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·Review·

M1 muscarinic acetylcholine receptor in Alzheimer's disease

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The degeneration of cholinergic neurons and cholinergic hypofunction are pathologies associated with Alzheimer's disease (AD). Muscarinic acetylcholine receptors (mAChRs) mediate acetylcholine-induced neurotransmission and five mAChR subtypes (M1–M5) have been identified. Among them, M1 mAChR is widely expressed in the central nervous system and has been implicated in many physiological and pathological brain functions. In addition, M1 mAChR is postulated to be an important therapeutic target for AD and several other neurodegenerative diseases. In this article, we review recent progress in understanding the functional involvement of M1 mAChR in AD pathology and in developing M1 mAChR agonists for AD treatment.

Keywords: agonist; Alzheimer's disease; amyloid; cholinergic hypofunction; M1 muscarinic acetylcholine receptor; tau

Introduction

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder afflicting millions of people. It is diagnosed by the progressive loss of cognitive function and behavioral deficits and is characterized by the presence of neurofibrillary tangles (NFTs), senile plaques, cholinergic neuron loss, and neuronal atrophy at autopsy[1, 2].

Senile plaques and NFTs are major pathological hallmarks of AD in the brain. Senile plaques consist of deposits of small peptides called β-amyloid (Aβ). Multiple lines of evidence suggest that the overproduction/aggregation of neurotoxic Aβ in vulnerable brain regions is the primary cause of AD[3-6]. NFTs are formed by accumulation of hyperphosphorylated tau protein[7, 8]. Tau is a microtubule-binding protein whose function is to stabilize microtubules and facilitate fast axonal transport. Once highly phosphorylated, tau dissociates from microtubules and is prone to aggregate, forming paired helical filaments that aggregate into NFTs[9, 10].

The third important hallmark of AD is cholinergic hypofunction. The neurotransmitter acetylcholine (ACh) exerts its physiological functions by activating either ionotropic nicotinic ACh receptors (nAChRs) or metabotropic muscarinic ACh receptors (mAChRs). It has been reported that in AD brains there are (1) reduced choline acetyltransferase levels accompanied by decreased ACh synthesis; (2) significant loss of cholinergic neurons; (3) reduction in the numbers of postsynaptic neurons accessible to ACh; (4) cholinergic neuronal and axonal abnormalities; and (5) reduction in nAChR levels[11-19]. Recent evidence indicates that cholinergic hypofunction is closely linked to Aβ and tau pathologies[20]. As a major receptor group for ACh, mAChRs have also been implicated in the pathophysiology of AD. In the present review, we focus on M1 mAChR, the dominant mAChR subtype involved in learning and memory, and discuss its involvement in AD.

Overview of the mAChR Family

mAChRs are seven-transmembrane G-protein-coupled receptors. Upon binding to the endogenous neurotransmitter ACh, mAChRs couple to G proteins to transduct signals[21-23]. So far, five mAChR subtypes (M1–M5) have been identified and are divided into two categories based on the manner of signal transduction: M1, M3, and M5 subtypes preferentially interact with the $G_{q/11}$ family of G proteins, activating phospholipase C and mobilizing intracellular calcium, while M2 and M4 subtypes couple to the $G_{o/i}$ family, inhibiting adenylate cyclases and reducing intracellular cAMP levels[24-26]. The amino-acid sequences of the five mAChRs are highly conserved, with an average sequence consensus of 56.6% (Fig. 1A, B). Phylogenetic analysis indicates that the relationship between the M2 and M4 subtypes is much closer than those among the M1, M3, and M5 subtypes (Fig. 1C). These mAChRs share a highly-conserved pocket deep within the transmembrane regions, and ACh binds to amino-acid residues on the outer region of the binding pocket with a critical asparagine (Asp105) residue[23, 27]. The similarity in ligand-binding sites across all five subtypes makes it difficult to design subtype-selective ligands. In addition, mAChR subtypes possess numerous allosteric sites at which compounds modulate the function of the receptor upon binding[28-30]. Importantly, most of the allosteric sites differ greatly among mAChR subtypes and thus provide opportunities to design highly subtype-selective allosteric modulators of mAChRs[30].

