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REVIEW ARTICLE

Cholinergic System and Post-translational Modifications: An Insight on the Role in Alzheimer's Disease

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Abstract: ***Background*:** Alzheimer's disease (AD) is the most common form of old age dementia. The formation of amyloid plaques (Aβ), neurofibrillary tangles and loss of basal forebrain cholinergic neurons are the hallmark events in the pathology of AD.

***Literature Review*:** Cholinergic system is one of the most important neurotransmitter system involved in learning and memory which preferentially degenerates in the initial stages of AD. Activation of cholinergic receptors (muscarinic and nicotinic) activates multiple pathways which result in post translational modifications (PTMs) in multiple proteins which bring changes in nervous system. Cholinergic receptors-mediated PTMs "in-part" substantially affect the biosynthesis, proteolysis, degradation and expression of many proteins and in particular, amyloid precursor protein (APP). APP is subjected to several PTMs (proteolytic processing, glycosylation, sulfation, and phosphorylation) during its course of processing, resulting in Aβ deposition, leading to AD. Aβ also alters the PTMs of tau which is a microtubule associated protein. Therefore, post-translationally modified tau and Aβ collectively aggravate the neuronal loss that leads to cholinergic hypofunction.

***Conclusion*:** Despite the accumulating evidences, the interaction between cholinergic neuro-transmission and the physiological significance of PTM events remain speculative and still needs further exploration. This review focuses on the role of cholinergic system and discusses the significance of PTMs in pathological progression of AD and highlights some important future directions.

Keywords: Acetylcholine, Alzheimer's disease, muscarinic receptors, nicotinic receptors, post translational modifications.

ALZHEIMER'S DISEASE

Neurodegenerative diseases are devastating conditions with progressive degeneration of nerve cells resulting in abnormal mental functioning specially dementia [1]. Degenerative diseases of the brain were long considered among the most ambiguous and troublesome of all diseases [2]. In 1906, Alois Alzheimer for the first time described the neuropathological features in the brain of a patient Auguste D., suffering from dementia. Later on, Emil Kraepelin in 1910 renamed the same pathology as "Alzheimer's Disease" to differentiate the general memory impairment from the common senile dementia [3].

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PATHOLOGICAL FEATURES OF ALZHEIMER'S DISEASE

Major hallmarks of AD include basal forebrain cholinergic hypofunction [4], extracellular accumulation of beta-amyloid known as amyloid or "senile" plaques [5] and intracellular neurofibrillary tangles (NFTs) accumulation [6, 7]. Senile plaques are the extracellular aggregates of beta-amyloid protein (Aβ) derived from cleavage of amyloid precursor protein (APP) *via* the action of β- and γ-secretase [8], while neurofibrillary tangles (NFTs) consist of hyperphosphorylated tau protein, present inside the neurons [9]. Beside plaques and NFTs, synaptic dysfunction is one of the most critical aspects of dementia [10, 11]. It has been found that synapses involving acetylcholine (ACh), glutamate and serotonin are primarily impaired in AD [12, 13]. The loss of basal cholinergic neurons is associated with severe neurodegeneration and cell loss in the nucleus basalis complex [14]. Cortex and hippocampus receive their major cholinergic input from nucleus basalis of Meynert and diagonal band of broncha, respectively [15]. The degeneration

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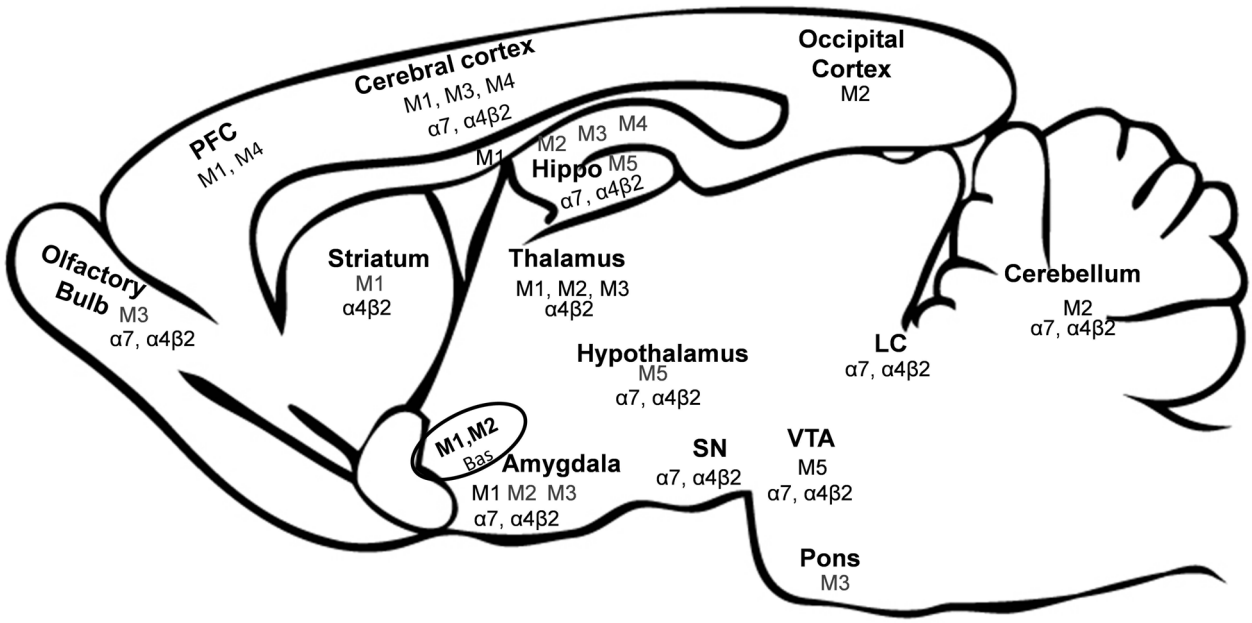

