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Roles of the M_4 Acetylcholine Receptor in the Basal Ganglia and the Treatment of Movement Disorders

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Abstract

Acetylcholine (ACh) released from cholinergic interneurons (ChI) acting through nicotinic (nAChR) and muscarinic acetylcholine receptors (mAChRs) in the striatum have been thought to be central for the potent cholinergic regulation of basal ganglia (BG) activity and motor behaviors. ACh activation of mAChRs has multiple actions to oppose dopamine (DA) release, signaling, and related motor behaviors, and has led to the idea that a delicate balance of DA and mAChR signaling in the striatum is critical for maintaining normal motor function. Consistent with this, mAChR antagonists have efficacy in reducing motor symptoms in diseases where DA release or signaling is diminished, such as in Parkinson's disease and dystonia, but are limited in their utility due to severe adverse effects. Recent breakthroughs in understanding both the anatomical sites of action of ACh and the mAChR subtypes involved in regulating BG function reveal that the M_4 subtype plays a central role in regulating DA signaling and release in the BG. These findings have raised the possibility that sources of ACh outside of the striatum can regulate motor activity, and that M_4 activity is a potent regulator of motor dysfunction. Here we discuss how M_4 activity regulates DA release and signaling, the potential sources of ACh that can regulate M_4 activity, as well as the implications of targeting M_4 activity for the treatment of the motor symptoms in movement disorders.

Keywords

Muscarinic; movement disorders; basal ganglia; cholinergic; motor deficits

Introduction

Acetylcholine (ACh) acting through both nicotinic (nAChR) and muscarinic acetylcholine receptor (mAChRs) has profound neuromodulatory capabilities throughout the central nervous system[1,2]. ACh can powerfully regulate brain circuits associated with learning, memory, and movement[3]. Within the basal ganglia (BG), ACh can substantially modulate dopamine (DA) release from terminals originating from the substantia nigra pars compacta (SNc)[4]. ACh acting through nAChRs can increase DA release by actions on DA terminals[5].

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However, ACh activation of mAChRs has more complex interactions on DA release and signaling, and activation of mAChRs has multiple actions to regulate DA release, signaling, and related motor behaviors[4]. This regulation of the effects of DA on BG output and locomotion has led to the idea that a delicate balance of opposing actions of DA and mAChR signaling in the striatum is critical for maintaining normal BG function[6].

Cholinergic neurons provide this important neuromodulatory control of the BG, and the major source of ACh to activate the mAChRs necessary to regulate BG function and DA has been thought to be large, aspiny cholinergic interneurons (ChI) that act locally in the striatum[6]. Striatal ChI are believed to be central to cholinergic regulation of BG signaling, DA, and related behavioral outputs[7,8] However, recent evidence has challenged and expanded our understanding of the sources of ACh capable of regulating BG output. We and others have recently shown that hindbrain cholinergic nuclei of the pedunculopontine (PPN) and laterodorsal tegmental area (LDT) are as capable as ChI of regulating locomotion, reward, and other BG-influenced behaviors[9,10]. This has raised the distinct possibility that non-striatal sources of ACh are important to regulating the BG, and that the PPN and LDT may be disturbed in, or influence disease states associated with the BG. Yet, the potential of the LDT and PPN to regulate BG nuclei in both normal and pathological states, as well as their therapeutic potential largely remain untested.

Because of the major modulatory role of ACh released from cholinergic nuclei onto mAChRs, there has been a great effort to understand the physiological role of each individual mAChR subtype[4]. Genetic, biochemical, immunohistochemical, and pharmacological studies have pointed to roles for multiple mAChR subtypes in regulating DA and BG output. These studies have pointed to major roles for M_1, M_4, and M_5 mAChR subtypes in regulating DA release, signaling, and related motor behaviors[4,11–14]. This raises the possibility that targeting of these receptors may be therapeutically beneficial[15]. Consistent with this, non-selective mAChR antagonists have efficacy in reducing motor symptoms in diseases where DA release or signaling is low, such as in Parkinson’s disease (PD) and dystonia, and may exacerbate symptoms in diseases where DA is elevated such as in certain symptom domains of schizophrenia[16,17]. Additionally, non-selective or only partially selective mAChR agonists show efficacy in reducing certain symptom domains in diseases with elevated DA, such as schizophrenia[18–20]. However, despite efficacy, the utility of non-selective mAChR therapeutics are limited due to their severe adverse effects[21]. Recent evidence suggests that mAChR subtype-selective drugs, likely M_4, may maintain the clinical efficacy of non-selective antagonists in movement disorders while avoiding adverse effects, but this remains to be tested[15]. Here we discuss how M_4 activity regulates DA release and signaling, the potential sources of ACh that can regulate M_4 activity, as well as the implications of targeting M_4 activity for the treatment of the motor symptoms in movement disorders.

The mAChR system

The mAChRs belong to the superfamily of G protein coupled receptors (GPCRs), and are prototypical family A or class 1 GPCRs[22]. The mAChR class of ACh receptors possesses five unique subtypes termed M_1-M_5. Each of the mAChR subtypes can be found expressed

in several brain nuclei. Other than M_5, whose expression is largely limited to midbrain dopaminergic nuclei, the other mAChRs can be found in several brain structures and cell types throughout the brain, but can be highly enriched in certain structures. For example, M_1 is most highly expressed throughout the cortex and hippocampus, with lower but abundant expression in basal ganglia and other brain regions. M_4 is most highly expressed throughout the striatum, but also has relatively high expression in cortical and other subcortical regions. M_2 and M_3 are not as heavily expressed in the CNS as are M_1 and M_4, but are present at lower levels throughout the brain (for extensive discussion of the expression of mAChR, see, [23–25]).

M_1-M_5 differ in the G proteins to which they couple[26]. M_1, M_3, and M_5 couple to G_q/G_{11} proteins which lead to the activation of phospholipase C, formation of the second messenger inositol triphosphate and other second messengers. These G_q-coupled mAChR subtypes induce mobilization of intracellular calcium, and typically increase the excitability of neurons through closure of potassium channels and activation of cation channels (see figure 1)[22,26]. In contrast, M_2 and M_4 couple to $G_{i/o}$ proteins, lead to the inhibition of adenylyl cyclase, decreasing production of the second messenger cAMP, and often decreases neuronal excitability and synaptic transmission (see figure 1)[22]. The actions of ACh through these distinct classes of mAChRs have powerful, and sometimes opposing, effects on the output of diverse neuronal populations[27].