Members of the mAChR family are widely expressed in various regions in the central nervous system (CNS) and in the peripheral system. They play crucial roles in diverse physiological processes such as memory, attention, nociception, motor control, sleep-wake

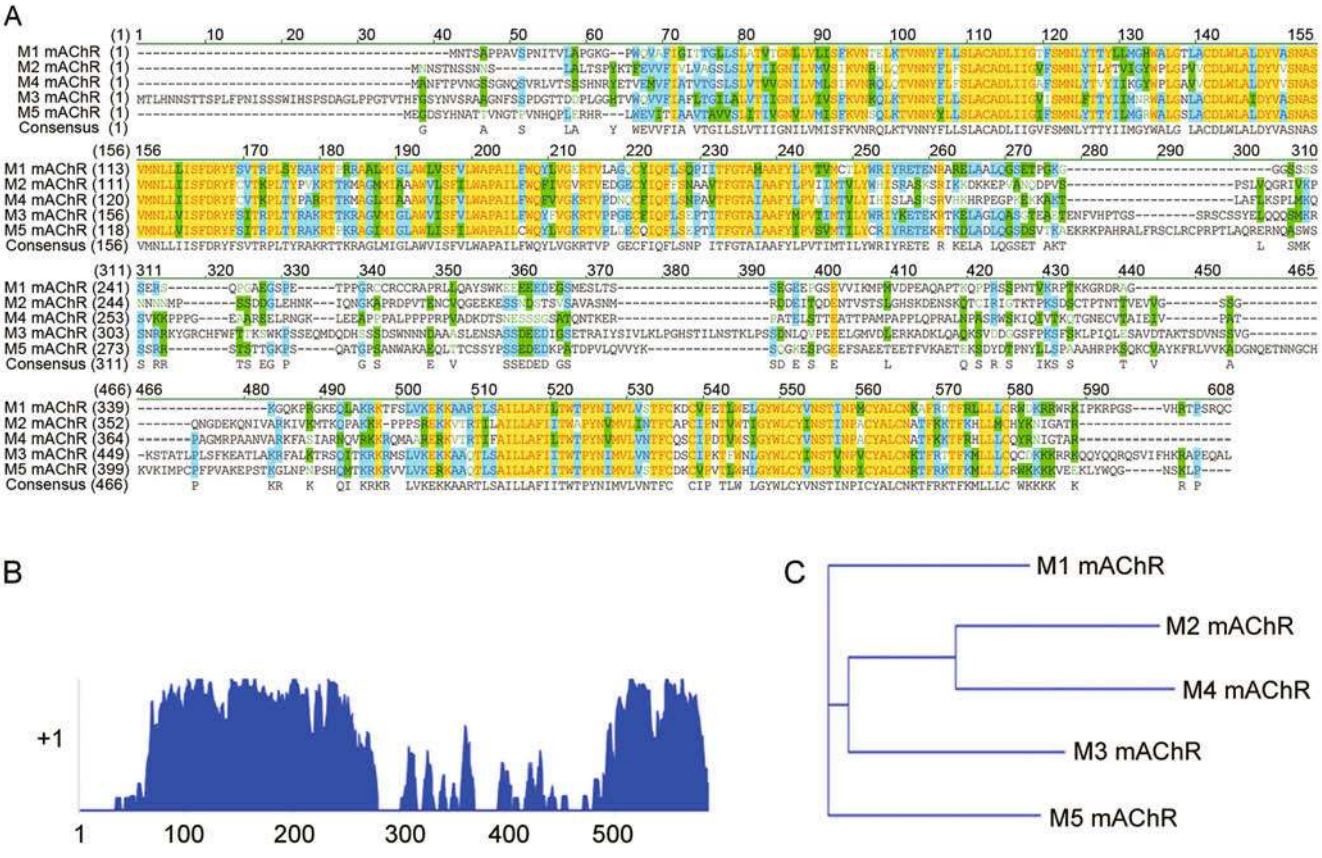

Fig. 1. Sequence comparison of mAChR subtypes. A: Amino-acid sequence alignment of the five subtypes. B: Conservation distribution pattern of mAChR sequences. X axis indicates the amino-acid numbering, and Y axis indicates the sequence consistency ratio. C: Phylogenetic analysis of the five subtypes.

cycles, and cardiovascular, renal, and gastrointestinal functions[24-26, 31-35]. Studies using *in situ* hybridization or immunohistochemistry with highly-specific antibodies to individual mAChR subtypes have revealed a unique yet somewhat overlapping distribution of these subtypes throughout the nervous system, being expressed both pre- and post-synaptically.

