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REVIEW

Outline of Therapeutic Interventions With Muscarinic Receptor-Mediated Transmission

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Summary

Muscarinc receptor-mediated signaling takes part in many physiological functions ranging from complex higher nervous activity to vegetative responses. Specificity of action of the natural muscarinic agonist acetylcholine is effected by action on five muscarinic receptor subtypes with particular tissue and cellular localization, and coupling preference with different G-proteins and their signaling pathways. In addition to physiological roles it is also implicated in pathologic events like promotion of carcinoma cells growth, early pathogenesis of neurodegenerative diseases in the central nervous system like Alzheimer´s disease and Parkinson´s disease, schizophrenia, intoxications resulting in drug addiction, or overactive bladder in the periphery. All of these disturbances demonstrate involvement of specific muscarinic receptor subtypes and point to the importance to develop selective pharmacotherapeutic interventions. Because of the high homology of the orthosteric binding site of muscarinic receptor subtypes there is virtually no subtype selective agonist that binds to this site. Activation of specific receptor subtypes may be achieved by developing allosteric modulators of acetylcholine binding, since ectopic binding domains on the receptor are less conserved compared to the orthosteric site. Potentiation of the effects of acetylcholine by allosteric modulators would be beneficial in cases where acetylcholine release is reduced due to pathological conditions. When presynaptic function is severly compromised, the utilization of ectopic agonists can be a thinkable solution.

Key words

Muscarinic receptors • G-proteins • Allosteric modulators • Ectopic agonists • Selectivity

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Physiology of muscarinic receptors

Muscarinic receptors belong to the family of G-protein coupled receptors (GPCR) that are the most abundant and pharmacologically targeted plasma membrane receptors (Lander *et al.* 2001, Fredriksson *et al.* 2003). A common structural feature of GPCR is the extracellular N-terminus, seven membrane spanning domains, three extracellular and three intracellular loops, and an intracellular C-terminus. Stimulation of various GPCRs leads to activation of particular G-proteins and their intracellular signaling pathways that play important regulatory roles in virtually all physiological functions. In addition to these well-established pathways, it has also been demonstrated that receptors also transduce non-G-protein-mediated signaling *via* arrestins and G-protein receptor kinases (Lefkowitz 1998, Lefkowitz and Shenoy 2005, Reiter and Lefkowitz 2006).

To date five subtypes of muscarinic receptors denoted as M_1-M_5 and encoded by five different genes have been discovered (Kubo *et al.* 1986a,b, Bonner *et al.* 1987, 1988, Peralta *et al.* 1987, Bonner 1989a,b). Muscarinic receptors are widely expressed in both the central and peripheral nervous system, with distinct cellular as well as tissue localization of individual subtypes. They mediate various physiological functions

of their natural agonist acetylcholine ranging from complex higher nervous functions such as arousal, memory and alertness to vegetative processes such as regulation of heart rate and cardiac output, blood pressure, temperature regulation, perspiration, secretion of exocrine and endocrine glands, and motility of the gastrointestinal tract (Eglen 2006, 2012). In addition to these functions mediated by neuronal acetylcholine, muscarinic receptors also play a role in mediating local responses of non-neuronally derived acetylcholine, e.g. modulation of immune responses or regulation of local circulation (Kawashima and Fujii 2004, 2008, Wessler and Kirkpatrick 2012). Non-neuronal acetylcholine has also been implicated in paracrine control influencing lung cancer growth through both nicotinic and muscarinic receptors signaling (Song *et al.* 2003a,b, Proskocil *et al.* 2004, Song *et al.* 2007, Schuller 2009).