Because of the neuromodulatory role imposed by mAChR subtypes, mAChRs have been viewed as promising targets for multiple CNS disorders[15]. Both agonists and antagonists of mAChRs, which are either non-selective or only partially selective for mAChR subtypes have been utilized in the clinic to treat CNS disorders. In schizophrenia, mAChR agonists that were only partially selective for M_1 and M_4 showed efficacy in reducing certain symptom domains of the disorder[20,28–30]. In movement disorders, such as PD and dystonia, non-selective mAChR antagonists relieve certain motor symptoms, and remain a mainstay of treatment for dystonia[16,31]. Despite their efficacy, both agonists and antagonists of mAChRs, induce severe adverse side effects have greatly limited their clinical utility. However, major efforts from both academic labs and pharmaceutical companies are focusing on the development of ligands that are truly selective for individual mAChR subtypes. These compounds are now being utilized to understand the roles individual of mAChRs in brain circuits, have yielded unique roles for individual mAChR subtypes, and have raised the possibility that subtype selective mAChRs ligands can maintain the efficacy of non-selective mAChR agents in treating CNS disorders while eliminating the adverse side effects [15,21,32].

M_4 is the primary mAChR responsible for regulating DA and locomotion

Outside of the important regulatory role of nAChR on DA release[5], the M_1, M_4, and M_5 mAChR subtypes have also been implicated in modulating DA and related behaviors[4]. While each of these mAChRs have a role in modulating DA release, several lines of evidence implicate M_4 as the primary mAChR subtype for regulation of DA signaling in the BG and related motor behaviors. M_4 is the most highly expressed mAChR subtype in the striatum, where it is most abundantly expressed in D_1 DA receptor (D_1DR)-expressing spiny projection neurons (SPNs) that comprise the BG direct pathway (D_1-SPNs), but is not, or

lowly expressed on indirect pathway spiny projection neurons[23,24,33,34]. This is a critical pathway for motor activation[35]. Genetically modified mice with M_4 deleted globally or specifically from D_1-SPNs are hyper-locomotive, have elevated baseline DA, and are more sensitive to dopaminergic stimulants than littermate controls[36,37]. Outside of D_1-SPNs, M_4 is also expressed pre-synaptically on glutamatergic cortical and thalamic inputs into the striatum as well as on ChI, but is not widely expressed on D_2 DA receptor containing spiny projection neurons or on DA terminals from the SNc (see Figure 2 for an overview of M_4 expression in the striatum)[23,24].

Further bolstering genetic and biochemical findings on the roles of mAChR subtypes have been the discovery of the first truly selective pharmacological tools for M_1, M_4, and M_5[15,21]. Using these novel probes, we and others have performed a series of electrophysiology, voltammetry, behavioral, and imaging experiments to provide compelling evidence that M_4 is the primary mAChR subtype responsible for regulation of DA signaling and related motor behaviors. M_4 activation can profoundly decrease DA release, DA receptor signaling, and locomotion, and these mechanisms are summarized in Figure 2[9,11,36,38–40]. Using pharmacological and genetic tools for M_1, the primary role for activation of M_1 appears to be pro-cognitive effects while having modest effects on locomotion[41–45]. Additionally, using selective tools for M_5, there have been more complicated findings with M_5 activation exerting excitatory effects on DA neurons in the SNc while reducing DA release from SNc terminals in the striatum[12]. Taken together, these data provide strong evidence that M_4 has powerful effects on DA and related behaviors, and a fine tuned balance of M_4 and DA are critical for normal BG function.

Mechanisms of M_4 activity in the basal ganglia

M_4 reduces glutamatergic signaling in the striatum

M_4 is located presynaptically on glutamatergic inputs into the striatum from cortical and thalamic projections[23,24]. When examining evoked excitatory post synaptic currents (eEPSCs) from cortical projections onto either D_1-SPNs or D_2-SPNs, potentiation of M_4 signaling using a M_4 positive allosteric modulator (PAM) can reduce eEPSCs by ~60% (summarized in Figure 2A)[46]. PAMs act by targeting allosteric sites on the receptor, rather than the orthosteric (neurotransmitter-binding) site. When bound, PAMs can increase the effect of the endogenous agonist for that receptor through increasing the receptor's affinity for the agonist and/or increasing the efficacy of the receptor's response (for an extensive review on allosteric modulators, see[21]). Additionally, PAMs can act by directly leading to the activation of intracellular signaling cascades in the absence of endogenous agonists (allosteric agonism, see Figure 3) Interestingly, even though M_4 is not expressed on D_2-SPNs, the magnitude of effect of M_4 activation on eEPSCs in these cells was comparable to the effect in M_4 expressing D_1-SPNs, suggesting that M_4 activation can regulate glutamatergic inputs into both the direct and indirect pathway equally[46]. Reduction of glutamatergic input into the striatum has been linked to deficits in goal directed actions. Additionally, M_4 potentiation has been shown to modulate cortico-striatal long-term depression[47]. This mechanism of M_4-mediated decreases in glutamatergic drive also appears to work in disease models as well, as M_4 potentiation has been shown to block excess

cortico-striatal glutamate release in mouse models of Huntington's Disease[48]. This indicates that in both normal and disease states M_4 activation can reduce glutamatergic drive into the striatum.

