Alzheimer's Disease: Interactions Between Cholinergic Functions and β-amyloid

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Abstract: Alzheimer's disease (AD) is characterized by two major features: (1) degeneration of basal forebrain cholinergic neurons and ensuing deficient cholinergic functions in cortex and hippocampus; (2) extracellular protein aggregates containing β-amyloid peptides (Aβ) in these cholinergic target areas. So far, the most effective therapy for AD is to enhance cholinergic transmission. Neuromodulatory functions of the cholinergic system are mainly mediated by muscarinic receptors (mAChRs). It has long been recognized that mAChRs are crucial for the control of high-level cognitive processes. Drugs that activate mAChRs are helpful in ameliorating cognitive deficits of AD. On the other hand, mounting evidence have established detrimental effects of Aβ to cognitive functions. Despite intensive research on AD, it remains unclear how these two prominent features of the disease may be linked to cause cognitive impairments. In this review, we will summarize a series of recent findings on the interactions between cholinergic functions and β-amyloid in normal animals and AD models, and discuss their potential implications in the pathophysiology and treatment of Alzheimer's disease.

Keywords: Alzheimer's disease**,** cholinergic, muscarinic receptors, β-amyloid peptides, insulin, GABAergic transmission, prefrontal cortex, protein kinase C.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive loss of memory and cognition [1, 2]. Its clinical symptoms have great variations depending on individual patients and different stages of the illness. Definitive diagnosis of AD usually can only be achieved from histological examination of postmortem brain tissues. From these tissues, it is apparent that AD-afflicted brains have two microscopic pathological hallmarks: extracellular plaques that are enriched in Aβ, a 42-residue hydrophobic peptide with an ominous propensity to assemble into oligomers and polymers, and intracellular neurofibrillary tangles composed of paired helical filaments assembled from hyperphosphorylated tau [3]. In addition to these abnormalities at the cellular level, there are widespread degeneration of neurons in many brain regions, including the frontal and temporal cortices, hippocampus, and the basal forebrain [3]. However, the atrophy only represents the end-stage of a long-term disease process. There are still ongoing debates about the potential link between the microscopic and gross pathological features of AD. It remains unsettled whether plaques and tangles are produced first, which eventually kill neurons, or degeneration of neurons in affected regions accelerates the formation of plaques and tangles.

This uncertainty is, to a large extent, caused by the lack of a clear understanding on the etiology of AD. The majority of AD is sporadic. Tremendous progress in the identification of genes involved in AD, particularly the rare familial forms, has led to the realization that the more prevalent, sporadic forms may be caused by the complex interplay between a number of genetic and environmental factors. Mutations on three genes (β-amyloid precursor protein, presenilin 1 and presenilin 2) have been definitively linked to early-onset familial AD [2]. A polymorphism in the apolipoprotein E gene (the E4 allele) has been associated with increased susceptibility to the more common, late onset AD [2]. However, these four genes may account for less than 30% of the genetic components of AD [4].

The complex nature of Alzheimer's disease makes it very difficult to deduce a common mechanism that can explain all different facets of the disorder. However, several consistent features of AD, combined with studies on cognition and memory in general, suggest that a coherent theme may link these apparently disparate aspects of AD.

DYSFUNCTIONAL CHOLINERGIC SIGNALING IN CORTEX IS INVOLVED IN AD

One of the most fundamental and consistent features of AD is the severe degeneration of cholinergic neurons projecting from basal forebrain to cortical and hippocampal areas [5, 6]. A 90% loss of basal forebrain cholinergic neurons has been found in AD patients [7, 5]. In contrast to the marked reduction of acetylcholine content in cholinergic target areas in AD brains, other transmitters, such as serotonin, norepinephrin, and dopamine, do not show a significant decrease [6]. The reduction of choline acetyltransferase, the rate-limiting enzyme in the synthesis of acetylcholine, correlates well to the severity of cognitive impairments and memory loss in AD patients [8]. Moreover,

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the degree of neuronal loss in basal forebrain has a good correlation with the severity of AD symptoms before death, especially among presenile cases of AD [9, 10]. Lesions of basal forebrain cholinergic neurons in rats with toxins also cause dose-dependent impairments in spatial memory tests and attentional functions [11-13]. So far, most of the effective drugs currently approved for the treatment of AD are inhibitors of acetylcholinesterase (AChE), such as aricept and tacrine. These drugs were developed based on the "cholinergic hypothesis" of AD [14]. From their clinical trials and later therapeutic use in AD patients, it is clear that these drugs can alleviate memory and cognitive problems in many AD patients, although the benefits are generally modest [15].

It has long been recognized that the cholinergic system is critically involved in the control of cognition [16]. Agents that block muscarinic cholinergic receptors (mAChRs) disrupt cognitive functions and cause transient loss of short-term memory [17-19, 6]. Drugs that potentiate central cholinergic functions enhance short-term memory and alleviate memory deficiencies caused by muscarinic blockers [20-22].