Fig. (1). Expression of Muscarinic and Nicotinic Receptors in Brain. Abbreviations: bas: Nucleus basalis, Hippo: hippocampus, LC: locus coeruleus, PFC: Prefrontal cortex, SN: substantia nigra, VTA: ventral tegmental area [39, 40, 44, 51, 52, 76].

of basal forebrain cholinergic neurons is considered to be the earliest pathological event along with plaque and tangle formation [16, 17]. Lesions of the cholinergic basal nuclei in rats result in a number of memory deficits [18] and affect memory and cognition in primates [19]. The basal forebrain cholinergic deficits positively correlate with cognitive [20] and non cognitive behavioral deficits [21] observed in AD patients. But the dilemma, that why the basal forebrain cholinergic neurons are among the first targets in AD pathology, still needs to be solved [22].

CHOLINERGIC RECEPTORS

The ACh receptor (AChR) is a vital membrane protein on which ACh acts as a neurotransmitter. The cholinergic receptors are broadly categorized as muscarinic ACh receptors (mAChR) and nicotinic ACh receptors (nAChR) on the basis of their exogenous agonists. The expression of these receptors varies in different brain areas (Fig. **1**). Nicotinic receptors are found in the neuromuscular junction, autonomic ganglia and various places in the CNS, though, with different composition. While muscarinic receptors are found to be expressed in the brain both at the pre-synaptic and post-synaptic nerve terminals and parasympathetic effector organs [23].

MUSCARINIC RECEPTORS: CLASSIFICATION, LOCATION AND BRAIN FUNCTIONS

The mAChR belong to the family of seven transmembrane receptors coupled to G-proteins (GPCRs), regulating a variety of physiological processes [24]. These receptors are comprised of single polypeptides which form seven transmembrane domains forming a central pore. ACh binds at a site inside this pore to activate the signaling cascade *via* G-proteins [25]. In the central nervous system (CNS), the muscarinic system plays important role in the regulation of many sensory, motor and autonomic processes [26]. Moreover, mAChRs have established roles in cholinergic transmission as well as learning and memory [12].

Muscarinic receptors are further sub-divided into five types M1-M5 encoded by five genes, m1–m5 [27]. The five mAChR subtypes are similar with the exception of the third intracellular loop while their signaling features are different, so these subtypes are further categorized into two groups [28-30] which determine the specific coupling preferences of these receptors [31]. The M1-like subfamily (M1, M3 and M5) is coupled to Gαq/11 protein which causes activation of phospholipase C. Stimulation of M1-like subfamily receptors leads to regulation of different proteins and their functions by the process of phosphorylation. Whereas the M2-like subfamily (M2 and M4) is coupled to Gi/o, which inhibits adenylate cyclase [32]. The stimulation of M2 and M4 receptors causes a reduced cytosolic cAMP level [33]. The intracellular muscarinic signaling responses include activation of protein kinases, phospholipases A2 and D (releasing arachidonic acid and choline, respectively) and regulation of calcium and potassium channels [34].

The mAChRs are widely distributed throughout the body peripherally as well as centrally. In the hippocampus and cerebral cortex, mAChRs are involved in cognitive processes such as memory [35-37]. While in the striatum and motor cortex, these receptors are involved in motor function [38].

M1 receptors are abundantly present in the hippocampus, neocortex, striatum, amygdala, thalamus [39] and prefrontal cortex [40]. M1 receptor knock-out mouse showed elevated levels of Aβ peptides in brain [41] and increased aggregation of amyloid plaques which leads to impaired memory consolidation in this model [42].

M2 receptors are auto-receptors for ACh release [43], present on presynaptic cholinergic neurons, and abundantly

found in the cerebellum, thalamus [44] and nucleus basalis while lower levels are found in the hippocampus, amygdala and caudate putamen [39, 45].

The expression of M3 is relatively low i-e; 5-10% of total mAChRs in brain [46]. It is found in several brain regions, including cortex, amygdala, hippocampus, olfactory bulb, striatum, thalamus and pons [39]. M3 is involved in the regulation of neurotransmitter release, including dopamine, GABA and glycine as well as endocannabinoids [47, 48], suggesting its critical role in regulating other types of neurotransmission and learning and memory.

Relatively low levels of M4 receptors are expressed in brain as compared to other mAChR subtypes and are localized to hippocampus, including CA1 region and dentate gyrus [39], while the highest levels are in the caudate putamen [45] and prefrontal cortex [40] where they play role in the feedback control of neurotransmitter release [49] and cognitive processes [50].

M5 mAChRs has been found at low levels in the brain, particularly in the ventral tegmental area [51, 52], hippocampus and substantia nigra [13]. These receptors play an important role in facilitating muscarinic agonist-induced dopamine release from the nerve terminal [53]. So as a consequence, presence of M5 receptor is important for dopamine release and might be involved in facilitating dopamine-mediated and reward related physiological functions.