M_4 regulates DA release in the striatum

Highly selective M_4 PAMs can induce profound inhibition of psychomotor stimulant-induced increases in motor activity, a mechanism known to be regulated by increases in DA release[4,28,32,49–51]. Additionally, M_4 PAMs are capable of blocking changes in fMRI BOLD responses caused by administration of psychomotor stimulants in both the dorsal and ventral striatum[49]. Interestingly, M_4 PAMs are no longer efficacious at blocking psychomotor stimulant induced increases in locomotor activity in animals where M_4 is selectively removed from D_1-SPNs (D_1-M_4 KO), suggesting that postsynaptic M_4 on D_1-SPNs can regulate presynaptic DA release on terminals from the SNc[11,36]. To assess the mechanism by which this happens we utilized a series of fast-scan cyclic voltammetry (FSCV) experiments. These studies showed, as the behavior and fMRI responses suggested, that M_4 activation induces a sustained inhibition of electrically and optically-evoked DA release in the striatum and this response is absent in M_4 knockout mice (summarized in Figure 2A)[11]. Further studies revealed that this sustained inhibition is mediated by a novel mechanism in which M_4 activation in D_1-SPNs induces release of an endocannabinoid (eCB), likely 2-AG, which activates CB_2 receptors on striatal DA terminals from the substantia nigra pars compacta (SNc)[11]. CB_2 activation then leads to sustained inhibition of DA release from SNc terminals (see Figure 2A) and effects of M_4 PAMs on DA release and some behavioral effects are blocked by CB_2 antagonists. While the effects of this mechanism of decreased DA release on the direct pathway are clear, potential effects of this mechanism on the indirect pathway remain to be determined. These data suggest that M_4 activation regulates motor function, in part, by modulation of DA release.

M_4 activation on D_1-SPNs directly inhibits D_1DR signaling.

In addition to blocking hyperlocomotor activity induced by DA release in response to psychomotor stimulants, M_4 activation also inhibits the effects of a direct acting D_1DR agonist on locomotor activity, suggesting that M_4 also acts by a mechanism that is independent of DA release and can directly inhibit D_1DR signaling[9]. DA stimulates motor activity in part by acting on D_1DR receptors, which couple to activation of AC through a unique GTP-binding protein, termed $G\alpha_{olf}$ (similar to $G\alpha_s$)[52]. Activation of D_1DR and $G\alpha_{olf}$ increases cAMP production and induces increased GABA release onto cells of the substantia nigra pars reticulata (SNr). This inhibits GABAergic cells of the SNr, disinhibiting the motor nucleus of the thalamus, exciting the motor cortex, and increases locomotion[35,53]. M_4 couples to $G\alpha_{i/o}$ G proteins, which can directly inhibit AC[54], and we have recently reported that M_4 activation can directly inhibit D_1DR/$G\alpha_{olf}$ signaling, and this appears to primarily occur at D_1-SPN terminals in the SNr. This M_4-mediated inhibition of D_1DR signaling decreases SNr GABA release, diminishes D_1-SPN activity, and decreases locomotor activity (summarized in Figure 2B)[9]. Interestingly, this M_4-mediated inhibition occurs tonically. When recording miniature inhibitory post synaptic currents (mIPSC) from cells of the SNr, genetic deletion of M_4 selectively in D_1-M_4 KO had a marked increase in baseline mIPSC frequency compared to controls, suggesting increased GABA release

probability and dysregulated D_1-SPN activity after genetic removal of M_4 activity[9]. Similar to the genetic findings, non-selective mAChR antagonists or M_4-selective peptide inhibitors led to a large increase in mIPSC frequency, and this effect was absent in D_1-M_4 KO animals, again suggesting a tonic inhibition of D_1-SPNs by M_4[9]. Additionally, fMRI studies looking at BOLD signaling after M_4 PAM and/or D_1DR agonist injection into rats recapitulates our electrophysiological findings that the primary anatomical site for the M_4 inhibition of D_1DR signaling is the SNr, and not in the striatum[9]. These findings suggest that, in addition to inducing a sustained inhibition of DA release, M_4 activation can directly and tonically inhibit D_1DR signaling at the level of the SNr. However, the mechanism, suggested to be competing actions on AC and cAMP production, require further experimentation, and the impact of this clinically remains to be determined. Taken together M_4 activation has multiple actions throughout the BG to oppose DA, glutamatergic drive in the striatum, and oppose locomotion.

Hindbrain cholinergic nuclei are capable of regulating DA and locomotion.— Unlike the M_4 effects on DA release in the striatum, the M_4-mediated inhibition of D_1DR/$G\alpha_{olf}$ occurs at the level of the SNr[9]. This action in the SNr cannot be mediated by striatal ChIs, suggesting that non-striatal hindbrain sources of ACh from the PPN or LDT are responsible for the M_4-mediated inhibition of D_1DR/$G\alpha_{olf}$ signaling and SNr GABA release. This is especially interesting given other labs recent preclinical findings that the PPN and the LDT, have discernible actions in regulating some classically BG influenced behaviors such as locomotion and reward[10]. The PPN and LDT are not only cholinergic, but also have glutamatergic and GABAergic projection neurons in these nuclei. These neurons project widely throughout the midbrain, BG, cerebellum, as well as several other structures[55]. Using genetic and viral technologies to selectively label cholinergic neurons, optogenetic stimulation of PPN terminals in the SNc or VTA could increase locomotion and reward respectively[10]. Additionally, optogenetic stimulation of the LDT terminals could only regulate reward as measured by conditioned place preference[10]. While these studies implicate the cholinergic cells in regulation of locomotion or reward, other recent evidence suggests that glutamatergic neurons of the PPN are also capable of modulating locomotion and locomotor speed[56]. Taken together, these studies implicate an emerging important role in the PPN and LDT regulating BG influenced behaviors.

Interestingly, in our recently published studies, selective lesions of cholinergic neurons in the PPN eliminated cholinergic modulation of D_1DR signaling and mIPSC frequency in the SNr[9]. This suggests that cholinergic projections from the PPN are responsible for tonic inhibition of GABA release from D_1-SPN terminals. Additionally, microinjection of non-selective mAChR antagonists directly into the SNr increases locomotor activity, and this effect is largely absent in D_1-M_4 KO animals[9]. These data suggest that, in addition to striatal ChIs, cholinergic projections from the PPN to the SNr acting through M_4 play an important role in regulating motor activity, and more broadly implicate that hindbrain cholinergic nuclei are as capable of regulating BG related behaviors as ChI in the striatum