Pharmacology of muscarinic receptors

Individual muscarinic receptor subtypes share a high degree of homology in the transmembrane domains while extracellular and intracellular loops are less well conserved (Hulme *et al.* 1990, 1991, 2003). The intracellular C-terminus may form the fourth intracellular loop by means of a glycosyl anchor. The N-terminal part of the third intracellular loop represents the contact domain for interaction with G-proteins (Wess *et al.* 1995, Hu *et al.* 2010). Higher variability of this domain enables selectivity of interaction with different G-proteins. The M_1, M_3, and M_5 receptor subtypes preferentially activate $G_{q/11}$ G-protein intracellular signaling while the M_2 and M_4 subtypes prefer $G_{i/o}$ G-proteins and activate their signaling pathways (Jones *et al.* 1991).

Muscarinic receptors have a classical (orthosteric) binding site for natural or exogenous agonists located deep in a pocket created by the transmembrane segments of the protein that are highly conserved among individual receptor subtypes (Hulme *et al.* 2003). Due to high conservation of the orthosteric site there are virtually no known selective orthosteric agonists. It is thus of prime importance to find out a way to influence selectively signaling pathways of individual muscarinic receptors. Apart from the orthosteric binding site that is naturally occupied by the endogenous agonist acetylcholine muscarinic receptors have allosteric binding sites located on less conserved extracellular loops. Allosteric ligands bind to an allosteric site on the receptor and may either activate the receptor by themselves or modulate receptor activation by acetylcholine. They exhibit subtype selectivity because they bind to less conserved receptor domains. Binding of allosteric ligands results in remarkable subtype selective influencing of orthosteric ligand binding that depends on the receptor subtype and the specific pair of orthosteric-allosteric ligands (Jakubik *et al.* 1995, 1997, 2005). Allosteric ligands (modulators) change receptor conformation and in this way increase, decrease, or have no influence (positive, negative, or neutral cooperativity) on the binding affinity of given orthosteric agonists, including the natural agonist acetylcholine (Jakubik and El-Fakahany 2010). The advantage of allosteric modulators is that their effect, with respect to the specific receptor-activated pathway, is given by the factor of cooperativity with orthosteric ligand that dictates a maximal degree of interaction of binding of both agents. This results in eliminating a danger of overdosing.

There are also so called ectopic ligands (Fig. 1 and 2) that attach to more distal parts of the receptor binding site pocket that is less conserved. Unlike allosteric modulators they prevent binding of orthosteric ligands to the orthosteric site. However, the selectivity of known ectopic ligands in terms of binding affinity to different receptor subtypes is generally poor. On the other hand, some of these compounds exhibit significant functional selectivity (e.g. N-desmethylclozapine, AC-42), which makes them good candidates for pharmacotherapy.

The next type of compounds that bind to muscarinic receptors are so called bitopic ligands. These agents can bind to two sites on a single receptor. An example is 77-LH-28-1 that was identified from a series of AC-42 analogs (Langmead *et al.* 2008) and shown to have selectivity for M1 receptors (Heinrich *et al.* 2009). *In vitro* studies indicated competitive interaction between the orthosteric antagonist scopolamine and 77-LH-28-1 (Langmead *et al.* 2008). Further functional and site-directed mutagenesis studies have demonstrated an allosteric mode of agonist action for this ligand. Another example of ligand that binds both to orthosteric and allosteric sites and can be labeled as bitopic is xanomeline (Jakubík *et al.* 2002). Xanomeline is one of few functionally selective muscarinic agonists. It preferentially activates M_1 and M_4 receptors while it has long-term antagonistic effects at M_5 receptors (Grant and El-Fakahany 2005, Grant *et al.* 2010). In addition, part of xanomeline binding that depends on the O-hexyl group of the molecule (Jakubik *et al.* 2004) is resistant to washing

(Christopoulos *et al.* 1998, Jakubik *et al.* 2002, 2006). Interestingly, wash-resistant xanomeline itself acts on the receptor both competitively and allosterically (Jakubik *et al.* 2002, Machová *et al.* 2007).