These lines of evidence indicate that acetylcholine plays a key role in normal cognition and memory. This role is greatly compromised in AD, most likely due to the selective degeneration of cholinergic neurons in the basal forebrain. Corresponding to the degeneration, there is a significant loss of nicotinic ACh receptors and certain types of muscarinic ACh receptors in the cortical and hippocampal regions of AD brains [23-25]. Although there is a general consensus that m1 muscarinic receptors are preserved in most AD patients, several studies suggest that m1/G-protein mediated signal transduction may be disrupted in AD [26-28]. These results suggest that m1 signaling could be a point of pharmacological intervention in the treatment of AD. As m1 receptors are largely intact, if dysfunctional downstream signaling cascade can be restored, there is a good chance to improve cholinergic functions. Thus, it is crucial to have a better understanding on muscarinic signaling in the normal situation and how it might be altered in AD models.

One of the major target areas of basal forebrain cholinergic neurons is the prefrontal cortex (PFC), a brain region strongly linked to high-level, "executive" processes needed for complicated goal-directed behavior [29]. Functional studies in primates have established that PFC is particularly critical for a form of short-term information storage described as "working memory" [30]. The firing of some PFC neurons increases throughout the delay period of a delayed-response task when information must be "held" momentarily and used subsequently to guide correct responses [30]. The damage of PFC in humans results in disturbances in a variety of functions, such as attention, memory, response selection and planning. Revealing the functions of muscarinic signaling in PFC is crucial for understanding the mechanisms of cognition and AD.

ACCUMULATION OF β-AMYLOID PEPTIDES IS ASSOCIATED WITH THE PATHOLOGY OF AD

In addition to cholinergic hypofunction, another prominent feature of AD is the accumulation of β-amyloid peptides (Aβ). Aβ is a major component of senile plaques [31], extracellular protein aggregates that are used as a histopathological hallmark for the terminal diagnosis of AD. Much evidence has suggested that Aβ is likely to make a direct contribution to the pathogenesis of AD. Aβ peptides are produced by a series of coordinated cleavages of the β-amyloid precursor protein (APP) [2]. Most of the mutations in the APP gene are clustered around the cleavage sites, which increases the rate of cleavage, thereby generating more Aβ [2, 4]. APP processing and Aβ generation are modulated by neuronal electrical activity [32, 33]. Mutations in presenilin 1 (PS1) and presenilin 2 (PS2) genes are also linked to increased secretion of Aβ [2]. Transgenic mice overexpressing mutant APP genes exhibit increased Aβ deposits and deficits in spatial learning and memory [34-36]. The situation becomes even worse when mutant APP and mutant PS1 are both expressed in transgenic mice [37, 38]. Immunization with $A\beta_{42}$ in transgenic mice expressing mutant APP [39] or mutant APP plus PS1 [40] abolishes β-amyloid plaques, neuritic dystrophy and astrogliosis. In the latter study, $A\beta_{42}$-vaccinated transgenic mice show significantly superior cognitive performance than control transgenic mice without immunization.

These lines of evidence unequivocally demonstrate the link between Aβ and AD. However, it is still debatable whether Aβ plaques actually cause AD, and whether all the clinical features seen in typical AD patients can be attributed to the actions of these senile plaques. The key concern is that Aβ plaques do not cause massive neuronal death in cortex and hippocampus when the clinical symptoms are manifested in AD patients, although many studies in cell lines and animal models show that Aβ is toxic at high concentrations [41, 42]. This is perhaps more clear in aged APP transgenic mice Tg2576, where numerous Aβ plaques are seen in cortical and limbic structures, yet no detectable loss of neurons is seen in these areas [43]. Cognitive deficits in old Tg2576 mice correlate to impaired hippocampal long-term potentiation [44], a synaptic model of memory. In these mice, no loss of presynaptic or postsynaptic elements is seen at ages when they exhibit problems in synaptic plasticity and spatial memory. These results suggest that, in the presence of Aβ, the dysfunction of cortical and hippocampal neurons, not their death, is largely responsible for the impairments in cognition and memory associated with AD. Currently the leading theory of causation for AD believes that synaptic dysfunction due to progressive Aβ accumulation is the most significant factor contributing to the initial stages of memory loss [45, 46]. Consistent with this notion, single systemic injections of an antibody to Aβ into 22-month-old APP transgenic mice essentially eliminate overnight their memory deficits [47], suggesting that antibodies to Aβ can interfere acutely with the Aβ-caused synaptic dysfunction.