These recent data implicating that ACh released into the SNr, and other nearby midbrain nuclei, can regulate DA and locomotion are especially interesting given the wealth of information known about ChI in the striatum. These early studies to mechanistically

understand how the hindbrain cholinergic nuclei regulate the BG suggest that the ability of ACh from the PPN and LDT to regulate BG nuclei outside the striatum are directly analogous to the role ACh from ChI in the striatum. With ACh from the PPN and LDT regulating BG output acting through multiple mAChRs and nAChRs to regulate BG nuclei outside of the striatum[7,8]. However, it may be possible that hindbrain versus ChI sources of ACh regulate specific aspects of BG function that are yet to be determined. Additionally, while these initial studies suggest that similar receptors underlie the mechanisms behind ACh regulation are mediated by similar mAChR and nAChR, recent evidence indicates that there may be different receptors or possibly different roles for the same receptor depending on brain region[9]. It is possible that these differences in expression or activity could be exploited to differentially target certain circuits or behaviors that may be influenced by ChI or hindbrain cholinergic nuclei. For example, based on fMRI data examining BOLD responses, M_4-mediated inhibition of D_1DR may be specific to the SNr, and, in other brain regions such as the hippocampus or cortex, M_4 may facilitate D_1DR activation rather than be inhibitory[9]. Future studies to understand the role of the PPN and LDT ACh, as well as the receptors through which they signal, in experimental models of movement disorders will be necessary to understand how these nuclei regulate motor function and dysfunction. Additionally, these studies will help elucidate if there are discernible actions of ACh released from these hindbrain nuclei verse ACh released from ChI.

Clinical evidence also suggests that hindbrain cholinergic nuclei may play a role in regulating motor deficits in movement disorders[57]. However, the significance of any hindbrain cholinergic mechanisms in movement disorders remains undetermined. Despite this, clinical and preclinical data suggests a role for the PPN or LDT in symptomology of movement disorders. In PD, degeneration of the PPN has been found in some patients, and these patients have more severe gait deficits, postural disturbances, and rigidity[58,59]. Importantly, the severity of the presentation of these symptom clusters correlates with loss of cholinergic neuron numbers and activity in the PPN[58,59]. Lesioning of the PPN cholinergic cells using a toxin based approach can recapitulate these clinical findings in pre-clinical models, as primates with chemical cholinergic lesions in the PPN have profound gait and postural disturbances[60]. Furthermore, in post-mortem tissue samples from dystonia patients, downregulation of ChAT activity or expression has been found, suggesting a dysregulation of PPN activity in dystonia patients[61]. These clinical findings in PD and dystonia suggest that intact hindbrain cholinergic activity is necessary for normal motor function, and shows the importance of dissecting out specific roles for the PPN in the expression of movement disorders.

Anti-muscarinic therapy is efficacious in treating movement disorders.—The use of anti-muscarinic agents, namely trihexyphenidyl, has a long established efficacy in reducing specific motor symptoms of PD and dystonia[16,62–65]. Additionally, trihexyphenidyl, and other poorly or non-selective anti-muscarinic compounds are effective at reducing abnormal movements in animal models of dystonia and PD with overt motor deficits[66–68]. The efficacy of anti-muscarinic compounds both pre-clinically and clinically, suggest that mAChRs are important regulators of certain symptom domains of movement disorders. However, the mAChR subtype or subtypes that mediate the efficacy of these

antagonists has remained elusive. Anti-muscarinic compounds largely display only modest selectivity *in vitro* in pharmacological assays, and, especially at doses needed to achieve efficacy, make it difficult to attribute the efficacy of the compound to a single mAChR[15,21,32,69]. However, recent pharmacological advances as well as the use of genetic tools in combination with non-selective mAChR antagonists, such as the studies we have outlined above, have raised the possibility that a single mAChR subtype may be responsible for the majority of efficacy seen clinically with current anti-muscarinic compounds[9,67].

Regardless of the source of ACh that contributes to the expression or treatment of specific symptoms of movement disorders, our data suggest that ChI or PPN sources of ACh acting through M_4 may be an exciting target to relieve certain symptoms of movement disorders. Our data showing M_4 has multiple actions to oppose DA release and signaling in the BG are especially interesting in light of the established efficacy of non-selective mAChR antagonists[16,17,64,70]. This raises the possibility that compounds selectively and specifically targeting M_4 may be able to maintain the efficacy of broad-spectrum anti-muscarinic therapeutics while avoiding the severe adverse effects. This notion is supported by previous studies which suggest that the peripheral adverse effects of mAChR antagonists are mediated by blockade of M_2 and M_3, and that the central adverse effects, centering on cognitive disruptions, are largely due to antagonism of M_1[15,21,62,71]. Additionally, while M_1 selective antagonists have been shown to normalize plasticity deficits in a genetic mouse model of dystonia[72], M_1 selective antagonists do not display robust anti-parkinsonian efficacy in animal models[27]. Furthermore, it is possible selective and specific M_4 compounds could have greater efficacy than non-selective mAChR agents, as the extent of potential efficacy of mAChR drugs has been difficult to establish due to limiting doses based on adverse effect liability[62]. Unfortunately, the lack of highly selective M_4 antagonists has limited the ability to test this hypothesis, and the development of these selective antagonists will be necessary to directly test this hypothesis in experimental models, and eventually advance these compounds into clinical populations. This evidence suggesting that selective M_4 antagonists or agonists will have robust efficacy in treating specific motor symptoms and signaling deficits associated with motor deficits while avoiding adverse side effects are discussed below.

M_4 antagonists may alleviate parkinsonian motor deficits.—The primary pathophysiological change giving rise to the motor symptoms of PD is the loss of DA neurons in SNc, which are critical in modulating the striatum and other BG nuclei[35,53]. Loss of DA neurons leads to diminished DA levels, which in turn decreases direct pathway and D_1DR/$G\alpha_{olf}$ activation, increases indirect pathway activity, and leads to an imbalanced BG activity[73]. Replacement of DA through administration of levodopa (L-DOPA), a precursor of DA and product of the rate limiting step in the catecholamine biosynthetic pathway, has become the mainstay of treatment for PD[74]. However, DA replacement can cause serious side effects, such as dyskinesia, and DA replacement is not effective in treating some PD symptoms, including tremor[75]. This highlights the critical need for other efficacious therapies in PD, especially in newly diagnosed patients. Our findings that M_4 activation leads to a sustained inhibition of DA release as well as inducing a tonic inhibition of D_1DR/$G\alpha_{olf}$ signaling raises the possibility that the tonic inhibition of DA release and signaling by

M_4 could play a major role in exacerbating motor disability. This also raises the possibility that selective M_4 antagonists could reduce parkinsonian motor disability by relieving M_4 mediated inhibition of DA release and signaling. This potentially could increase the efficacy of remaining DA fibers as well as normalize the balance of direct and indirect pathway activity by removing the tonic inhibition of the direct pathway by M_4. However, M_4 activity in parkinsonian animal models remains unexplored, and the potential for modulating M_4 activity for clinical benefit remains theoretical due to lack of selective pharmacological agents. None the less, acting by these mechanisms, M_4 antagonists could possibly be a standalone therapy or could possibly be an L-DOPA sparing therapy to limit the potential negative impacts of L-DOPA therapy.

