periods [^{3}H]pirenzepine shows a 75% selectivity for CHRM1 [11], and (2) the ratio of CHRM1 to CHRM4 is high in human cortical regions [54]. This hypothesis is supported by studies investigating the expression of human muscarinic receptors by measuring levels of mRNA and protein, which show deceased cortical expression of CHRM1 [43, 55] but not CHRM4 [43], CHRM2 or CHRM3 [56] in tissue from subjects with schizophrenia. The exception to this theory is the hippocampus, where the decrease in [^{3}H]pirenzepine-binding density is accompanied by a decrease in mRNA for the CHRM4 [46]. This suggests that the affected member of the muscarinic receptor family may be dependent on the region being studied, adding a new level of complexity to our understanding of the pathophysiology of schizophrenia.

A recent, large study showed that the decreased cortical CHRM1 densities are restricted to a sub-group of people with schizophrenia [57]. This sub-group (muscarinic receptor deficit schizophrenia) comprises approximately 25% of the subjects with schizophrenia and the decrease in CHRM1 could not be accounted for by any known factors such as duration of illness, medication history or suicide. This is the first report of the syndrome of schizophrenia being separated on the basis of a biochemical marker, raising the likelihood that this group will have a different pathophysiology to other people with schizophrenia who do not have a deficit in cortical CHRM1 levels. The ability to separate the syndrome of schizophrenia into subgroups with distinct pathophysiologies may, in turn, result in improved clinical outcomes; with therapies being designed to redress specific neurochemical imbalances rather than treating the spectrum of the syndrome with a single drug.

Potential Outcomes of Decreased Cortical CHRM1 Receptors

The outcome of these decreases in cortical CHRM1 expression in subjects with schizophrenia will depend on the phenotype of the cells they are normally expressed by and the neuronal circuits they are associated with. To date, most localisation studies have been conducted using antibodies for the muscarinic receptors, this approach is confounded by the recent publication showing a lack of specificity of such antibodies [58]. However, with a lack of information pertaining to the cortical distribution of mRNA for these receptors, such studies can be used as a guide to the distribution of muscarinic receptors, but additional support should be drawn from other investigations.

Immunohistochemical studies in the cortex of rhesus monkeys indicate that CHRM1 is expressed predominantly in pyramidal cells of cortical layers III and V/VI of the prefrontal cortex [59] and in the presynaptic elements of non-cholinergic synapses, proposed to be axons of the corticocortical and thalamocortical pathways due to their localisation and nature. Thus, it is inferred that acetylcholine has the potential to modulate cortical excitatory transmission via the CHRM1. Indeed, studies in the mouse visual cortex indicate that acetylcholine modulates the functional dynamics of the cortical network [60] giving some support to the proposed interactions between acetylcholine and glutamate. In the hippocampus, it has been demonstrated that not only do CHRM1s colocalise with the obligate NMDA receptor subunit, but they also potentiate NMDA receptor currents [17, 61], suggesting that acetylcholine can modulate synaptic plasticity. Further support for a modulatory role of acetylcholine on glutamatergic activity comes from an electrophysiological study showing that the selective M1 agonist, 1-[3-(4-butyl-1-piperidinyl)propyl]-3,4-dihydro-2(1H)-quinolinone (77-LH-28-1), stimulated pyramidal cell firing and induced gamma oscillations [62], a synchronous event proposed to be fundamental to perceptual processing and reported to be disrupted in patients in schizophrenia [63]. Furthermore, gamma oscillations are lacking in the CHRM1$^{-/-}$ mouse [64], suggesting that the depolarisation of pyramidal neurons by CHRM1s plays a significant role in this network synchrony. In addition, microdialysis studies have shown that activation of CHRM1 causes an increase in acetylcholine and dopamine efflux in the rat cortex and hippocampus [65]. The increases in acetylcholine release might be mediated via a feedback loop to the nucleus basalis which may or may not involve glutamatergic transmission [66]. However,

the increased dopamine efflux apparently involves serotonin 1A receptor activation since it can be blocked by both serotonin 1A receptor and CHRM1 antagonists. It is possible that these are 5-HT1A autoreceptors, resulting in a reduction in the inhibitory serotonergic tone on mesocortical dopaminergic neurons and thus increased cortical dopamine efflux [67]. Together, these data suggest that a loss of cortical CHRM1 could have profound effects on the functionality of excitatory cortical networks and thus processing of perceptual information, possibly resulting in the cognitive deficits that have been linked to cholinergic dysfunction.