Among the mAChR family members, the M1 subtype makes up 50–60% of the total and is predominantly expressed in all major areas of the forebrain, including the hippocampus, cerebral cortex, corpus striatum, and thalamus[36-38]. M1 mAChR-knockout mice show a series of cognitive deficits and impairments in long-term potentiation, indicating that the M1 subtype is physiologically linked to multiple functions such as synaptic plasticity, neuronal excitability, neuronal differentiation during early development, and learning and memory[38-44]. At the cellular level, M1 mAChR is highly expressed in striatonigral, striatopallidal, and glutamatergic pyramidal neurons, especially in extrasynaptic regions. This localization of M1 mAChR is consistent with the cholinergic modulation of glutamatergic neurotransmission[45, 46].

M2 mAChR is expressed throughout the brain, including the hippocampus and neocortex, and is abundant in non-cholinergic neurons that project to these areas. In the caudate-putamen, M2 mAChR acts as an inhibitory modulator on dopaminergic terminals[46-48]. Therefore, selectively blocking M2 mAChR may provide an approach for the treatment of schizophrenia, a neuronal disorder associated with excessive dopamine neurotransmission. Mice deficient in M2 mAChR also show a striking reduction in muscarinic-dependent antinociceptive responses[49], suggesting a general antinociceptive effect.

M3 mAChR is widely distributed in the CNS, although at a lower level than other mAChR subtypes. M3 mAChR is expressed at a relatively high level in the hypothalamus, but is also found in many other regions including the hippocampus[47]. Mice lacking M3 mAChR appear hypophagic and lean, suggesting a general function of M2 mAChR in regulating food intake[50]. Consistently, mice with conditional knockout of M3 mAChR in the brain exhibit a dwarf phenotype. They also exhibit hypoplasia of the anterior pituitary gland and significantly decreased hormones including pituitary prolactin and growth hormone[51]. These findings indicate that M3 mAChR plays a critical role in promoting body growth.

M4 mAChR is mainly expressed in the corpus striatum in the CNS and on various prejunctional nerve terminals in the periphery. M4 mAChR has been suggested to play a role in psychosis and to be a promising target for the treatment of schizophrenia[52]. Indeed, the mixed M1/M4 mAChR agonist xanomeline has antipsychotic effects, and M4 mAChR-knockout mice display increased sensitivity to the disruptive effects of phencyclidine, a drug of abuse[53, 54]. M4 mAChR is also involved in the pathology of Parkinson's disease, which is associated with the loss of dopaminergic neurons projecting to the striatum and an imbalance between cholinergic and dopaminergic systems. In the corpus striatum, M4 mAChR is closely co-localized with dopamine receptors on striatal-projecting neurons and the striatal M4 mAChR inhibits dopamine D1 receptor function. Mice lacking M4 mAChR show increased locomotor activity and enhanced dopamine D1 receptor-mediated effects[55]. Consequently, selective M4 mAChR antagonists, such asbenzoxazines, have been developed for the treatment of Parkinson's disease[56].

M5 mAChR is predominantly distributed in the pars compacta of the substantia nigra, a structure that provides dopaminergic innervation to the striatum, and in the ventral tegmental area, a structure providing dopaminergic innervation to the nucleus accumbens and other limbic areas[26, 57]. These areas are well known to play a critical role in the rewarding effects of several drugs of abuse. M5 mAChR-knockout mice are less sensitive to addictive drugs such as morphine and cocaine[58]. Therefore, M5 mAChR antagonists may be important candidates for the treatment of drug addiction.

M1 mAChR in Alzheimer's Disease

Aβ, an important player in AD, is derived from β-amyloid precursor protein (APP) through sequential cleavages by β- and γ-secretases: APP is cleaved by β-secretase (BACE1) to generate the large secreted derivative sAPPβ and the membrane-bound APP C-terminal fragment-β; the latter can be further cleaved by γ-secretase to generate Aβ and APP intracellular domain. Alternatively, APP can be cleaved by α-secretase within the Aβ domain, which precludes Aβ production and instead generates secreted sAPPα that has been shown to be neuroprotective[59, 60]. Interestingly,

stimulation of M1 mAChR by agonists has been found to enhance sAPPα generation and reduce Aβ production[61-70]. Protein kinase C (PKC) is well-known to be activated upon stimulation of M1 mAChR. PKC may promote the activity of α-secretase[71] and the trafficking of APP from the Golgi/trans-Golgi network to the cell surface[72]. Some studies suggest that M1 mAChR stimulation also leads to activation of ERK1/2, which can modulate α-secretase activity and APP processing[67, 73], though there are contradictory findings showing that the α-secretase-mediated APP processing *via* M1 mAChR stimulation is not modulated by the ERK1/MEK cascade[71]. On the other hand, loss of M1 mAChR increases amyloidogenic APP processing in neurons and promotes brain Aβ plaque pathology in a mouse model of AD[74].