There is accumulating evidence that muscarinic receptors can be activated *via* several different allosteric sites (Jakubík *et al.* 1996, Lebois *et al.* 2010) and ectopic sites (Langmead *et al.* 2008). Thus regardless of the binding mode (orthosteric, ectopic, allosteric or bitopic; Fig. 1 and 2) ligands can act as agonists (induce response like natural neurotransmitter) or neutral antagonists (produce no response on their own but block activation by agonists) or inverse agonists (induce response opposite to the natural neurotransmitter).

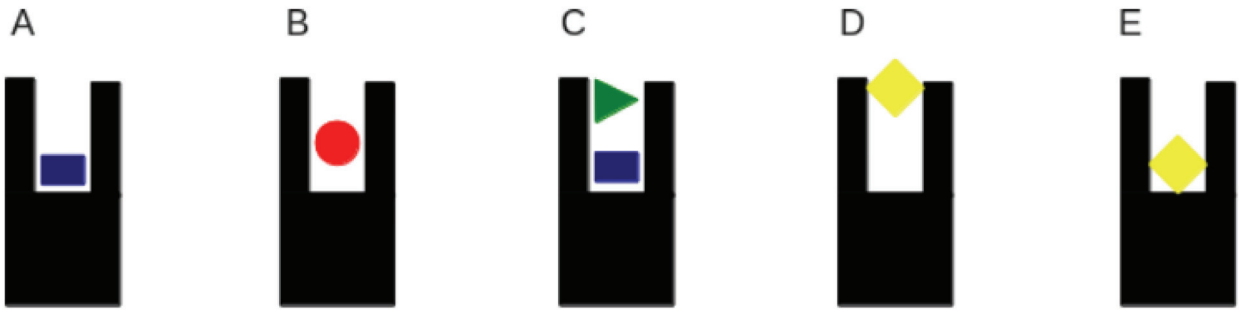

Fig. 1. Schematic representation of ligand binding modes. Binding of the orthosteric ligand (blue rectangle) to the othosteric site **(A)**, binding of the ectopic ligand (red circle) to the ectopic site that is different from the orthosteric site but prevents binding of the othosteric ligands **(B)**, allosteric ligand (green triangle) binds to the allosteric binding site concurrently with the orthosteric ligand **(C)**, bitopic ligand (yellow diamod) can bind to the allosteric binding site **(D)** as well as to the orthosteric binding site **(E)**.