Muscarinic Receptors in Bipolar Disorder

There has been considerably less research into the pathophysiology of BD than into schizophrenia. However, there are still some data to support a role of muscarinic receptors in this disorder. A positron emission tomography (PET) study using [^{18}F]FP-TZTP, a selective CHRM2 ligand, reported decreased receptor availability in the anterior cingulate of patients with BD in the depressive phase of the illness compared to control subjects [68]. Furthermore, the decreased CHRM2 availability correlated with the severity of the depressive symptoms. This finding was supported by a post-mortem study, which showed that [^{3}H]AF-DX 384-binding density was decreased in the dorsolateral prefrontal cortex of subjects with BP [69]. However, an earlier post-mortem study in the anterior cingulate had shown that there was no difference in [^{3}H]AF-DX 384-binding density in the anterior cingulate [52]. The discrepancies between post-mortem studies could be due to regional variation, since the more recent study found no difference in [^{3}H]AF-DX 384-binding density in other cortical regions, implying that this is not a generalised effect. The major difference between the studies in the anterior cingulate is that the PET study used an agonist, the binding of which would be influenced by both receptor state and local levels of acetylcholine. Thus, although the data indicate that CHRM2 may be involved in BD, particularly in the depressive phase of the illness, more work is required to fully elucidate the nature of these alterations.

To date, levels of CHRM3 have only been measured once in tissue from subjects with BD [69]. This study found that there was a decrease in the binding density of [^{3}H]4-DAMP between tissue from the frontal, but not dorsolateral prefrontal or parietal, cortex of subjects with BD compared to that of controls. Since this study was conducted in the same cohort used for the [^{3}H]AF-DX 384 study, these data suggest that the change in CHRM2 and CHRM3 is region specific. Of particular note is the fact that none of the studies investigating levels of CHRM1 have found differences between binding in tissue from BD and that from controls [44, 69].

Muscarinic Receptors in Major Depressive Disorder

Much of the data supporting a role for muscarinic receptors in the pathophysiology of MDD come from psychopharmacological studies, with patients showing increased physiological responses to cholinergic agonists [70]. As with BD, there has been relatively little research providing direct evidence for a role of muscarinic receptors in the pathophysiology of MDD. [^{3}H]AF-DX 384-binding density has been reported to be decreased in the dorsolateral prefrontal, but not frontal or parietal, cortex from subjects with MDD compared to that from control subjects [69]. The same study found [^{3}H]4-DAMP- and [^{3}H]pirenzepine-binding densities to be unaltered in all three cortical regions from subjects with MDD, indicating that this is a specific deficit, rather than a generalised effect on CHRMs. A post-mortem study in the anterior cingulate found there were no differences in [^{3}H]AF-DX 384-binding density in tissue from subjects with MDD compared to that from controls [52]. Taken together, these data suggest there are highly region-specific changes in levels of CHRM2 in patients with MDD. As with BD, there are no reports of altered [^{3}H]pirenzepine-binding density in MDD [44, 69].

In summary, although the data implicating a role for muscarinic receptors in the pathophysiology of psychiatric disorders are strongest in schizophrenia, there is evidence to suggest they may also be involved in the processes underlying both BD and MDD, particularly the depressive symptoms. Thus, it may prove to be worth assessing their viability as drug targets for the development of novel pharmaceuticals.

Potential Outcomes of Decreased Cortical CHRM2 Receptors

As discussed for the CHRM1 receptor, the outcomes of decreased cortical expression of CHRM2s will be dictated by the phenotype of the cells expressing them and the neural circuits they impact upon.