M1 mAChR also affects BACE1, the rate-limiting enzyme for Aβ generation[75, 76]. When APP/PS1/tau triple transgenic (3×Tg) AD mice are treated with the selective M1 mAChR agonist AF267B, the endogenous level of BACE1 decreases *via* an unclear mechanism, accompanied by a decreased Aβ level[77]. However, another study found that stimulation of M1 mAChR upregulates BACE1 levels in SK-SH-SY5Y cells *via* the PKC and MAPK signaling cascades[78]. We recently found that M1 mAChR directly interacts with BACE1 and mediates its proteasomal degradation[79]. These results suggest that M1 mAChR modulates BACE1 in a mixed manner.

In addition to inhibiting Aβ generation, activation of M1 mAChR counteracts Aβ-induced neurotoxicity through the Wnt signaling pathway, as Aβ impairs the Wnt pathway and M1 mAChR stimulation inactivates GSK-3β *via* PKC activation, stabilizes β-catenin, and induces the expression of Wnt-targeting genes engrailed and cyclin-D1 for neuron survival[80].

The involvement of M1 mAChR in AD is also manifested by its amelioration of tau pathology. Stimulation of M1 mAChR by two agonists, carbachol and AF102B, time- and dose-dependently decreases tau phosphorylation in PC12 cells[81]. Chronic treatment with AF267B also alleviates tau pathology in 3×Tg AD mice, possibly by activating PKC and inhibiting GSK-3β[77, 82].

Activation of M1 mAChR also protects against apoptotic factors in human neuroblastoma SH-SY5Y cells, such as DNA damage, oxidative stress, caspase activation, and mitochondrial impairment[83]. In addition, apoptosis induced by serum deprivation is blocked by M1 mAChR activation in a phosphoinositide 3-kinase- and MAPK/ERK-independent manner[84].

The M1 mAChR cascade may also be involved in counteracting decreased cerebral blood flow, which is one of the most consistent characteristics in pathological conditions such as AD, ischemic brain injury, intracerebral hemorrhage, and cognitive dysfunction[85, 86]. In mice with scopolamine-induced deficits, PQCA, a selective M1 mAChR positive allosteric modulator[87], improves not only recognition memory, spatial working memory, and executive function, but also blood-flow in the frontal cortex, though the mechanism is not yet clear.

Although the post-synaptic M1 mAChR level is relatively unaltered in AD[88-90], there are reports suggesting an uncoupling of M1 mAChR from G-protein in the postmortem brains of AD patients, especially in the hippocampal area, which is the most affected by Aβ[91-95]. In fact, Aβ has been shown to induce the uncoupling of M1 mAChR from G-protein, antagonizing the function of M1 mAChR under the pathological conditions of AD[96, 97]. Such an uncoupling may result in decreased signal transduction, reduced levels of sAPPα, and increased production of Aβ, triggering a vicious cycle. Although the mechanism by which Aβ disrupts mAChR-G-protein coupling is unclear, this uncoupling is palliated by antioxidants, implicating the involvement of free radicals[96]. A summary of the involvement of M1 mAChR in AD is illustrated in Fig. 2.

M1 mAChR Drugs

Because M1 mAChR plays a crucial role in learning and memory and is closely associated with AD, it has long been postulated as a therapeutic target. However, although stimulation of M1 mAChR is advantageous for cognitive improvement in AD patients, co-activation of other mAChR subtypes leads to side-effects[98]. Hence, an ideal M1 mAChR agonist should possess a high selectivity for the M1 subtype, desirable pharmacological properties, and favorable CNS penetration. So far, three types of M1 mAChR-targeting drugs have been developed: orthosteric agonists, M1 positive allosteric modulators (M1 PAMs), and allosteric agonists.