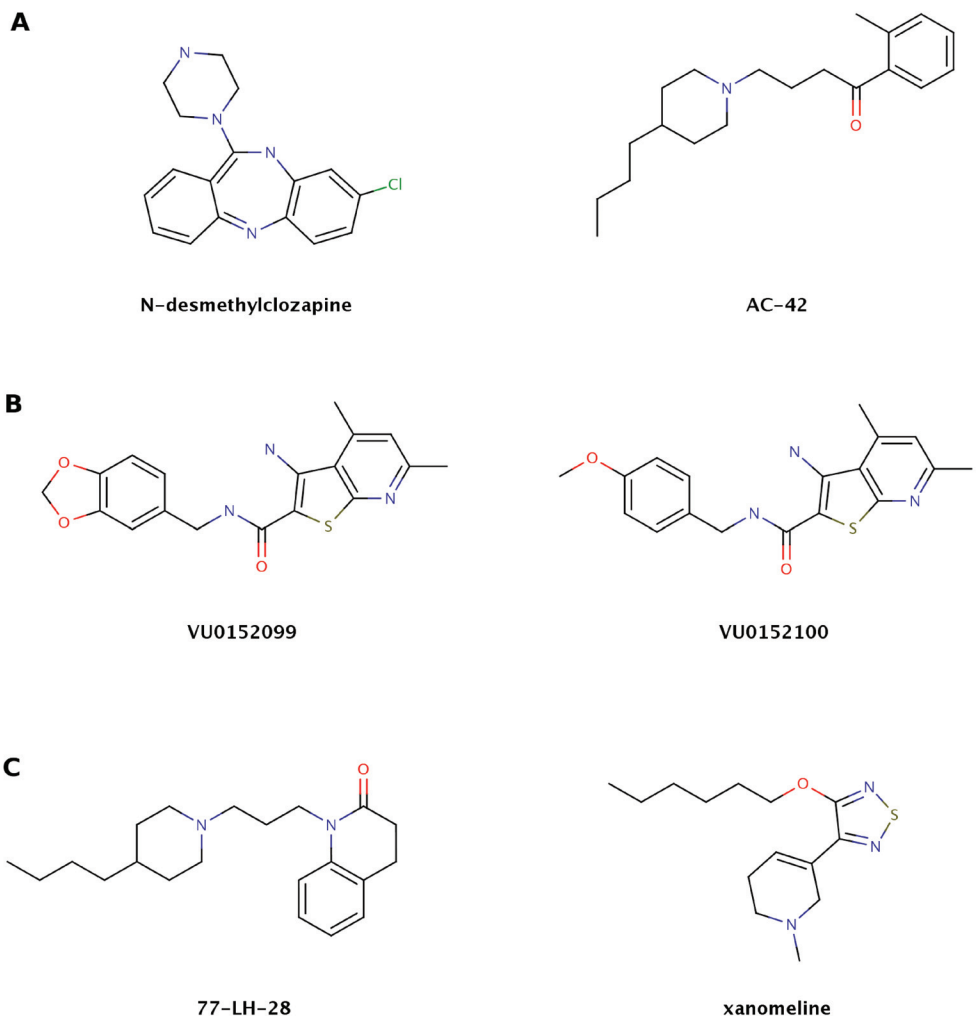

Fig. 2. Structures of atypical muscarinic ligands. **A**, ectopic ligands N-desmethylclozapine and AC-42; **B**, allosteric agonists VU0152099 and VU0152100; **C**, bitopic agonists 77-LH-28-1 and xanomeline.

Alzheimer's disease

Alzheimer´s disease (AD) is the most widespread dementing neurodegenerative disease. It was described in 1907 by Alois Alzheimer and since then enormous efforts have been exerted to find out how it originates and explore possibilities of an efficient treatment. Original pathological findings of amyloid plaques, neurofibrillary tangles, and impairments of the brain cholinergic system led to the formulation of the "cholinergic hypothesis" of AD (Bartus *et al.* 1982). Later on fragments of the amyloid precursor protein (APP), a major protein isolated from amyloid plaques (Masters *et al.* 1985a,b), were discovered (Kang *et al.* 1987). Proof of increased accumulation of these fragments in Alzheimer's brains gave rise to the "amyloid cascade hypothesis" (Hardy and Higgins 1992). Overproduction of Aβ fragments in hereditary cases of the disease is due to known defects of genes for APP localized on chromosome 21, presenilin 1 on chromosome 14 (Sherrington *et al.* 1995), and presenilin 2 on chromosome 1 (Levy-Lahad *et al.* 1995a,b). However, the reason for their increased production in sporadic cases representing the majority (up to 98 %) of cases is largely unknown. Allelic polymorphism of the ApoE gene is a major genetic risk factor in sporadic early onset AD that can nonetheless account for no more than 5-15 % of cases. By far the major risk factor of the disease is increasing age yet it is not known how it contributes to development of the disease. It has been suggested that exposure to a variety of insults during life cycle may lead to the gradual accumulation of native β-amyloid (Aβ) fragments and finally to the common clinical and pathological picture of Alzheimer´s disease (Mesulam 1999, Selkoe 2001, 2002, Kukar *et al.* 2005, Turner and Nalivaeva 2007, Karran *et al.* 2011).