In the rhesus monkey, immunohistochemical studies showed that cortical CHRM2s are located both pre- and post-synaptically [59]. As expected, presynaptic CHRM2s were located in the axons of symmetric synapses – confirming their role as autoreceptors of the cortical cholinergic system [71]. Somewhat more surprising

was the existence of CHRM2s in a subset of glutamatergic synapses as well as neurons that appeared to be interneurons and thus GABAergic in nature. These findings were expanded by a more detailed study, measuring levels of CHRM2 protein and mRNA in the cortex of rhesus monkeys [72]. This study found that CHRM2 protein and mRNA were localised primarily in pyramidal neurons but also in interneurons. In pyramidal neurons the apical dendrites were prominently stained whilst in the interneurons the immunoreactivity was present on the cytoplasmic surface of cell bodies, the initial axon segment and dendrites. The density and distribution of these CHRM2 markers were not affected by immunotoxic lesions of the nucleus basalis, which resulted in significant cholinergic denervation [72]. These data indicate that CHRM2s may also play a postsynaptic role in the cortex, modulating both excitatory input and inhibitory control. Support for modulation of excitatory transmission via CHRM2 comes from an electrophysiological study showing that the cholinergic facilitation of cortical extracellular field potentials is significantly reduced in $CHRM2^{-/-}$ mice [60]. In addition, it has been shown that basal forebrain neurons, which project to the cortex and hippocampus, release both acetylcholine and glutamate with presynaptic CHRM2s controlling the release of each transmitter [73]. Thus, through the CHRM2s, acetylcholine has the potential to regulate the release of glutamate as well as facilitating the activation of glutamatergic neurons. With regard to CHRM2 and interneurons, further immunocytochemical studies in the macaque primary visual cortex found that approximately a third of the GABAergic neurons expressed CHRM2s [74]. The concept of acetylcholine modulating inhibitory interneurons is supported by an electrophysiological study which showed that CHRM2 could reduce the amount of GABA released in the mouse auditory cortex [75]. Given the recent discovery that the axons of GABAergic interneurons can project beyond the region the cell body is located in [76], the potential impact of this cholinergic control of cortical GABAergic transmission would be significant. Overall, the combined effect of CHRM2s under normal circumstances would be to enhance the excitatory tone of cortical circuits. Thus, it is possible that a decrease in the density of these receptors would result in a reduction in this excitatory tone, severely affecting the normal activation of cortical networks.

Muscarinic Receptors as Therapeutic Targets

The relative paucity of data on the role of muscarinic receptors in both MDD and BD, combined with the problems of targeting only central CHRM2 and CHRM3 in order to avoid undesirable side-effect profiles means that most of the investigations into whether muscarinic receptors are viable drug targets in psychiatric disorders has focused on schizophrenia. In turn, much of the work has centred on developing ligands for the CHRM1s. This focus is due to a number of factors: (1) the potency of CHRM agonists to enhance cortical dopamine release correlates with their CHRM1 affinity [77]; (2) CHRM1s modulate hippocampal glutamate receptor-driven currents [17, 78], and (3) CHRM1s are thought to play a major role in cognition [79, 80]. Both dopamine and glutamate are critical components of the cortico-striato-thalamo-cortical feedback loop; abnormal activity of this neuronal circuit has been proposed to underpin the development of psychoses [81]. The fact that acetylcholine can modulate the activity of both of these neurotransmitter systems, via the CHRM1, make it a prospective target for future antipsychotic drug development, particularly since mice that lack CHRM1s show some similarities to animal models that are predictive of antipsychotic efficacy, such as increased locomotor activity in response to amphetamine [19]. Finally, it is now recognised that the cognitive deficits associated with schizophrenia are the most debilitating symptom cluster of schizophrenia, to the extent that the ability of a person with schizophrenia to reintegrate back into society can be predicted by the severity of their cognitive deficits [82]. Therefore, ameliorating the cognitive deficits associated with the illness should lead to an improvement in the quality of life of patients with the disorder. The role of CHRM1s in cognition makes them a viable target for such drug development [29, 83, 84].

Despite the evidence implicating muscarinic receptors in psychiatric disorders, the outcomes of efforts to ameliorate the symptoms of these disorders by modulation of the receptors have been modest rather than impressive. Development of drugs to target specific CHRMs has been severely hampered by the high degree of homology seen at the orthosteric binding sites [12]. The inability to target specific CHRMs has meant that the drugs often have side-effect profiles, mediated predominantly by peripheral CHRM2 and CHRM3s [85], that limit their usefulness. However, there are clinical data supporting the concept that targeting CHRMs may prove to be beneficial for patients suffering from schizophrenia. The antipsychotic