Orthosteric Agonists

Orthosteric agonists, the first-generation M1 mAChR-

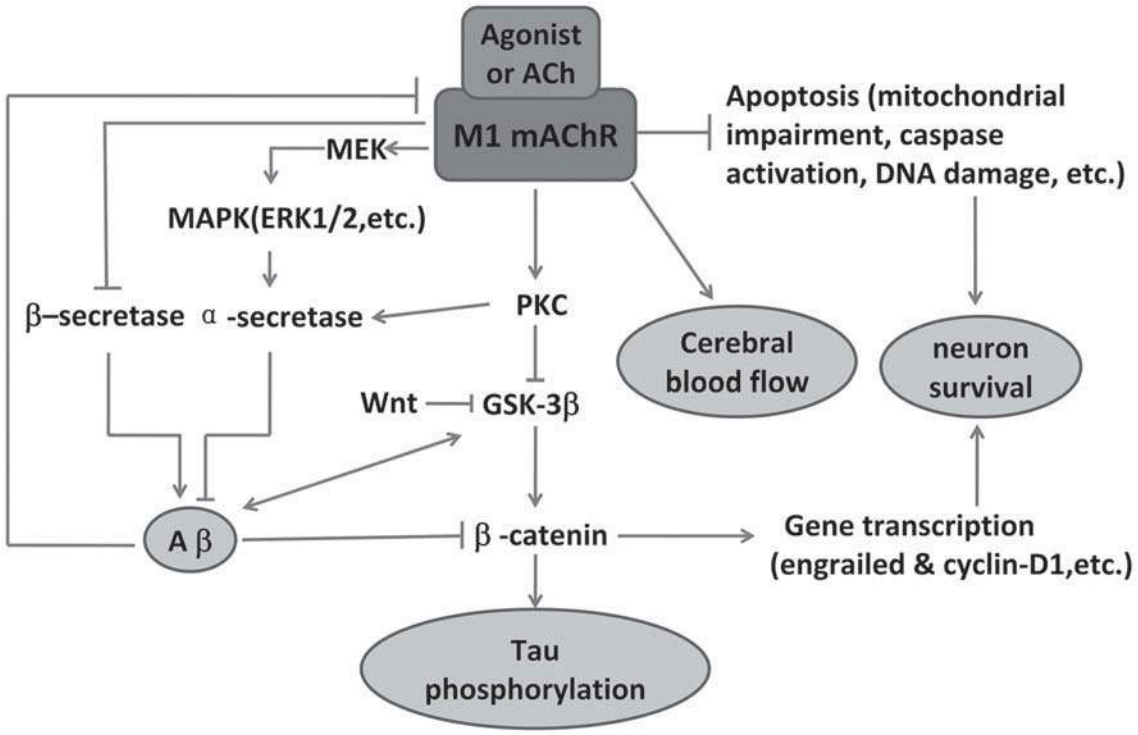

Fig. 2. The involvement of M1 mAChR in AD. Stimulation of M1 mAChR by agonists or ACh enhances sAPPα generation and reduces Aβ production. PKC and MAPKs (such as ERK1/2) are reported to be involved in this process by activating α-secretase. M1 mAChR activation also alters β-secretase (BACE1) levels *via* uncertain mechanisms. In addition, M1 mAChR stimulation counteracts Aβ-induced neurotoxicity through the Wnt signaling pathway, as Aβ impairs this pathway by destabilizing β-catenin, and M1 mAChR stimulation inactivates GSK-3β *via* PKC activation, thus stabilizing β-catenin and inducing the expression of the Wnt-targeting genes engrailed and cyclin-D1 for neuronal survival. In contrast, Aβ may induce the uncoupling of M1 mAChR from G-protein, antagonizing the function of M1 mAChR under the pathological conditions of AD. As GSK-3β is a major kinase for tau phosphorylation, stimulation of M1 mAChR inhibits tau phosphorylation as well. Moreover, activation of M1 mAChR protects cells against apoptotic factors such as DNA damage, mitochondrial impairment, caspase activation, and oxidative stress. Finally, the involvement of M1 mAChR in AD is indicated by its amelioration of decreased cerebral blood flow that has been reported as one of the most consistent characteristics in AD.

selective agonists, bind directly to the highly-conserved orthosteric ACh-binding site. Unfortunately, the similarity of orthosteric agonist-binding sites among all five mAChR subtypes makes it difficult to develop compounds that specifically target M1 mAChR. This may at least partly explain the failures of such agonists in clinical trials[99, 100]. One example is xanomeline, an mAChR agonist with selectivity for the M1 and M4 subtypes. Xanomeline improves working memory in rodents and improves cognition and reduces psychotic episodes in AD patients, but it failed during phase-II clinical trial because of serious side-effects, probably due to simultaneous activation of M1 and M4 mAChRs (M4 > M1)[53, 101-106]. AF267B and AF102B provide another example. Chronic treatment with AF267B reduces Aβ plaques and tau hyperphosphorylation and rescues learning and memory impairments in 3×Tg AD mice[77]. However, although AF267B is a selective M1 mAChR agonist and has ~30-fold selectivity for M1 *versus* the M2, M4, and M5 subtypes, it has no better selectivity for M1 *versus* the M3 subtype[107]. Administration of AF267B and AF102B (Cevimeline, EVOXAC™), an M1 mAChR-selective agonist once prescribed for the treatment of Sjogren's syndrome, decreases Aβ42 levels in the cerebral spinal fluid (CSF) of rabbits without affecting APP[108]. Moreover, AF102B administration decreased the total CSF Aβ levels by 22% in 14 of 19 AD patients without