The amyloid cascade hypothesis postulates that the primary event in the pathogenesis of AD is the overproduction of Aβ fragments as a result of known genetic defects in hereditary cases of the disease (Hardy 1997). It is now generally accepted that the causal agent that triggers and drives the disease progression is increased concentration of small soluble oligomers of Aβ, mainly fragment $A\beta_{1-42}$ (Selkoe 2002, Lesne *et al.* 2006, 2008, Maezawa *et al.* 2011, Shankar *et al.* 2011). However, familial AD disease represents only about 1 % of all cases. This has urged for investigations of the physiological function of Aβ that should help to explain the high prevalence of the disease in sporadic cases. The fragments of Aβ that are generated by sequential cleavage at the β and γ sites of APP have been reported to have both neuroprotective and neurotoxic effects (Whitson *et al.* 1989, 1990, Yankner *et al.* 1990, Pike *et al.* 1991). More recently, the specific physiological role of major Aβ fragments connecting APP and lipid metabolism has been demonstrated. Fragment $A\beta_{1-40}$ downregulates cholesterol synthesis by inhibiting hydroxymethylglutaryl-CoA synthase whereas fragment $A\beta_{1-42}$ decreases sphingomyeline levels by activating neutral sphingomyelinase (Grimm *et al.* 2005, 2007). In turn, changes in membrane lipid composition influence APP processing (Kojro *et al.* 2001, Grimm *et al.* 2008, 2011). The amyloid precursor protein is a receptor-like membrane protein. Tuning of proteolytic amyloidogenic/ nonamyloidogenic processing depends on plasma membrane properties and localization in membrane domains (Schneider *et al.* 2006, 2008, Hicks *et al.* 2012) and the same may be true for other transmembrane proteins including G-protein-coupled receptors (Rudajev *et al.* 2005, Michal *et al.* 2007, 2009).

Original neurochemical findings in Alzheimer´s disease brains pointed out disturbances of acetylcholine metabolism (Bowen *et al.* 1976, Davies and Maloney 1976, Perry *et al.* 1977a,b, Sims *et al.* 1981, Francis *et al.* 1985, 1999). Since then a large body of evidence supporting as well as questioning this hypothesis has accumulated (Bartus and Emerich 1999, Bartus 2000). Several lines of evidence argue for viability of the cholinergic hypothesis. Cholinergic muscarinic transmission plays an important role in mental functions like attention, learning, and memory (Peralta *et al.* 1988, Ehlert *et al.* 1994, Lahiri *et al.* 2004, Koch *et al.* 2005). These functions decline in the course of natural aging and more so in AD. In primates such a decline correlates with a decrease in the number of cholinergic neurons in the basal forebrain and treatments that rescue these neurons lead to improvement of cognitive performance (Smith *et al.* 1999, Conner *et al.* 2001). Cholinergic neurons are very sensitive to changes in homeostasis and disturbances of cognitive performance also accompany various insults like head trauma, intoxications, and hypoxia. Up to now the major therapeutic interventions that demonstrate certain benefits target the cholinergic system (e.g. clinically approved cholinesterase inhibitors). Conversely, it has been shown that application of antimuscarinic treatment in patients with Parkinson´s disease results in a significant increase in the probability to develop Alzheimer´s disease (Perry *et al.* 2003). In line

with this finding is an enhancement of amyloid pathology in transgenic APPswe/ind mice that express low levels of M_1 muscarinic acetylcholine receptors (Davis *et al.* 2010).

Aging is by far the most imporatant risk factor in sporadic Alzheimer´s disease. A decline of cholinergic transmission naturally occurring during aging is dramatically accentuated in Alzheimer´s disease and underlies cognitive symptoms of this devastating disorder. Up to now the only treatment of this disease that shows certain benefit is the use of cholinesterase inhibitors (Wilkinson *et al.* 2004). These drugs prevent hydrolysis of the endogenous muscarinic agonist acetylcholine and can thus be effective only when the presynaptic component of cholinergic synapses is operating. This is often not the case in clinically manifested stages of Alzheimer's disease. Moreover, preservation of synaptic acetylcholine by these compounds results not only in beneficial memory enhancing effects (through M_1 muscarinic receptors), but also significant side effects (mediated by other subtypes of muscarinic receptors). Muscarinic receptors are rather well preserved even in the late state of the disease although their activation appears somewhat compromised in the course of healthy aging and more so during disease progression (Tsang *et al.* 2006, Machová *et al.* 2008, 2010, Janickova *et al.* 2013). Thus M_1 selective agonists bear therapeutic potential for treatment of Alzheimer's disease. Recently, systemically active M_1 allosteric agonists VU0152099 and VU0152100, were synthesized at the Vanderbilt Center for Neuroscience Drug Discovery (Lebois *et al.* 2010).