clozapine, a CHRM1 antagonist, has been credited with being more efficacious at improving cognitive symptoms than other antipsychotic medications [for review, see 86]. It has since been shown that the major metabolite of clozapine, *N*-desmethylclozapine, is a CHMR1 agonist and that the cognitive improvement comes from activating, rather than inhibiting the receptor [87]. However, recent phase IIb clinical trials reported that *N*-desmethylclozapine (ACP-104, Acadia Pharmaceuticals) is not an effective antipsychotic agent in its own right [88]. More recently, a pilot study showed that administering xanomeline, predominantly a CHRM1/CHRM4 partial agonist [89] although it is also active at serotonergic receptors [90], to medication-free, treatment-resistant patients improved their ratings on the Brief Psychiatric Rating Scale and the Positive and Negative Syndrome Scale as well as improving their cognitive function compared to patients who had received placebo [91]. It has since been shown that the CHRM4 activity of xanomeline is the dominant contributor to its anti-psychotic profile, with CHRM1s playing a lesser role [92]. However, as discussed at the beginning of this section, the CHRM-driven side-effect profile of xanomeline renders it unsuitable for further development.

The development of CHRM-specific drugs was revitalised by the discovery that neuromuscular blockers, which by their nature are nicotinic antagonists, could bind to CHRMs, particularly CHRM2 [93], and modulate the effects of muscarinic agonists. The neuromuscular blockers did not bind to the orthosteric-binding site, instead binding to a site proposed to regulate accessibility to the orthosteric site, the allosteric-binding site. The nature (positive or negative) and size of the allosteric interaction was found to be dependent on the combinations of orthosteric and allosteric ligands [for review, see 94]. It has since been shown that there are a number of allosteric ligands for CHRMs, with some such compounds being isolated from snake venoms [95]. One of the first positive allosteric ligands was 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine (AC-42) [96, 97]; although it binds to all muscarinic receptors, it selectively activates CHRM1s in the absence of an orthosteric agonist. There are now a number of positive allosteric ligands in development. These fall into 2 classes: positive allosteric modulators (PAMs) which have no agonist activity in their own right, and allosteric agonists which can modulate the effects of orthosteric ligands as well as activating the receptor in their own right [98]. Structural analogues of AC-42 have been shown to be central CHRM1 agonists capable of crossing the blood-brain barrier (77-LH-28-1 [62]), which reduce the hyperactivity induced by both dopaminergic and glutamatergic manipulations as well as improving performance in a spatial memory paradigm (4-[3-(4-butylpiperidin-1-yl)-propyl]-7-fluoro-4H-benzo[1,4]oxazin-3-one; AC-260584 [99]). 1-(1′-2-methylbenzyl)-1,4′-bipiperidin-4-yl)-1H-benzo[d]imidazol-2(3H)-one (TBPB) is an unrelated CHRM1 allosteric agonist, which has also been shown to be efficacious at reducing amphetamine-induced hyperactivity [100]. There are also a number of PAMs in development, some of which have been shown to potentiate the binding of orthosteric agonists and cause leftward shifts in acetylcholine response curves: cyclopentyl 1,6-dimethyl-4-(6-nitrobenzo[d][1,3]-dioxol-5-yl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (VU0090157) and (E)-2-(4-ethoxyphenylamino)-N′-((2-hydroxynaphthalen-1-yl)methylene)acetohydrazide (VU0029767) [101]. Together, these preclinical data support the proposal that activation of CHRM1s may be beneficial in treating cognitive deficits seen in patients with schizophrenia, possibly with the added advantage of exerting an antipsychotic effect.

Ligands have also been developed which selectively target the CHRM4, in the hope of producing a drug with a stronger antipsychotic effect than is caused by activating the CHRM1. 3-Amino-*N*-[(4-chlorophenyl)methyl]-4,6-dimethylthieno[2,3-b]pyridine-2-carboxamide (VU100010) is a highly selective CHRM4 modulator [102], but problems with the physiochemical properties of the compound precluded its further development [103]. However, 3-amino-5-chloro-6-methoxy-4-methyl-thieno(2,3-b)pyridine-2-carboxylic acid cyclopropylamide (LY2033298), a selective PAM for CHRM4s, was shown to be effective in two behavioural paradigms used to screen for molecules with anti-psychotic efficacy; reducing condition avoidance responses and apomorphine-induced prepulse inhibition deficits, when administered with a sub-effective dose of oxotremorine [104]. Furthermore, two analogues of VU100010, 3-amino-*N*-(benzo[d][1,3]dioxol-5-ylmethyl)-4,6-dimethylthieno[2,3-*b*]pyridine carboxamide (VU0152099) and 3-amino-*N*-(4-methoxybenzyl)-4,6-dimethylthieno [2,3-*b*]pyridine carboxamide (VU0152100), are centrally active CHRM4 modulators which have been shown to reduce amphetamine-induced hyperlocomotion in rats [103]. Together, these data suggest that selective positive modulation of the CHRM4 may indeed prove to be an alternative approach to the traditional dopamine D2 receptor antagonism used in the development of antipsychotic drugs.