INVOLVEMENT IN ALZHEIMER'S DISEASE

Normal processing of the APP is under the control of cholinergic inputs [54, 55]. So altered cholinergic inner-vations could lead to abnormal processing of β-amyloid and possible formation of potentially neurotoxic fragments leading to neuritic plaque formation [56-58]. It has been observed that ACh esterase (AChE) accelerates the aggregation of Aβ into insoluble amyloid fibrils *via* unknown mechanism [59]. Basal forebrain cholinergic neuronal fibres are lost in AD at a later stage [60, 61]. This neuronal loss may be a result of Aβ neurotoxicity to the cholinergic terminals followed by retrograde degeneration [62]. Long-term exposure to micromolar concentration of Aβ is toxic to cholinergic neurons [63]. There are several reports on loss of cholinergic fibers and nerve terminals in AD and reduced cholinergic receptors [12, 64] but the relationship between Aβ and cholinergic deficit is poorly understood [62]. Due to the deterioration of cholinergic neurons in the brain of AD patients, there is a considerable loss of nicotinic receptors and certain muscarinic receptors particularly in the cortex and hippocampus [65-67], leading to impaired neurotransmitter release. In the cortical pyramidal neurons the activation of muscarinic receptors is known to enhance GABAergic transmission. The GABAergic inhibition is cruical for execution of certain memory forms by controlling the information flow in cortical circuits. Therefore, cholinergic hypofunction leads to cognitive impairment in AD patients [68].

Cholinergic hypofunction is a hallmark of AD [69, 70]. Specially, M1 and, M2 are down-regulated in hippocampus and cortex [45] and M4 appears to be down-regulated in cortex [71]. Down-regulation of M1, M2 and M4 is responsible for cognition deficits as well as impaired ACh release which exacerbates the AD symptoms. In another study, M1 receptors remained unchanged in AD patients, but M1/G-protein coupling was considerably decreased in the frontal cortex which was linked with the progression of cognitive impairment [72]. M1 receptors being involved in modulation of cognition, and are found to be the therapeutic targets for AD treatment. M1 agonists may alter the proteolysis of APP resulting in significantly reduced Aβ levels in cortex and hippocampus [73]. The M1/M3 activation increases non amyloidogenic pathway of APP processing. Therefore, the hypofunction of these receptors might increase Aβ generation leading to severe AD pathology [74]. It is reported that M1 receptor signal transduction-related functions are compromised in AD [72, 75, 76] but in another study cortical M1 receptor was increased in AD [77]. M2 receptor is increased in AD suggesting, that the presynaptic M2 receptors are preserved or up-regulated resulting in reduction in neurotransmitter release [71], but in another study M2 receptors were significantly reduced in hippocampus of AD brains [78]. Learning and memory associated with fear conditioning was declined in M3 knock-out mice [79]. M3 receptor levels were found to be decreased in the entorrhinal cortex and hippocampus [78]. Alteration in mAChR subtypes has important implications in cognitive control as well as ACh regulation. Impaired M1 receptor exacerbates AD-related cognitive decline, while disruption of M2/M4 receptors negatively regulates the ACh release as well the cognitive learning.

Among the cholinergic markers, the activity of choline acetyltransferase (ChAT) is greatly reduced in AD and is related to severity of disease [62, 80, 81]. Reduction in ChAT activity has been correlated with the numbers of neurofibrillary tangles in AD [82], suggesting a strong relationship between cholinergic transmission and AD. Loss of cholinergic neurons causes a significant reduction of ChAT activity (up to 95%) in the neocortex [83, 84] and hippocampus [85], that has been related to a marked decrease of ACh levels in these regions [86].

Agents that block mAChRs disturb cognitive functions and cause temporary loss of short term memory [12, 87-89]. Recently, muscarinic receptor family has shown clinical effectiveness in recovering cognitive impairment associated with AD [90, 91]. The role of Gq-coupled M1 and M3 receptors has been confirmed in modifying non-amyloidogenic pathway of APP processing while Gi-coupled M2 and M4 receptors promote amyloidogenic pathway [54, 92]. It reflects that the muscarinic receptors are among the excellent targets for the treatment of AD.

MUSCARINIC AGONISTS FOR ALZHEIMER'S DISEASE TREATMENT

mAChR are considered to be of prime importance among key drug discovery targets for the cure of AD (Table **1**) [93-95]. Muscarinic agonists delay the progression of AD by decreasing β-amyloid aggregation, reducing tau phosphorylation and improving cognitive behavior [73, 94, 96, 97]. Cholinergic system modulation improves synaptic

function by increasing synaptic protein expression at various stages of disease [98], improves synaptic plasticity [99] and also suppresses inflammatory response [100].

M1 receptor is considered to be an important therapeutic target as it is abundant in the hippocampus and cerebral cortex, where the cholinergic hypofunction is well-defined in AD. This receptor subtype is concerned with short-term memory [13]. Moreover, stimulation of M1 muscarinic receptors reduces the production of β-amyloid by activating α-secretase as this leads to non-amyloidogenic pathway [101].

NICOTINIC ACETYLCHOLINE RECEPTORS

The nicotinic ACh receptors (nAChRs) are ligand gated ion channels. These receptors are formed by assembly of five subunits, around a central pore, in homomeric or heteromeric conformation [106]. The standard subunits include α2-α9 and β2-β4 [107]. The neural subunits capable of forming heteromeric nAChRs with αβ subunit combinations are α2-α6 and β2-β4. Whereas subunits α7-α9 make functional homomeric nAChRs [106]. The β subunits alone are incapable of forming functional receptor while α2-α6 alone can only make receptors with very weak response to ligand. This indicates that only the combination of α and β receptors make a fully functional receptor [108]. It is also reported that α subunits contain agonist recognition and binding site. The β subunits are helpful to increase affinity towards agonist and to stabilize the whole receptor [108]. Individual nAChR subunits can combine in different stichiometries but, $(\alpha7)_5$, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ nAChR are the most common receptor types in central nervous system [109]. Each subunit of nAChRs contains four transmembrane domains (M1-M4), two hydrophilic extracellular segments (N- and C- terminals) and an intracellular loop between M3 and M4 transmembrane domains [110]. This intracellular loop has putative phosphorylation sites [110]. The second transmembrane domain, M2, aligns along the centre to make the central pore [106]. Two molecules of ligand must bind to the receptor to allow opening of the central pore and permeation of cations (Ca^{+2}, Na^{+}, K^{+}) [110].