The cholinergic and amyloid hypotheses are not mutually exclusive (Isacson *et al.* 2002). As mentioned above, the increase in Aβ concentration in hereditary cases is due to known gene defects. The link between cholinergic neurotransmission and increase in Aβ concentration has been demonstrated *in vitro*. Stimulation of $G_{q/11}$ G-protein coupled M_1 and M_3 muscarinic receptors increases non-amyloidogenic cleavage of APP at the α site by α-secretase and in this way prevents amyloidogenic processing of APP (Buxbaum *et al.* 1992, Nitsch *et al.* 1992). Weakening of cholinergic muscarinic signal transduction may thus lead to an increase in Aβ production and consequently to the acceleration of disease progression (Doležal and Kašparová 2003). Indeed, inhibition of $G_{q/11}$ G-protein function has been demonstrated in rodent primary cultures as a reduction of muscarinic receptor-induced GTPase activity (Kelly *et al.* 1996), and as a decrease in $G_{q/11}$ G-protein concentration (Kelly *et al.* 2005) and attenuation of muscarinic receptor-stimulated phosphatidylinositol hydrolysis in plasma membranes prepared from *post mortem* brain samples of Alzheimer's patients (Jope *et al.* 1997, Thathiah and De Strooper 2009).

Schizophrenia

Schizophrenia is a diagnosis that covers a set of disorders of different etiologies with the same symptoms. This disorder can be divided based on the presence or absence of negative symptoms or according to DSM-IV (The Diagnostic and Statistical Manual of Mental Disorders) to paranoid, disorganized, catatonic, undifferentiated, and residual types. Schizophrenia is characterized by faint pathology and has both sporadic and hereditary forms. The common pathologic aspect of schizophrenia is excessive dopaminergic transmission in striatal and mesolimbic areas that can be abated by dopamine D_2 receptor antagonists, and deficit of dopamine signaling in prefrontal cortex (Karam *et al.* 2010). An alternative hypothesis for the development of schizophrenia symptoms involves muscarinic receptors. Clinical trials provided evidence that muscarinic agonists are moderately effective as antipsychotic agents (Biel *et al.* 1962, Mego *et al.* 1988). Moreover, it has been shown that the levels of both M_1 and M_4 receptors are reduced in the prefrontal cortex, hippocampus, caudate and putamen in *post mortem* samples from schizophrenic patients (Dean *et al.* 1999, 2002, Crook *et al.* 1999, 2000, 2001). From studies in knockout mice, the M_1 receptor subtype has been viewed as the most likely candidate for mediating effects on cognition, attention mechanisms, and sensory processing so reduction in M_1 receptors may be the cause of cognitive symptoms of schizophrenia. The M_4 receptor is localized in dopamine rich brain regions (the mesolimbic dopaminergic pathway), and regulates dopamine levels in this region (Tzavara *et al.* 2004). Thus the "dopamine hyperfunction hypothesis" and the "cholinergic hypothesis" of schizophrenia are compatible.

The importance of the cholinergic system in schizophrenia has been further validated clinically by the use of clozapine, one of the most clinically useful atypical antipsychotics (Kane *et al.* 1988, Hagger *et al.* 1993, Goldberg and Winberger 1994). Numerous studies suggest that the unique efficacy of clozapine is due to its major circulating metabolite, N-desmethylclozapine (NDMC) acting as an M_1 ectopic agonist (Weiner *et al.*

2004, Burstein *et al.* 2005, Davies *et al.* 2005) in combination with its inhibition of D_2 receptors. Taken together M_1 and M_4 selective agonists have a potential to alleviate cognitive deficits and positive symptoms of schizophrenia. The studies with positive allosteric modulators of acetylcholine at M_4 receptors VU0152099 and VU0152100 (Brady *et al.* 2008, Shierey *et al.* 2008, Byun *et al.* 2011) provide further support for the "cholinergic hypothesis" of shizophrenia.