Levodopa-induced dyskinesia may be treated by M_4 activators.—While, as discussed above, M_4 antagonists may be beneficial in treating PD in newly diagnosed patients, M_4 antagonists could not be efficacious and potentially cause adverse effects in patients that have been treated with L-DOPA, and especially those who have developed L-DOPA induced dyskinesia (LID). In LID, despite the loss of DA neurons in the SNc, treatment with L-DOPA can cause a local hyper-DA state in the remaining DA neurons, and possibly release of DA through other neurotransmitter fibers[75,76]. Therefore, M_4 antagonists could cause an increase in an already hyper-DA state by further increasing DA release in SNc fibers. However, this remains to be tested as a lack of selective M_4 antagonists has hindered testing this hypothesis.

Conversely, M_4 PAMs have been tested in experimental models of LID, and support the notion that M_4 antagonists may exacerbate already established LID[47]. In both rodents and primates, M_4 PAMs could decrease the behavioral expression of the LID phenotype and decrease abnormal involuntary movements in both species[47]. Additionally, M_4 PAMs normalize electrophysiological correlates of LID, such as normalizing deficits in cortico-striatal long-term depression in the direct pathway[47]. These studies suggest that M_4 antagonists may not be beneficial in PD patients with LID, may be limited to patients that do not express LID symptoms, and may represent that there may be a switch in the role of M_4 activity in pathological states depending on DA levels.

Dystonic motor phenotypes may be relieved by M_4 antagonists.—Some genetic mutations that are causative for dystonia, such as mutations in the *GNAL gene* (encoding $G\alpha_{olf}$, the major signal transduction protein for D_1DR) or in the gene encoding tyrosine hydroxylase, suggest that diminished DA release, synthesis, or D_1DR signaling underlie the pathophysiology of dystonia in a subset of patients[77–80]. In addition, some dystonia-linked mutations that are not directly linked to the DA system, such as mutations in DYT1, may also show diminished *GNAL* and D_1DR levels[81,82]. Thus, while the pathophysiology underlying different forms of dystonia is complex and diverse, and some forms of dystonia are not likely to involve disruption of DA signaling, pathophysiological changes in DA signaling possibly contribute to several forms of dystonia

Discovery of the *GNAL* link to primary dystonia has contributed to a resurgent interest in examining the DA system in the pathophysiology of dystonia[79]. Interestingly, due possibly to common pathophysiological changes induced by low DA in PD and disturbances in the

dopaminergic system in some dystonia patients, up to 40% of PD patients also have a co-morbidity of dystonia[83,84]. *GNAL* mutations associated with dystonia are believed to be loss of function mutations[80], and initial animal studies modeling mutations in *GNAL* indicates that loss of $G\alpha_{olf}$ can lead to decreased motor coordination and can lead to evoked dystonic like movements[85]. Recent findings from our lab that M_4 directly and tonically inhibits $D_1DR/G\alpha_{olf}$ signaling is interesting given the recent genetic studies showing mutations in *GNAL* are a major cause of adult onset primary dystonia[9,80]. This suggests that loss of function in $G\alpha_{olf}$ induced by mutations in *GNAL* may allow tonic inhibition of $D_1DR/G\alpha_{olf}$ by M_4 to predominate and possibly lead to or exacerbate dystonic motor phenotypes. However, this hypothesis remains to be directly tested in relevant animal models. However, experimental evidence suggests that this may be the case as mice that are heterozygous for *GNAL* have multiple DA deficits, including loss of hyper-locomotive response to psychostimulants and modest motor disturbances[85–87]. Thus, supporting the possibility that loss of $G\alpha_{olf}$ results in a dominant effect of M_4 signaling to induce a dystonic motor phenotype, and that selective antagonists of M_4 may alleviate these symptoms by removing inhibition of $D_1DR/G\alpha_{olf}$ signaling.

Huntington's Disease symptoms may be delayed by M_4 activation.—In Huntington's Disease (HD), before the onset of motor symptoms in experimental models of HD, there is excessive cortico-striatal drive and increased DA release in the striatum of many mouse models of HD[88,89]. This increased glutamatergic and DA drive eventually switches to decreased cortico-striatal drive and decreased DA release by the time that motor symptoms appear[48]. This increased release of neurotransmitter may underlie or exacerbate the degeneration present in HD, or could drive the circuitry changes that lead to the alterations of motor behaviors. If this increase in DA and glutamate release could be normalized, this may delay the onset of symptoms or possibly be neuroprotective. Our mechanistc data suggests that M_4 is well suited to possibly normalize this excessive release, as, in wildtype animals, we have already shown that M_4 can achieve this[11,46]. When mice bearing the mutant *huntingtin* gene are treated daily with an M_4 PAM from the time of increases in neurotransmitter release to when motor symptoms would normally appear, treated mice have delayed motor symptom onset and are indistinguishable from littermate control animals[48]. When chronically treated mice are examined at the electrophysiological level, M_4 PAM treated mice have comparable levels of DA and glutamate release to littermate controls while untreated HD mice have severely decreased neurotransmitter release[48]. This suggests that normalization of this excessive glutamatergic and DA release present in pre-symptomatic time points in HD mice can delay motor symptom onset. Future studies will be required to test whether this treatment mechanism is efficacious in multiple HD models, and if chronic treatment with M_4 PAMs may be neuro-protective in addition to delaying symptom onset.