LOCATION AND FUNCTION OF NICOTINIC ACETYLCHOLINE RECEPTORS

Among many different possible combinations of the nicotinic receptor subunits the α4β2 and α7 type receptors are most abundant in the mammalian brain [107]. In the rodent brain α4β2 receptor type is reported to be the most abundant of all the nicotinic receptor subtypes and is found to be expressed in all layers of cerebral cortex and hippocampus in rat [111]. The α7 receptor subtype is highly expressed by basal forebrain neurons, and the innervations projecting towards hippocampus from basal forebrain [112, 113].

The function of the nAChRs is regulated by the binding of ligand, nicotine or ACh, to the receptor. ACh binds to extracellular N-terminal domain of receptor at boundary between α and non-α subunits [114]. The binding causes an influx of different cations, especially Ca^{+2} ion, inside the cell [115]. This nAChR mediated Ca^{+2} entry causes a marked increase in intracellular Ca^{+2} concentration, which is adequate to initiate Ca^{+2} sensitive processes [110].

INVOLVEMENT IN ALZHEIMER'S DISEASE

There are contradictory reports about the expression of most abundant nAChR subtypes, α4β2 and α7, during AD in different brain areas. Some studies report an increase in the expression of nAChRs in AD [116-118] while others report nAChRs decrease during progression of AD [108, 119-121]. It was reported in a study that at mRNA level, the expression of both receptor types remains the same in control and AD patient brain cortices while at protein level there is a 30% decrease in the expression of α4β2 and α7 receptor subtypes. The difference observed in α4β2 and α7 receptor expression at protein level and mRNA level might be due to a change at translational or post translational level during nAChR biosynthesis [122], but the exact mechanism is not known and needs to be investigated. Similar observation was made when autopsy samples of cerebral cortex from AD patients were studied [123]. But they reported a 40% decrease in α4 receptor expression and 17% decrease in α7 receptor expression. Decreased expression of nAChRs causes a

Table 1. M1 Allosteric modulators/agonists under clinical trials.

M1 Agonist	Therapeutic Effects	Refs.
AF150(S)	Decreases β-amyloid levels in CSF	[102]
AF267B	Restores cognitive and behavioural impairments, decreases Aβ aggregation and tau hyperphosphorylation	[61]
AF102B	Decreases CSF β-amyloid level in AD	[84]
TBPB	Activation of non-amyloidogenic pathway for APP processing and reduced Aβ synthesis *in vitro*.	[81]
BQCA	Restores discrimination-based learning in a transgenic mouse model of AD, control non-amyloidogenic pathway of APP *in vitro*	[103]
AC-260584	Improves cognitive performance in animal model	[104]
77-LH-28-1	An agonist at rat hippocampal M1 receptors, increases cell firing	[105]

AD: Alzheimer's disease, APP: Amyloid Precursor Protein, CSF: Cerebrospinal fluid, M1: Muscarinic receptor 1, BQCA: Benzylquinolone carboxylic acid.

deficiency in the binding sites for nicotine and ACh which leads to cognitive deficit in AD [123]. Contrary to these observations that report a decrease in nicotinic receptor expression, another study [124] reported an increase in α7 mRNA expression in patients of AD, while no difference in the expression of α4 was observed by them. An increased expression of nicotinic receptor protein was also reported in animal models [125, 126]. This difference in expression studies might be due to age dependent biphasic effect on nicotinic receptor expression, during AD, in animal model. As it is reported that nAChR expression increases 3-4 fold at 9 months of age and then a decrease in expression is observed at 12 months of age [127].

It is reported that in early AD the initial Aβ aggregation overlaps with the α7 receptor expression in basal forebrain cholinergic system [128]. This early co-localization of the α7 receptors and Aβ may be due to high affinity binding between these two components [117, 129]. Receptor binding experiments have also shown co-precipitation of Aβ1-42 and α7 nAChRs [113]. Because these co-precipitates are resistant to detergent treatment which shows that a high affinity binding takes place between Aβ1-42 and α7 nAChRs [117]. Aβ1-42 also binds to heteromeric nAChRs but with 5000 times lesser affinity as compared to α7- and is known to block whole cell and single channel currents in CA1 stratum radiatum interneurons in rat hippocampal slices [130].

It is now known that Aβ1-42 at high (nM) concentrations leads to nAChR inactivation and thus can disrupt synaptic plasticity and cognitive functions [131]. However, at low (pM) concentration the Aβ1-42 plays neuromodulatory role and activate nAChRs, thus modulating synaptic plasticity and enhancing cognitive functions [132]. Low levels of Aβ and short exposure time help to activate different neuro-modulatory pathways in nAChR dependant manner but extended exposure at higher doses causes a dysregulation of these signal transduction pathways, possibly through desensitization of receptor, leading to cell death which in turn impairs learning and memory [133].