Overactive bladder

Current therapy of overactive bladder relies on inhibition of M_3 (and M_2) receptors of lower urinary tract smooth muscles by long acting muscarinic antagonists (LAMAs) (Smith and Wein 2010). LAMAs produce symptomatic improvement by decreasing detrusor overactivity, increasing bladder capacity, and reducing urgency and urge of urinary incontinence and frequency (Smith and Wein 2010). LAMAs, however, exert adverse effects, mainly dry mouth and constipation, probably due to the lack of binding selectivity. Their effect is primarily based on slower kinetics at M_3 receptors (Hegde 2006, Sykes *et al.* 2012). Thus, there is room for improvement of LAMAs in binding selectivity that would be beneficial in dose lowering and diminution of side effects. Importantly, currently available LAMAs do not possess the O-hexyl group that is responsible for xanomeline wash-resistant binding (Jakubík *et al.* 2004). Combination of potential M_3 selective antagonists with O-hexyl groups may thus open an avenue to synthesize new classes of LAMAs.

Drug addiction

Drug addiction is a disease that is not primarily caused by cell damage. Addictive drugs impact regular learning to reinforce their own intake. In general, addictive drugs increase dopaminergic transmission in the striatum (Sulzer 2011). Blocking of M_5 receptors has been shown to reduce reinforcement and withdrawal symptoms of morphine (Basile *et al.* 2002) as well as cocaine addiction (Lester *et al.* 2010). Occurrence of M_5 receptors in the body is limited to cerebral blood vessels (Yamada *et al.* 2001) and neurons of specific regions of brain-ventral tegmental area of substantia nigra, hippocampus, and striatum (Yamada *et al.* 2003, Raffa 2009). In the striatum M_5 receptors located on dopaminergic nerve terminals facilitate muscarinic agonist-induced dopamine release, a key process of drug addiction events of reward, reinforcement and withdrawal (Koob and Volkow 2010, Morales and Pickel 2012). Moreover, striatum innervating dopaminergic neurons almost exclusively express the M_5 receptor subtype (Yamada *et al.* 2001). Therefore M_5 antagonists have potential therapeutic use for treatment of drug addiction and abuse with minimum side effects. No M_5 selective antagonists are known so far (Eglen *et al.* 2006, Raffa 2009, Stahl *et al.* 2010). Search for ectopic antagonists that bind to the less conserved parts of the receptor but still effectively block the receptor by interaction with the orthosteric site may be a way to obtain potent M_5 selective antagonists.

Conclusions

The major problem of muscarinic pharmacotherapy is the paucity of targets influencing of muscarinic neurotransmission. The use of anticholinesterases to strengthen transmission, e.g. in treatment of Alzheimer´s disease, by prolonging the presence of the natural agonist acetylcholine in the synaptic cleft does not discriminate among various signaling pathways activated by various muscarinic receptor subtypes and consequently suffers of many side-effects and a peril of overdosing. Despite this disadvantage cholinesterases inhibitors are up to now the only approved drugs for Alzheimer´s disease that demonstrate marked therapeutic benefits. Provided that presynaptic function is at least partially preserved, allosteric modulators of acetylcholine binding provide unusual selectivity and may serve as a drug for selective activation (e.g. in Alzheimer´s disease) or attenuation (e.g. in Parkinson´s disease) of neurotransmission mediated by different muscarinic receptors. When presynaptic function is severly compromised, the utilization of ectopic agonists can be a thinkable solution. Unfortunately, in either case, no clinically exploitable drugs have been generated yet.

Conflict of Interest

There is no conflict of interest.

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