affecting sAPPα levels. However, AF102B has serious side-effects including gastrointestinal symptoms, diaphoresis, confusion, diarrhea, and asthenia[109, 110]. Another M1 mAChR-selective agonist, talsaclidine, enhances non-amyloidogenic processing of APP, resulting in increased sAPPα release from both a transfected human astrocytoma cell line and rat brain slices in a dose-dependent manner, as well as significantly decreasing CSF Aβ in AD patients[111]. However, talsaclidine at high doses had several side-effects such as sweating and salivation[101]. Similarly, the M1 agonist WAY-132983 at a low dose improves cognitive status in animal models but at a high dose causes side-effects such as salivation and hypothermia[102, 112]. The advantages and disadvantages of various M1 mAChR agonists are listed in Table 1 and their chemical structures are shown in Figure 3.

Allosteric Compounds

An alternative approach to design selective M1 mAChR agonists is to develop compounds that bind to the less-conserved allosteric or ectopic binding sites. Since these regions are not highly conserved among mAChR subtypes and are topographically distinct from the orthosteric binding site[28-30], allosteric compounds may have better selectivity for the M1 subtype. Based on their activation mechanisms, allosteric compounds can be further classified into regular agonists and M1 PAMs. PAMs cannot activate receptors directly. Instead, their binding modifies the receptor conformation and changes the ligand-binding and functional properties of M1 mAChR[110]. Thus, PAMs are inactive in the absence of the endogenous neurotransmitter ACh and only exert their effect in its presence. On the other hand, regular allosteric agonists activate the receptor directly, independent of the presence of the endogenous agonist[110]. Since cholinergic neurons degenerate in specific brain areas and thus cause a decrease of presynaptic ACh release in AD, M1 mAChR allosteric agonists may have unique advantages for AD treatment because they selectively activate the M1 subtype when the endogenous ligand Ach is insufficient. Over the years, major advances have been made in developing selective allosteric agonists and PAMs of M1 mAChR. These molecules are now being optimized for use and tested in animal models.

Brucine, the first reported M1-PAM, when it binds to M1 mAChR simultaneously with orthosteric ligands, potentiates the binding affinity of the ligands. However, brucine only induces a modest increase in ACh affinity and has its effect at relatively high doses[113, 114]. Brucine failed in a preclinical test for further application. Nevertheless, the high selectivity of brucine for M1 mAChR shed light on the possibility of developing agents with absolute selectivity for mAChR subtypes. After brucine, several other M1-PAMs have been discovered, including VU0029767, VU0090157, and benzyl quinolone carboxylic acid (BQCA)[115-117]. These compounds do not activate M1 mAChR directly but greatly increase the affinity of ACh for the M1 subtype. In addition, BQCA is effective in restoring discrimination reversal learning in a mouse model of AD and regulating non-amyloidogenic APP processing[117]. These positive roles of M1-PAMs make them potentially useful for AD treatment.

Besides M1-PAMs, several allosteric agonists of M1 mAChR have been discovered. A novel compound, AC-42, was found to be able to activate M1 mAChR at a region clearly distinct from the orthosteric ACh-binding site and this region is not conserved among mAChR subtypes, explaining its unprecedented selectivity for M1 mAChR. The highly anticipated AC-42 was also confirmed to be active in cell lines by monitoring intracellular calcium release and inositolphosphate accumulation. Unfortunately, AC-42 failed to activate M1 mAChR in more complex systems such as brain slices[118, 119]. Other disadvantages of many allosteric agonists developed earlier include off-target activity and poor solubility in physiological buffer systems, preventing their application *in vivo*[110]. Nevertheless, a compound developed later, TBPB, selectively activates M1 mAChR in cell lines and shows no agonist activity in any other mAChR subtype. Interestingly, TBPB also potentiates the NMDA-evoked current in hippocampal pyramidal neurons, which is considered to be important for the effect of M1 mAChR on improving cognition. In addition, TBPB shifts the processing of APP in the non-amyloidogenic direction and thereafter decreases neurotoxic Aβ production *in vitro*[120]. Further studies demonstrated that TBPB is also systemically active and crosses the blood-brain barrier[110]. All these encouraging data support the potential of using M1 allosteric agonists in the treatment of AD. The allosteric agonists 77-LH-28-1 and AC-260584 were synthesized as structural analogs of AC-42. Compound 77-LH-28-1 shows relatively higher selectivity for the M1 than for the M2, M4, and M5 subtypes, but retains weak agonist activity for M3 mAChR