α7 nAChRs are reported to be involved in induction of long term potentiation (LTP) and long term depression (LTD), two forms of synaptic plasticity. As reported by Gu and Yakel schaffer collateral (SC) CA1 plasticity is dependent on α7 receptor. In this experiment when septal cholinergic input was activated 100ms or 10ms prior to SC stimulation caused induction of LTP or LTD which was blocked by α7 antagonist MLA but not by non-α7 antagonist DHβE. Moreover, this synaptic plasticity was disrupted by 10nM or 100nM Aβ. These results suggest that inactivation of α7 receptors by Aβ has negative effects on synaptic plasticity [134] which results in impaired learning and memory.

The fact that α7 nAChRs are present in glial cells, particularly astrocytes, suggests that these receptors also have an important role in inflammation process. Work by Nagele *et al*. show that α7 nAChRs and Aβ were found to be intensely co-localized with green fluorescence activated protein (GFAP) positive (activated) astrocytes in AD brains. Since the authors also found ChAT, they proposed a model that α7 and Aβ are phagocytized by activated astrocytes in the vicinity of neural remnants. As a result astrocyte viability is compromised with increased accumulation of neuronal debris in astrocytes. This results in selective lysis of the astrocytes lead to astrocyte derived amyloid plaque formation [135]. The Aβ peptide is also reported to activate caspase 3 and induce astrocyte apoptosis [136] leading to higher rate of astrocyte apoptosis as compared to neuronal cells [137]. Thus, apoptosis of astrocytes may positively contribute to pathogenesis of AD [138].

α7 NICOTINIC ACETYLCHOLINE RECEPTOR TARGETING DRUGS FOR TREATMENT OF ALZHEIMER'S DISEASE

Although there is no preventive treatment available for AD but there is a continuous urge in the scientific community for the search of novel therapeutic strategies that can alleviate pathological symptoms of AD. The α7 nAChRs are pentameric ligand gated ion channels with selective permeability to Na^{+} and Ca^{+2} ions [139]. These receptors have exceptionally high Ca^{+2} permebility [140] as compared to other ligand gated ion channels. The Ca^{+2} acts as a second messenger and activates many signaling pathways in the cell and also mediates neurotransmitter release [141]. Due to high vulnerability of cholinergic neurons (specifically those having high amount of α7 nAChRs), high affinity binding between α7 nAChRs–Aβ and exceptionally high calcium ion selectivity of α7 nAChRs these receptors have become an attractive target for the treatment of AD [140].

For drug target both α7 nAChR agonists and antagonists are under investigation. The α7 nAChR agonists are of more therapeutic interest for the pharmacologist. Antagonists of α7 nAChRs have lower practical impact as compared to its agonists [109]. Table **2** lists some of the agonists, targeting α7 nAChR, under clinical trials for treatment of AD.

AMYLOID PRECURSOR PROTEIN AND ITS POST TRANSLATIONAL MODIFICATIONS AND PROCESSING IN ALZHEIMER'S DISEASE

Although the relationship between protein dysfunction and neurodegeneration remains elusive [146-148], protein aggregation has evolved as an emerging theme in diseases such as AD. Several heavily debated hypotheses exist to sequentially interlink all these phenomena under one event; aggregation of toxic Aβ is considered to be the driving force of AD pathology. Aβ, peptides of 40 or 42 amino acids, are derived from the sequential proteolytic cleavage of β-amyloid precursor protein (APP). Two mutually exclusive pathways exist for proteolytic processing of APP; while cleavage at residue Lys 16 by α-secretase results in the generation of soluble APP (sAPP) peptides, altered cleavage by β- and γ-secretases results in the formation of the 40-42 amino acid which coalesces to form insoluble, extracellular Aβ [149].

With recent paradigm shift, post-translational modifications (PTMs) of pathology associated proteins have become a valuable tool in the evaluation of the structural and functional alterations governing the neurodegenerative diseases [150]. PTMs significantly contribute to proteome expansion with each variant displaying a starkly different

Table 2. α7 Nicotinic acetylcholine receptor agonists and antagonists under clinical testing for the treatment of AD.

α7 Nicotinic Acetylcholine Receptor Agonists		
Name	**Therapeutic effects**	**Refs.**
EVP-6124	Activates α7 nAChRs and is used for the treatment of mild to moderate AD, under phase 3 clinical trials	[142]
AZD-0328	Activates α7 nAChRs and enhances dopamine release. Used for the treatment of AD and is under clinical testing.	[143]
ABT-107	Treatment of AD and cognitive deficits associated with schizophrenia, under testing, not comercially available	[144]
GTS-21	Treatment of AD and cognitive deficits associated with schizophrenia, experimental testing for anti-inflammatory potency, under clinical testing	[145]

property such as phosphorylation of Tau protein in neurofibrillary tangles (NFT) or alternative cleavage of post-translationally modified APP into different forms of Aβ [150]. It comes in good agreement with studies implicating aberrant PTMs in AD pathogenesis [151].

APP is post-translationally modified by sulfation, phosphorylation, glycosylation, including both N- and O-linked glycosylation and proteolytic processing. In fact it is the O-glycosylated version of APP that is preferentially secreted. The most interesting correlation of PTMs with AD pathophysiology is "glycosylation" whereby oligosaccharide side chains attach themselves at N&O- linked sites on the nascent APP, in the endoplasmic reticulum. Two putative N-linked oligosaccharide attachment sites (Asn 467 and Asn 496) have been identified. Accumulating evidence proposes that only the former is occupied under normal conditions [152]. It has been suggested that the oligosaccharide side chains have a pivotal role in the protein processing. In AD, the major lesion associated proteins, APP and Tau, and their respective metabolites undergo altered N- and O-glycosylation [153].