Conclusions

While nAChRs and multiple mAChR and subtypes regulate DA release and BG function, recent evidence suggests that the M_4 mAChR as the primary mAChR subtype responsible for regulation of DA signaling and related motor behaviors in the BG. We have found that

there are three primary mechanisms behind how M_4 can regulate the BG, one centering on reducing glutamatergic drive onto both the direct and indirect BG pathways[46], and two pathways that center on M_4 reducing DA release and D_1DR signaling in direct pathway SPNs[9,11]. Interestingly, we have found that these mechanisms surrounding M_4 modulating DA and related behaviors are not necessarily restricted to the striatum, and M_4 acting in the SNr through hindbrain cholinergic nuclei are as capable as striatal ChI in regulating direct pathway output[9]. These hindbrain cholinergic nuclei remain largely unexplored in their role at regulating motor behaviors in normal states as well as motor disabilities present in movement disorders. While animal models and clinical evidence suggests that these hindbrain nuclei may play a critical role in regulating the expression and severity of certain aspects of motor disabilities, their role and possible manipulation for therapeutic benefit remain largely unexplored [58,59].

Our findings of the mechanisms of M_4 regulation of DA and BG related behaviors have major possible implications for the treatment of movement disorders, especially given the established efficacy of anti-mAChR compounds at reducing certain motor symptoms of movement disorders (summarized in table 1)[7,16,17]. This raises the distinct possibility that selective and specific M_4 modulators may retain the efficacy seen with non-selective compounds while avoiding the adverse side effects. Broadly, we believe that in hyper-DA states such as in schizophrenia, LID, and HD M_4 activation (either through PAMs or orthosteric agonists) will be beneficial, while conversely in states of low DA release or signaling, such as in PD and dystonia, M_4 antagonists will be beneficial.

Our hypothesis surrounding modulation of M_4 being beneficial in diseases where DA is disturbed will likely be tested clinically in hyper-DA states first. This is due to the discovery of M_4 PAMs that are highly subtype-selective being used widely in preclinical animal models, especially for schizophrenia, and to a lesser extent LID and HD. These studies have provided the preclinical rationale to develop molecules that are advancing as drug candidates for future clinical trials. These molecules, once tested in clinical trials, will ultimately be critical in providing evidence that this target is safe and devoid of central or peripheral adverse effects. For example, based on the actions of M_4 PAMs in pre-clinical animal models, it is possible that activation of M_4 could decrease motivation and locomotion while antagonism of M_4 may have modest cognition-impairing effects and could induce dyskinesia or worsen LID. However, it is possible that activation of M_4 will have an adverse effect profile that is not mechanistically related the adverse effects of inhibition of M_4.

Unlike our hypothesis of M_4 PAMs being useful in hyper DA states, testing our hypothesis surrounding M_4 antagonists being useful for states of low DA has been more difficult. This is due to a lack of truly selective M_4 antagonist tool compounds, and most studies to date have relied upon the use non-selective mAChR antagonists combined with genetic tools to remove M_4 expression or activity to show specificity of the non-selective antagonist to M_4. M_4 antagonists that are selective and specific, however, have been harder to achieve, and compounds that have been published have not achieved suitable selectivity. Development of the first truly selective M_4 antagonists will represent a major breakthrough, and will allow for the direct testing of our hypothesis that M_4 antagonists will retain or exceed the efficacy of non-selective mAChR antagonists in symptomatically treating the motor symptoms of

movement disorders in relevant pre-clinical animal models of disease. These compounds will allow both the testing of our hypothesis that M_4 antagonists will be beneficial for certain symptom domains of movement disorders, but also test for on-target adverse effects of M_4 modulation. For example, based off the expression profile of M_4 and our pre-clinical evidence, it is possible that inhibition of this receptor may modestly disrupt cognition and motivation. However, these potential effects remain to be tested both pre-clinically and clinically. Studies with selective and specific M_4 antagonists will provide critical pre-clinical evidence of the role of M_4 verse other subtypes, such as M_1, in the expression of motor deficits, and the primary mAChR subtype or subtypes responsible for the efficacy of non-selective anti-muscarinic therapeutics.

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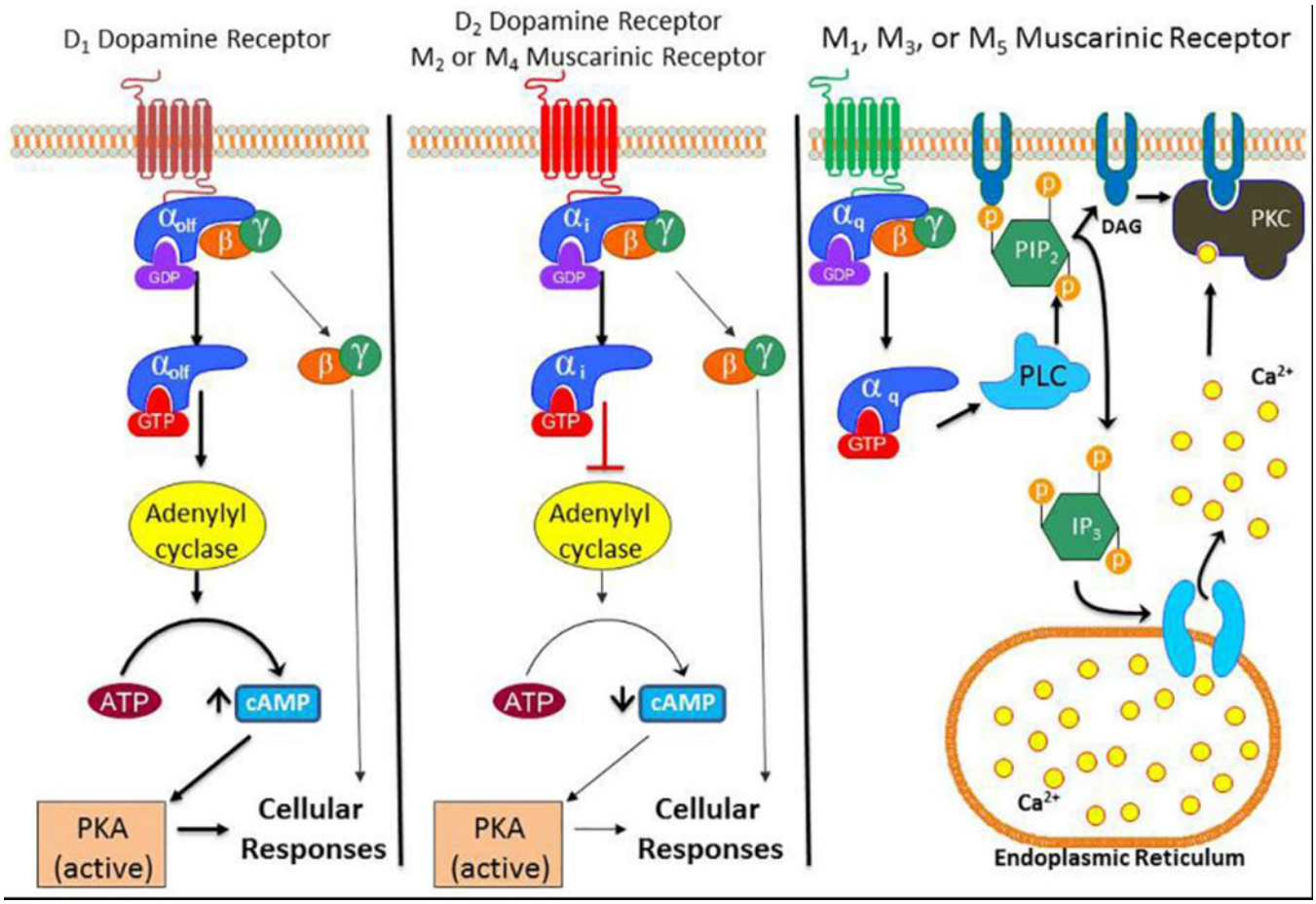