Table 1. M1 mAChR-based drugs for AD treatment

Orthosteric agonists of M1 mAChR		
Drugs	Potential roles in AD	Disadvantages
Xanomeline[53, 101-105]	Improves cognition and reduces psychotic symptoms in both preclinical and clinical studies.	Failed during Phase II clinical test due to dose-dependent side-effects - nausea and diarrhea.
Talsaclidine[101, 111]	Enhances non-amyloidogenic processing of APP and decreases CSF Aβ level in AD patients.	Discontinued due to side-effects - sweating and salivation.
AF102B[108-110]	Reverses cognitive impairments at low dose and reduces CSF Aβ in AD patients. The first drug ever shown to have such an effect in human patients.	Discontinued due to side-effects - gastrointestinal symptoms, diaphoresis, confusion, diarrhea, and asthenia.
AF267B[77, 98, 107, 108]	Improves cognitive function, decreases Aβ, hyperphosphorylated tau, and BACE1 levels in AD mice.	Inactive in clinical trial and discontinued.
WAY-132983[102, 112]	Enhances performance memory and cognition in animal models.	Discontinued.
M1 positive allosteric modulators		
Drugs	Potential roles in AD	Disadvantages
Brucine[113, 114]	The first reported M1-PAM. Potentiates ACh affinity to M1 mAChR.	Only induces a modest increase of ACh affinity. Relatively high dose required to elicit effect.
VU0029767[115]	Increases ACh affinity to M1 mAChR.	Unreported
VU0090157[115]	Increases ACh affinity to M1 mAChR.	Unreported
BQCA[115-117]	Reverses learning impairment in an AD mouse model.	Unreported
Allosteric agonists of M1 mAChR		
Drugs	Potential roles in AD	Disadvantages
AC-42[118, 119]	The first confirmed allosteric agonist of M1 mAChR and selectively activates M1 mAChR at an allosteric binding site in cell lines.	Failed to activate M1 mAChR in brain slices.
TBPB[110, 120]	Highly selective agonist for M1 mAChR subtype. Shifts APP processing toward the non-amyloidogenic pathway in cells and appears to have antipsychotic-like effects.	May also bind allosteric sites shared by other G protein-coupled receptors.
77-LH-28-1[121]	Highly selective and highly efficient agonist of M1 mAChR. Promotes several physiological functions related to cognition.	Unreported
AC-260584[118, 122]	Orally bioavailable with favorable antipsychotic and cognitive enhancing effect.	Not specifically selective to M1 mAChR.
VU0184670[110, 123]	Highly selective to M1 mAChR and excellent pharmacokinetic profile. Also potentiates NMDA receptor-mediated current in hippocampal neurons.	Unreported
VU0357017[110, 123]	Highly selective to M1 mAChR and excellent pharmacokinetic profile. Reverses cognitive deficits induced by mAChR antagonist scopolamine in animal models.	Unreported