Since Aβ can be produced by cultured cells, this has left us with a powerful model system for analyzing the aberrant PTMs, leading to Aβ formation in cells. Mutant Lec 8 strain, CHO cell lines are reported to have a defect in the CMP-NeuNAc transport system, which has shown an increase in asialo-oligosaccharide expression [154]. Tunicamycin and brefeldin A, soluble inhibitors of glycosylation have demonstrated to reduce APP secretion when N-glycosylation and sialylation were inhibited [152]. Altered protein glycosylation, *via* tunicamycin or mannosidase inhibition, has shown the disruption of axonal sorting in both *in vitro* and *in vivo* [155]. A similar result was observed with another model, when the asparagine residues, *i.e*. the sites of N Glycosylation, were removed [156]. McFarlane *et al*., [157] used mannosidase I and II inhibitors, 2-deosxymannojirimycin (dMan) and swainsonine respectively, for the investigation of the different effects of mannose and other complex sugars on APP processing. They observed that the treatment of AtT-20 mouse pituitary cells with dMan or swainsonine *in vitro*, prevented the N-linked sugars to mature which resulted in a significant decrease in APP secretion from the cell to the cell membrane.

Taken together these results confirm the previous lectin studies that suggested the preferential transfer of mature APP from the perinuclear region to cell membrane, where the high-mannose containing forms were retained in the ER/Golgi complex. If the APP is retained in the perinuclear region, it may have implications in its processing and the generation of Aβ [158]. Hence we can say that the impairment of APP maturation of the oligosaccharide chains, causing the retention of APP in the perinuclear region leads to an uprise in Aβ concentration in the cell due to holoprotein buildup in cellular compartments [159].

Decreased secretion of sAPP has been associated with generation of oligomannosyl oligosaccharides mediated altered APP glycosylation state. This was observed to be coupled with a parallel increase in the deposition of the cellular protein within the cell [154, 160]. Conversely, conjugation of terminal sialic acid residues to the glycan was shown to increase sAPP levels [161, 162]. Activation of Protein kinase C (PKC) has been widely reported to alter APP processing [163, 164]. Sialyltransferase enzyme transfected cells have demonstrated a direct relationship between the sialylation potential of APP and the fold stimulation of sAPP, following PKC activation [154]. Mutations altering the APP glycosylation state have been linked to an increased Aβ 42/Aβ 40 ratio, such as Swedish and London mutations. Both of these mutations account for altered N-glycosylation of APP, with an increased content of bisecting GlcNAc [165]. In accordance with this, GlcNActransferase III mRNA expression has reportedly been increased in AD brains [161].

Several studies have reported the presence of O-glycosylation sites and their functional role in APP [166-169]. Though elusive, the role of O-Glycosylastion has been proposed in proteolytic processing of APP by α-secretase, β-secretase and γ-secretase. In addition, studies have shown that it is the O-glycosylated APP that is preferentially secreted [168, 170]. Tyrosine O-glycosylation has been reported in Aβ 1–15 to Ab1–20 but not in full-length (Aβ1–38 to Aβ 1–42) Aβ fragments [167]. An increase in the shorter Aβ fragments cerebrospinal fluid (CSF) from AD patients and non-demented controls showed to carry the tyrosine-linked glycan in AD patients. APP is also O-GlcNAcylated [171], which affects proteolytic processing of APP, thereby increasing sAPP and decreasing Aβ secretion [172]. These results suggest that the post-translational modification of APP by glycosylation is a key event in determining the processing of the protein and may have significant implications in understanding the initial

deposition and kinetics of amyloid aggregation in a pathological situation like AD.

ROLE OF CHOLINERGIC SYSTEM IN APP POST TRANSLATIONAL MODIFICATIONS

It has been established that APP, γ-secretase and altered glycosylation can lead towards misfolding and AD pathology. Moreover, cholinergic system has a pivotal role in learning and memory, and its deficits are also part of AD pathology. But the relationship between APP and cholinergic neurons has not been elucidated. However, recent evidence obtained from mice and cell lines implies that the cognitive decline occurs due to loss of cholinergic neurons and APP processing [173]. Experimental evidence obtained from the studies on these model organisms suggests that activity of cholinergic neurotransmission might have an impact on APP processing. Moreover, the APP phosphorylation on threonine 668 (P-APP) may also influence the APP metabolism. The Aβ production significantly reduced due to mutation or inhibition of T668 kinase inhibitors. It is suggested that the T668 phosphorylation may facilitate the β-secretase (BACE) 1 cleavage of APP to increase Aβ generation [174]. In addition, p35- and p25-mediated Cdk5 activities lead to discrete APP (Thr668) phosphorylation, where the overexpression of both p35 and p25, increases the secretion of Aβ, as well sAPP (beta), and sAPP (alpha) [175]. The APP T688 phosphorylation also regulates the nuclear translocation of APP intracellular domain that also contributes towards neurodegeneration [176].

Alterations in APP metabolism significantly aids in the long-lasting effects of AChE inhibitors. However, complete inhibition is lethal as the natural physiology of the neuron will also be inhibited [177]. The potentiation of the central cholinergic system can be a potential tool and a promising strategy for therapeutics by modulating AChE that ultimately increases the ACh concentration in the brain [178]. However, despite the accumulating evidences, the interaction between cholinergic neurotransmission and APP processing and the physiological significance of PTM events remain speculative and still needs further exploration.