Figure 1. Canonical Signaling Pathways of Dopamine and Muscarinic Receptors.

The D_1 Dopamine Receptor couples to $G\alpha_{olf}$ which replaces $G\alpha_s$ in the striatum. $G\alpha_{olf}$ will dissociate from the β and λ subunits of the heterotrimeric G protein complex upon dopamine binding the D_1 receptor and $G\alpha_{olf}$ switching GDP with GTP, and becoming active. $G\alpha_{olf}$ will then bind and activate adenylyl cyclase which will stimulate the conversion of ATP to cAMP. cAMP is a key second messenger that lead to the activation of protein kinase A (PKA), which, when active, will cause a number of intracellular responses, including increasing neurotransmitter release. Conversely, the D_2 Dopamine Receptor, and the M_2 and M_4 muscarinic couple to $G\alpha_{i/o}$. After dissociation like above, when active, $G\alpha_{i/o}$ will lead to the inhibition of adenylyl cyclase and decreasing cAMP and subsequent PKA activation. M_1, M_3, and M_5 couple to a different G protein termed $G\alpha_q$. Upon activation, after acetylcholine binds the receptor, $G\alpha_q$ will cause the activation of phospholipase C (PLC). PLC will cause the cleavage of PIP_2 in IP_2 and diacylglycerol (DAG). IP_2 will bind receptors on the endoplasmic reticulum that will lead to Ca2+ into the cytoplasm. Ca2+ and DAG will then bind to and activate protein kinase C (PKC), which will then lead to a number of intracellular responses.

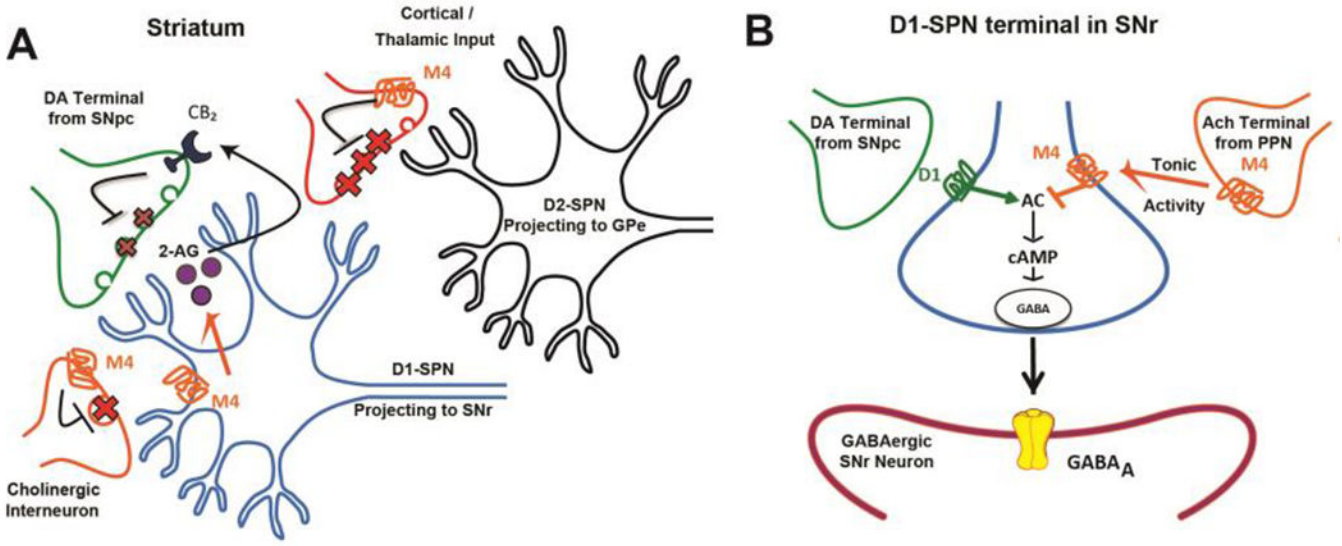

Figure 2. Model of M_4 Regulation of the Basal Ganglia

(A) In the striatum, M_4 has multiple actions on the circuitry of the BG. On cortical and thalamic inputs into the striatum, activation of presynaptic M_4 on these terminals can decrease glutamate release and promote long-term depression in the striatum. This change in glutamatergic drive occurs on direct and indirect pathway spiny projection neurons (SPNs). M_4 activation specifically on D_1-SPNs causes the release of an endocannabinoid, likely 2-AG, (purple circles), which acts on cannabinoid receptor 2 (CB_2) receptors on SNc dopaminergic terminals to induce a sustained inhibition of DA release. The effects of decreased DA release from direct pathway SPNs modulating indirect pathway SPNs remains unclear. M_4 activation on cholinergic interneurons is suggested to decrease tonic firing and ACh release, however, no studies have directly examined this using selective pharmacological or genetic tools. (B) In the SNr, M_4 decreases GABA release probability from D_1-SPNs onto GABAergic cells of the SNr. This likely occurs through D_1DR and M_4 having competing actions on adenylate cyclase (AC), cAMP production, and downstream cAMP signaling. Additionally, this M_4 mediated inhibition of D_1DR signaling in D_1-SPN terminals occurs tonically. However, it remains unclear whether this is caused by tonic activity of the M_4 receptor, or tonic release of ACh from cholinergic cells of the PPN.