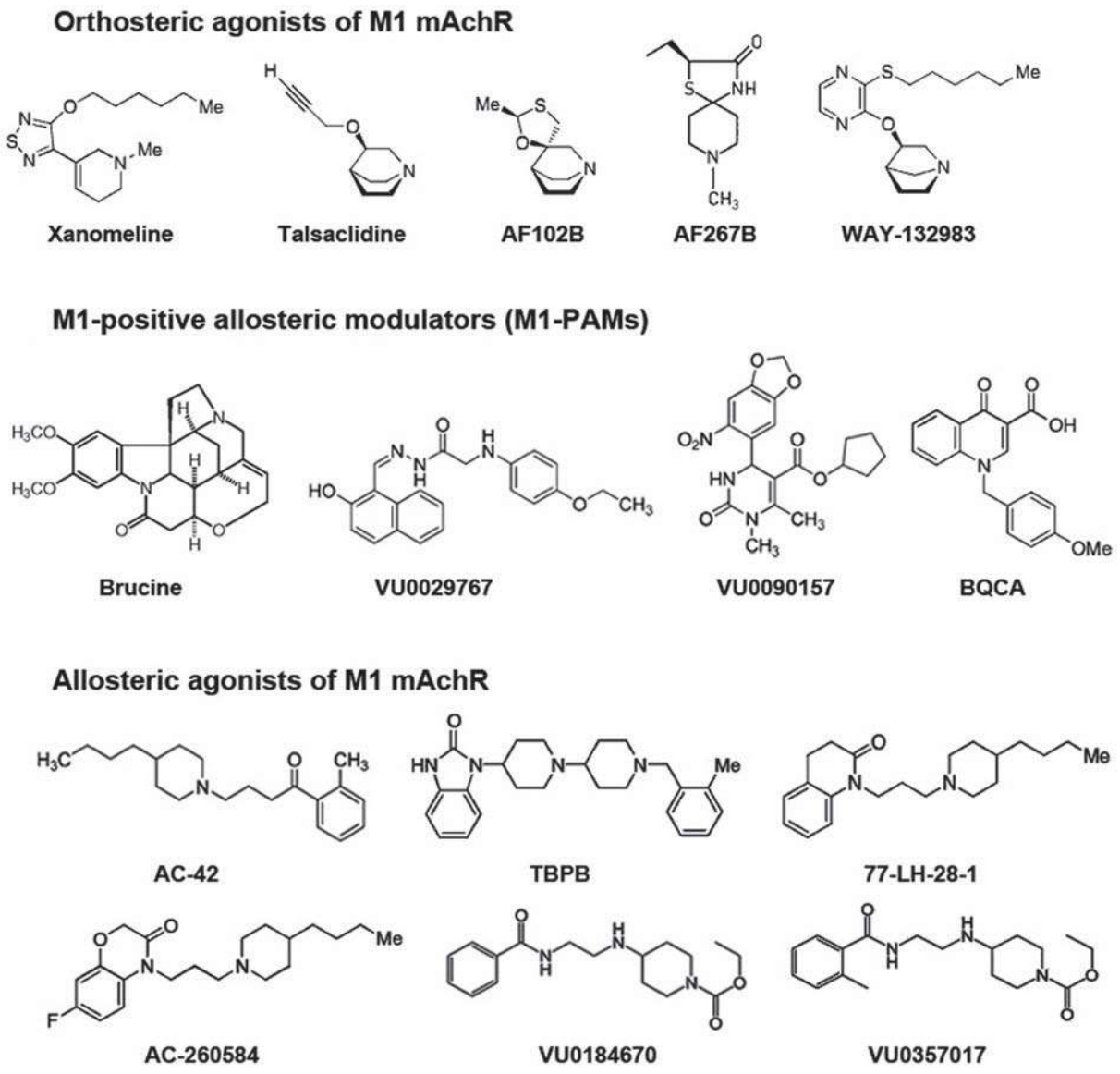

Fig. 3. Chemical structures of M1-selective agonists.

at high doses. Electrophysiological studies indicate that 77-LH-28-1 increases the activity of hippocampal CA1 pyramidal cells both *in vitro* and *in vivo*. Interestingly, unlike other normal orthosteric agonists, 77-LH-28-1 appears to selectively activate M1 mAChR in a distinct signaling pathway[121]. Such a difference requires more caution in determining the potential of 77-LH-28-1 for the treatment of AD. The M1 mAChR agonist AC-260584 was recently reported to be orally bioavailable with favorable antipsychotic and cognition-enhancing effects[118, 122]. However, the lack of absolute M1 mAChR selectivity of AC-260584 and other related compounds may limit their therapeutic use. During the past few years, the M1 mAChR allosteric agonists VU0184670 and VU0357017 have been screened out, and have more exciting properties. Both compounds have high solubility in aqueous solutions as well as good CNS penetration, without any agonist or antagonist activity for the M2 and M5 subtypes. Moreover, VU0184670 potentiates neuronal NMDAR-mediated currents in hippocampal brain slices and VU0357017

reverses the cognitive deficits induced by an mAChR antagonist in a contextual fear conditioning paradigm, exhibiting improvement of hippocampus-dependent learning[110, 123]. These results implicate the two compounds as a highly potent, selective, and systemically active new generation of M1 allosteric agonists.

Conclusion

M1 mAChR plays a crucial role in cognitive functions like learning and memory. Dysregulation of M1 mAChR contributes to AD and the specific activation of the M1 subtype is considered to be a promising strategy for AD treatment. A full elucidation of the network interactions between the M1 mAChR and other AD core factor-mediated signaling pathways will facilitate the development of effective therapeutics.

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