POST TRANSLATIONAL MODIFICATIONS AND TAU

Several studies have suggested and proposed two major hypotheses about the relationship of tau and Aβ through which tau may facilitate Aβ-induced impairments [179]. According to the first hypothesis, physiological forms of tau may cause abnormal neural network activity *via* diverse pathogenic triggers [180, 181], while the second hypothesis proposed that the Aβ might change the PTM or distribution of tau, making it an active mediator of Aβ-induced neuronal dysfunction [182]. A detailed characterization of tau PTMs may assist to strategize the plausible mechanisms to combat the consequences of the pathological processes associated with tau.

Although the phosphorylation of tau is well understood, with identified phosphorylated sites *i.e.* Ser-68, Thr-69, and Thr-71 [183], it also involves cross talk among diverse and sometimes competing PTMs which have still not been well studied including O-glycosylation, ubiquitination, acetylation and methylation [179].

Methylation of tau on lysine and arginine residues has been recently reported [184, 185]; however, the functional effects are still unknown. Methylation inhibits tau aggregation by increasing the amount of proteins required to form aggregates by increasing tau's dissociation rate from fibrils and decreasing the flexible extension rate. Moreover, methylation also delays the aggregation rate by diminishing the filament nucleation, which is the rate limiting step in the formation of neurofibrillary tangles [184]. Interestingly, the increased demethylation of protein phosphatase 2A (PP2A) (L309) results in reduced PP2A activity in AD brain, mediated by Aβ overproduction (or estrogen deficiency in mice), leading to compromised dephosphorylation of abnormally hyperphosphorylated tau [186].

In addition, tau phosphorylation which is directly mediated by phosphotransferases, there is a complex regulatory control on tau aggregation by competing modifications like O-linked β-N-acetylglucosaminylation (OGlcNAcylation). The OGlcNAcylation significantly regulates tau phosphorylation by decreasing the phosphorylation levels, thus depressing the neurofibrillary lesion formation and aggregation [187].

Furthermore, the ubiquitination of tau at Lys-6, Lys-11 and Lys-48 also modulates the intracellular tau levels in AD brains [188]. Studies have also shown significant regulating effects of increased acetylation on tau in AD which also counteracts the ubiquitination and degradation of phosphorylated tau [189].

MUSCARINIC ACETYLCHOLINE RECEPTOR AND TAU HYPERPHOSPHORYLATION

Post-translational modifications play an important role in the structure and function of GPCRs. Although N-linked glycosylation is the most common posttranslational modification of GPCRs but limited data is available regarding their role in mAChRs [190]. Among all mAChRs, M3 undergoes few important modifications, such as N-glycosylation and disulfide bond formation [191]. The absence of M3 N-glycosylation promotes receptor trafficking impairment, generates ER stress and thus leads to an increased susceptibility for cell disruption [191]. Impaired neurotransmission which is highly evident in many neurodegenerative disorders is also perturbed due to lack of N-glycosylation of M3 [192]. On the contrary, N-glycosylation of the M2 is not required for cell surface localization or ligand binding [193].

It has been observed that activation of M1 receptor decreases tau phosphorylation. Fisher *et al*., demonstrated the plausible mechanism of M1-mediated decrease in tau phosphorylation [194]. M1- agonists improved cognition and behavior, decreased the hyperphosphorylated tau and the number of neurons containing aggregated tau and paired helical filaments (PHFs), and decreased the inflammation [194].

NICOTINIC ACETYLCHOLINE RECEPTOR AND TAU HYPERPHOSPHORYLATION

The α7-Aβ binding activates α7 nAChR and increases tau hyperphosphorylation *via* ERK-MAPK and JNK-1-MAPK activation [195]. The ERK-MAPK activation leads to