One immediate outcome of the development of numerous allosteric ligands for CHRMs is the revelation that there appears to be more than one allosteric-binding site on these receptors. Once more most of the work has been conducted on the CHRM1, but there is also evidence to suggest that the same is true of CHRM2. Including the orthosteric-binding site, there are three distinct modes of activation for the CHRM1, the other modes are AC-42-like compounds and clozapine-like compounds [105]. AC-42 and its related compounds showed clear allosteric binding and point mutation studies showed that they seemed to occupy distinct sites to that occupied by carbachol, the orthosteric ligand. *N*-Desmethylclozapine also showed some allosteric properties but not as clearly as the AC-42 family. Point mutation studies suggest that *N*-desmethylclozapine occupies a space which substantially overlaps that occupied by orthosteric ligands, suggesting that there are at least 2 non-orthosteric-binding sites on CHRM1 [105]. There is also evidence to indicate that TBPB and 77-LH-28-1 activate CHRM1s by different mechanisms, with 77-LH-28-1 acting at the same site as AC-42 [106]. In addition, it has been shown that the VU CHRM1 allosteric potentiators, VU0090157 and VU0029767, are mechanistically distinct, possibly acting at different sites [101]. However, it has recently been postulated that rather than there being multiple allosteric-binding sites on CHRMs, the different allosteric agonists act at overlapping sites and that there is no stringent 'pharmacophore' which can be associated with selective interaction at this site [98]. In addition to the need to understand the exact nature of these allosteric interactions and whether they occupy spatially distinct-binding sites on CHRMs, the endogenous ligands for these sites have yet to be identified.

Can We Mimic Normal Muscarinic Function?

With the progress that is being made in the development of selective agonists comes the question of whether stimulating muscarinic receptors with exogenous ligands will accurately reflect the activation that follows stimulation with the endogenous ligand, acetylcholine and the, as yet, unidentified ligand for the non-orthosteric sites?

The prevailing credo is that CHRM1, 3 and 5 couple to $G\alpha_{q/11}$ proteins, stimulate PLC and thus, mobilise intracellular calcium; whilst CHRM2 and 4 couple to $G\alpha_{i/o}$ proteins, inhibit adenylyl cyclase and therefore reduce intracellular levels of cAMP [10]. However, it would now appear that this is a rather simplistic view of the interactions that potentially occur between the CHRMs and G proteins. It is now postulated that GPCRs can assume multiple receptor conformations; these conformations can be adopted either spontaneously or induced/stabilised by the interaction of the receptor with ligands for either ortho- or allosteric-binding sites [107]. For example, CHRM3, stably expressed in HEK-293 cells, have been shown to stimulate PLC via $G\alpha_q$ and phospholipase D (PLD) via $G\alpha_{12}$ [108], with the investigators suggesting that nearly every GPCR which activates PLC is capable of stimulating PLD. Furthermore, different ligands seem to be able to facilitate different conformations, in turn binding to different G proteins; CHRM3 expressing CHO cells activate $G\alpha_{i/o}$ when stimulated with pilocarpine but activate both $G\alpha_{i/o}$ and $G\alpha_{q/11}$ when treated with methacholine [109]. Pilocarpine and methacholine both bind to the orthosteric site; thus, these data suggest that the ligands induce different conformations, which in turn stimulate different G proteins. Although much of the work on G-protein simulation has been conducted in cell lines stably expressing the GPCR of interest, work done in native tissue supports the hypothesis that the conformations adopted by GPCRs are governed, in part, by the orthosteric ligand that binds to them [107]. This downstream signalling promiscuity is not particularly novel in GPCRs and certainly is not limited to CHRMs. For example, the serotonin 2A receptor activates PLC via $G\alpha_q$ but it also releases arachidonic acid – possibly by the activation of PLA. Furthermore, different ligands for the serotonin 2A receptors have been shown to have different efficacies for the activation of these downstream effectors [110, 111]. To date, the structural characteristics of ligands responsible for governing the activation of one signalling pathway rather than another have not been determined.