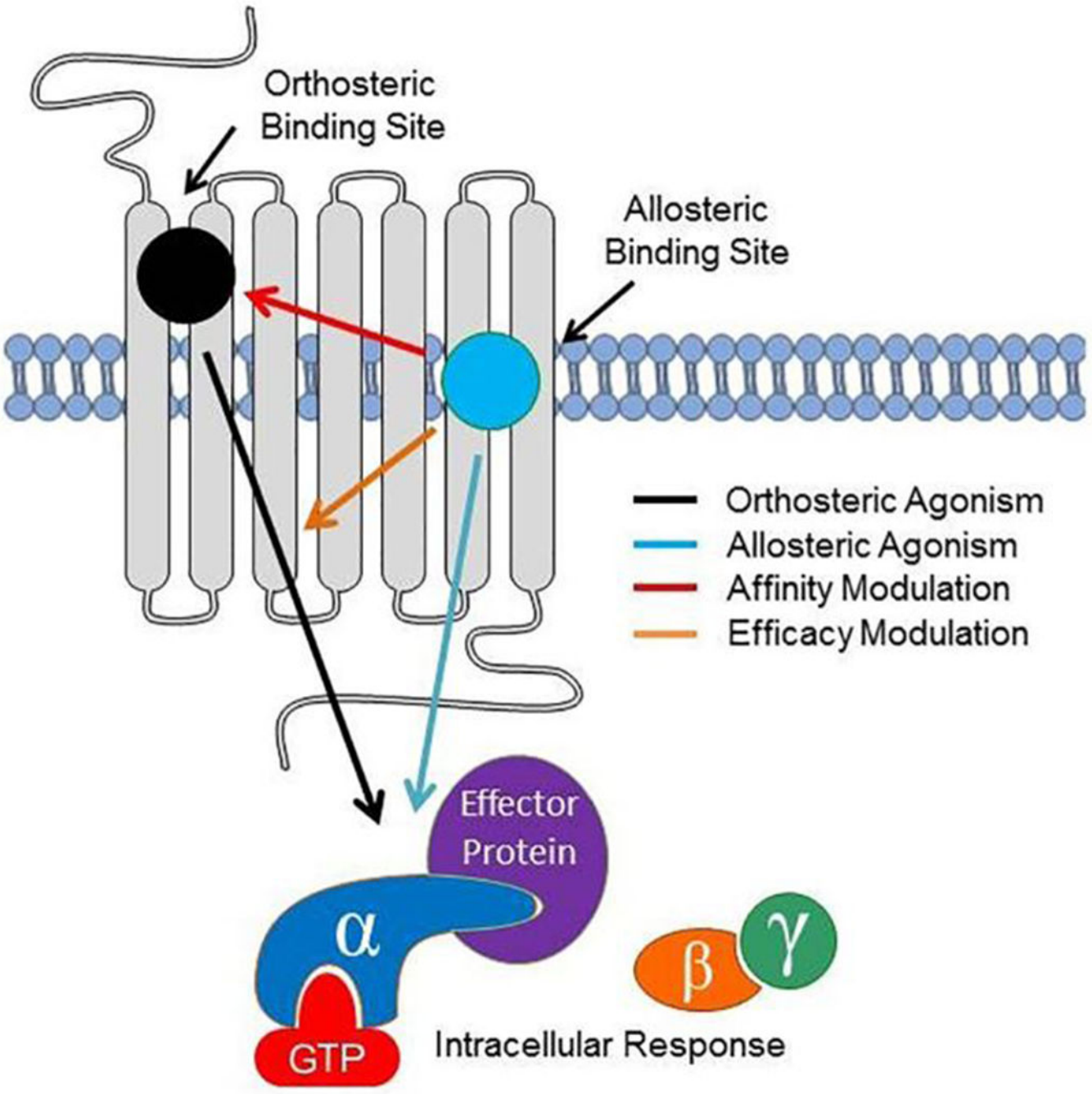

Figure 3. Mechanisms of allosteric modulators.

Binding of the endogenous agonist to its receptor at the orthosteric site leads to the activation of intracellular responses. Allosteric modulation of the receptor can occur through several mechanism. An allosteric modulator can lead to the direct activation of an intracellular response in the absence of an endogenous agonist (allosteric agonist). Additionally, an allosteric modulator can alter the response of an endogenous agonist

through modulation of the receptor's affinity of the endogenous agonist or through modulating the efficacy of the receptor's response to the endogenous agonist.

Table 1.

Summary of proposed mechanisms of action for M_4 selective drugs in movement disorders with hyperdopaminergic and hypodopaminergic states.

	Disease	Proposed Mechanism	Direction of Activity	Disease Modifying	Refs
Hyperdopaminergic	Huntington's Disease	Normalizing increased cortico-striatal glutamate release and increased dopamine release at pre-symptomatic timepoints	Activation or Potentiation	Possibly	48
	Levodopa Induced Dyskinesia	Reducing hyper-dopaminergic state in remaining dopamine fibers	Activation or Potentiation	Symptomatic	47
Hypodopaminergic	Dystonia	Removing muscarinic mediated inhibition of dopamine release and signaling of the basal ganglia direct pathway	Inhibition	Symptomatic	9, 11
	Parkinson's Disease	Removing muscarinic mediated inhibition of dopamine signaling and release	Inhibition	Symptomatic	9, 11, 67