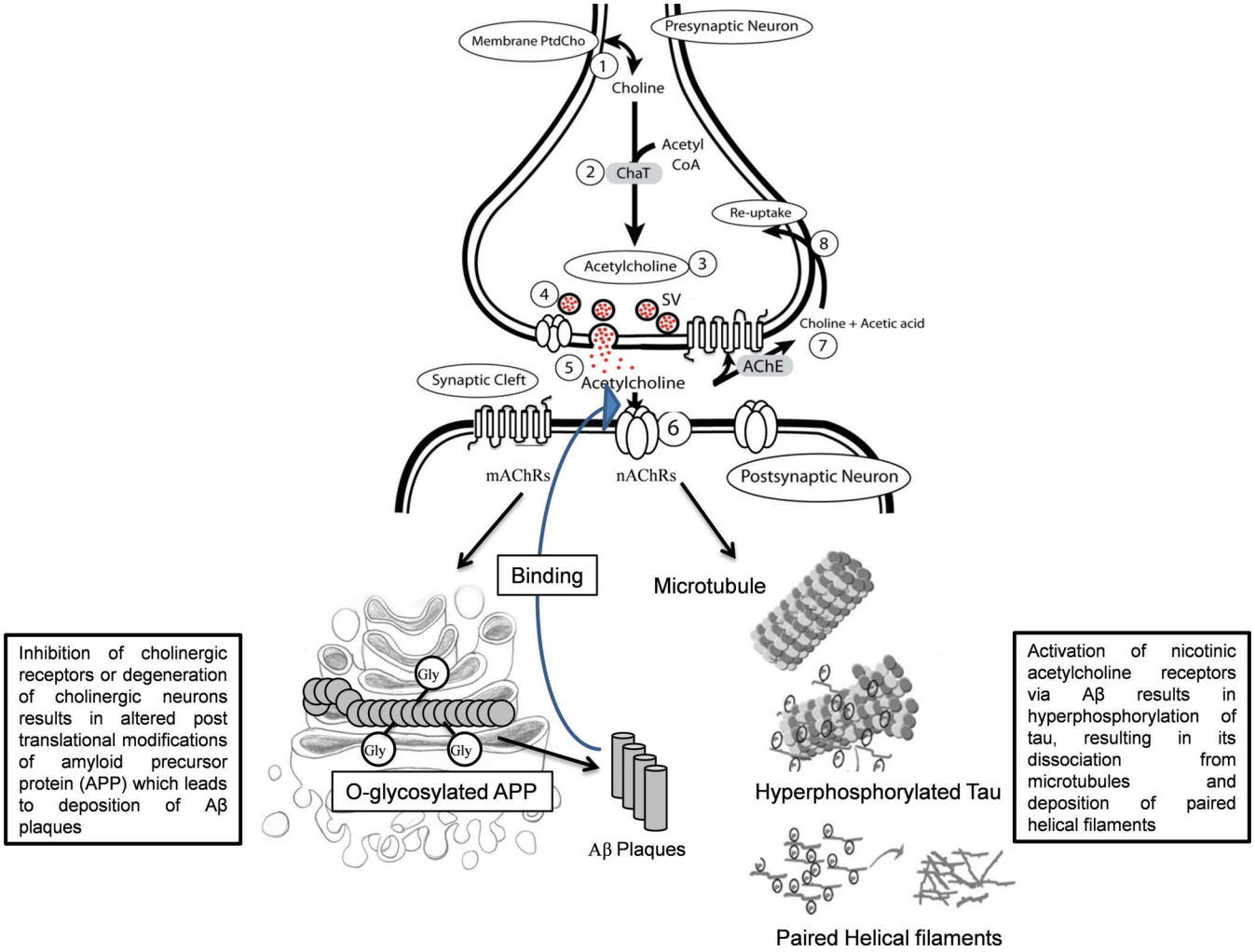

Fig. (2). Cholinergic Synapse and PTMs of APP and Tau. Abbreviations: ChaT: Choline acetylTransferase, PtdCho: Phosphotidylcholine, SV: Synaptic vesicles, AChE: Acetylcholine esterase, ACh: acetylcholine, mAChR: muscarinic ACh receptor, nAChR: nicotinic ACh receptor, 1) PtdCho provides the choline precursor and acetyl moiety from acetyl CoA, 2) in the presence of ChaT 3) the ACh is synthesized, 4) Storage of ACh in synaptic vesicles, 5) Release of ACh in the synaptic cleft, 6) Action of ACh on postsynaptic cholinergic receptor, 7) Degradation of ACh *via* AChE into choline and acetate ion, 8) Reuptake of choline into the presynaptic nerve terminal by choline transporter.

phosphorylation of two proline directed MAPK-targeted serine and threonine residues (S202, T181) while the activation of JNK-1-MAPK pathway phosphorylates the T231 along with S202 and T181 residues on tau [195]. Activation of α7 nAChR *via* Aβ mediates the phosphorylation of GSK 3β at tyrosine 216 which also results in phosphorylation of S202 on tau [196]. The phosphorylated S202 and T181 residues can lead to microtubule instability as they are involved in binding kinetics of tau-microtubule, resulting in the formation of NFTs [197]. The Aβ induced tau hyper-phosphorylation can be blocked by α7 nAChR selective antagonist, methyllycaconitine (MLA) [133].

Moreover, increase in tau phosphorylation was observed after nAChR activation *via* application of nAChR agonists. It is evident that chronic nicotine treatment in transgenic model of AD causes an upregulation of nAChRs which results in activation of p38 MAP kinase which in turn phosphorylates tau and exacerbates tau pathology [198]. Similarly, increase in tau phosphorylation was also observed after activation of nAChRs *via* AChE inhibitors and nAChR agonists, however, this requires nAChR mediated Ca^{+2} entry inside the cell as Ca^{+2} removal by ethylene glycol tetra acetic acid (EGTA) prevents increased tau hyperphosphorylation [199]. Taken together these studies highlight the nAChR-Aβ interaction, mediating tau hyperphosphorylation and neurofibrillary tangle formation (Fig. **2**).

CONCLUSION

AD, despite been extensively studied for the last many years, is a constellation of consequences which is still to be explored, to devise effective therapeutic strategies. The dysfunctional cholinergic system in AD has been linked to ACh deficits, which emphasizes therapeutic research to focus on effective approaches for maintaining its level in

brain. Muscarinic cholinergic receptors play a significant role in regulating CNS circuits, particularly involving learning and memory. Muscarinic receptor subtypes with different distributions in CNS, provides the opportunity to take each receptor as a drug target. The available cholinergic compounds lack the subtype-specificity and effectiveness that favors the side effects and may influence cognitive effects because of weak or differing actions. However, some selective allosteric modulators of ACh have demonstrated therapeutic potential that can be used as a better therapeutic approach. Additionally, a rather unique aspect of involvement of PTMs and their potential effect on muscarinic and nicotinic cholinergic receptors, gives a new dimension to study the pathological consequences where these cholinergic receptors are involved. The distinct and influential association of several competing PTMs with cholinergic receptors provide a detailed understanding of complex regulation of cholinergic system under several PTMs that may govern the diverse pathological mechanisms and associated consequences in AD. Future studies are required to focus on the specific role of cholinergic neurotransmission and PTMs in AD, to further validate drug targets and effective therapeutics.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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