This complex scenario is further exacerbated by the discovery that the allosteric ligands also appear to be able to activate select G-protein populations. In CHO cells stably expressing CHRM1s, AC-42 and 77-LH-28-1 stimulated $G\alpha_{q/11}$- and $G\alpha_s$-dependent signalling as did the orthosteric agonists, oxotremorine-M, arecoline, and pilocarpine [112]. However, whilst the orthosteric ligands also stimulated $G\alpha_{i1/2}$-dependent signalling, AC-42 and 77-LH-28-1 were ineffective. In addition, the CHRM1 modulator, VU0090157, has been shown to potentiate activation of both PLC and PLD whilst VU0029767 was less effective at potentiating stimulation of PLC and had almost no effect on orthosteric activation

of PLD [101]. Whether these differences in receptor coupling following allosteric modulation are due to the ligands binding to different sites on the receptor or facilitating different receptor conformations remains to be determined. To date, there have been no published reports of differential receptor coupling following allosteric activation or modulation in native tissues, thus it is possible that some of the couplings reported above might be artefacts of cells that have been manipulated to express non-native receptors.

Conclusions

There is strong evidence to support the hypothesis that CHRMs play a role in the pathophysiology of schizophrenia. Additional support comes from studies on genetically modified mice, which suggest that CHRM1 may be involved in the cognitive deficits and contribute to the positive symptoms observed in patients with the disorder and that CHRM4 may play a more prominent role in the chemical imbalance that leads to psychosis. To date, the outcomes of clinical studies have been promising rather than authoritative. In part, this is probably due to issues associated with attempts to generate ligands that target specific muscarinic receptors in order to improve the side-effect profile of muscarinic agonists. The small amount of data available for BD and MDD also suggest that CHRMs may play a role in these disorders, with the CHRM2 appearing to be associated with the presentation of depressive symptoms in both disorders. As yet, the implications of decreased CHRM3 in BD have yet to be elucidated. However, the likelihood of either of these receptors constituting a viable drug target is extremely low since the only means of avoiding the unacceptable muscarinic side effect profile which is associated with the activation of peripheral CHRM2 and 3s is to develop ligands which are active only in the CNS.

The development of CHRM-specific drugs was boosted by the discovery of allosteric-binding sites on the receptors, allowing selective functional modulation. To date, the preclinical studies with agonists and modulators have yielded very promising data, suggesting that increasing the activation of central CHRM1 may have beneficial effects for cognition as well as showing efficacy in models that are used to identify antipsychotic activity. Similarly, increasing the activation of central CHRM4 is also efficacious in models used to predict antipsychotic action. These findings are in agreement with the 'proof of principle' clinical study using xanomeline, where patients who received the drug showed an improvement on cognitive tests and decreased positive symptoms compared to the patients on placebo [91]. Furthermore, it is hypothesised that using allosteric modulators to enhance cholinergic transmission is likely to prove safe due to the pharmacodynamic limits inherent in doing so and the fact that the effect will be activity-dependent thereby avoiding inappropriate activation of the system [113]. Modulating the system in this manner will allow the temporal and spatial integration of the endogenous system rather than imposing constraints on these fundamentals of neurotransmission, potentially resulting in a more 'healthy' cholinergic system in the CNS.

However, the fact that different ligands appear to couple the receptors to diverse signalling systems complicates the situation. It would appear that the 'normal' activation of effector systems depends on the presence of the endogenous ligand and any number of allosteric ligands. Furthermore, alterations in these components may result in the activation of alternative effector systems and therefore fail to produce the desired physiological outcome. Whilst the allosteric ligands offer an alluring means of 'tweaking' a system that is operating in a suboptimal manner, it is also possible that such a pharmacological intervention will result in the receptor adopting a conformation which does not couple appropriately to downstream events. In order for us to realise the full potential of the allosteric ligands as therapeutic agents, it is vital that we first identify the consequences of CHRM activation in the native system and fully evaluate the physiological effects of modulating the system artificially.

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