THE GABA SYSTEM IS POTENTIALLY ONE OF THE KEY CELLULAR TARGETS FOR MUSCARINIC SIGNALING IN COGNITION AND MEMORY

Given the key role of muscarinic receptors in cognition and memory, one of the important questions yet to be answered is the targets of muscarinic signaling that are involved in cognitive processes. Aβ accumulation may

impair muscarinic regulation of these targets, therefore contributing to the cognitive deficits associated with AD. At the cellular and circuit levels, cognition and memory rely on the processing of information encoded in synaptic transmission. As ion channels are the information processors in the network, regulations of ion channel activities should be able to significantly affect the generation and integration of neural signals. Under this context, muscarinic modulation of ion channels and synaptic transmission in cortex may underlie the crucial roles of acetylcholine in cognition and memory. Consistent with this, patients taking AChE inhibitors often report immediate improvement in thinking and memory, suggesting an acute action of ACh, perhaps on targets directly linked to synaptic transmission.

Recent studies suggest that GABAergic inhibition in frontal cortex of monkeys is critical for controlling the timing of neuronal activities during the thought process, which is fundamental for processing ongoing information and planning appropriate actions at a future time [48]. Injection of the $GABA_A$ receptor antagonist bicuculline into dorsolateral PFC disrupts the performance of a delayed response task – a measurement of spatial working memory [49, 50]. Another study using a similar paradigm further demonstrates that $GABA_A$ receptors-mediated inhibition plays a key role in the construction of the memory fields of cortical neurons [51]. Furthermore, clinical studies have found that benzodiazepines, agents that enhance GABAergic inhibitory transmission, protect against AD [52]. The critical involvement of cortical muscarinic signaling in cognition and AD, combined with the central role of GABAergic inhibition in working memory, makes it reasonable to hypothesize that the GABA system might be one of the key cellular targets for muscarinic signaling in cognition and memory, and its dysregulation by mAChRs in AD might contribute to the cognitive impairment.

In CNS, the inhibitory synaptic transmission is mediated by $GABA_A$ receptors. The $GABA_A$ receptor contains an intrinsic chloride channel. It is thought to be a hetero-pentameric structure, assembled by combining homologous subunits from five different classes: α (1-6), β (1-4), γ (1-3), δ and ε (1-2). The subunit composition of $GABA_A$ receptors critically affects the functional properties of $GABA_A$ channels [53]. Multiple PKA, PKC and tyrosine phosphorylation sites have been identified in several $GABA_A$ receptor subunits, and protein phosphorylation exerts a powerful regulation of GABA-activated currents in recombinant and native $GABA_A$ channels [54, 55].

Recently, the muscarinic regulation of $GABA_A$ receptor currents in PFC pyramidal neurons has been examined [56]. Activation of mAChRs with carbachol produces an enhancement of $GABA_A$ receptor currents in acutely-dissociated cells following a short treatment with insulin. Inhibiting phosphoinositide 3-kinase (PI3K), a downstream target of insulin signaling, eliminates this effect, as well as the carbachol-induced enhancement of GABAergic miniature inhibitory postsynaptic current (mIPSC) amplitudes in PFC slices. The muscarinic potentiation of $GABA_A$ currents is blocked by PKC inhibitors, broad-spectrum protein tyrosine kinase inhibitors, and specific inhibitors of the non-receptor tyrosine kinase Src. Furthermore, muscarinic receptors in PFC slices activates PKC and the focal adhesion kinase Pyk2 (a potential molecular link between PKC and Src) in a PI3K-dependent manner. These results (Fig. **1**) show that mAChR activation in PFC pyramidal neurons enhances $GABA_A$ receptor functions through a PKC-dependent, Src-mediated signaling cascade. More interestingly, this muscarinic regulation of $GABA_A$ receptors is gated by insulin, another key player implicated in cognitive functions.

EMERGING EVIDENCE INDICATES THAT INSULIN SIGNALING IS INVOLVED IN AD

Impairment of cerebral insulin signaling causes similar behavioral abnormalities as disruption of the cholinergic function [57], implying the potential interaction between the two systems. Emerging evidence suggests that insulin has important functions in brain regions involved in cognition, and insulin dysfunction in these areas can result in memory loss and even AD [58-62]. Insulin receptors are highly expressed in CNS neurons [63, 64] and localized to synapses [65, 66]. The expression of insulin receptors is found to be increased in postmortem brain tissues from patients with sporadic AD, compared to age-matched controls. However, there is a significant reduction in the kinase activity of insulin receptors in these AD brains [67]. It suggests that insulin signaling may be compromised in AD. Consistent with this, a severe reduction in cerebral glucose utilization is found in late-onset sporadic AD patients, although their glucose supply to the brain is normal [59]. Moreover, it has been shown that the increase in blood insulin, not glucose level, significantly improves memory in AD patients [61], suggesting that insulin has a glucose-independent mechanism of regulating cognition.

In addition to the above evidence, many other studies also show that insulin is involved in processes related to AD. For example, insulin and insulin-like growth factor I (IGF-I) stimulation reduces tau phosphorylation through a PI3K-dependent mechanism [68]. Disruption of the insulin receptor substrate-2 (Irs2) gene promotes tau phosphorylation and causes the accumulation of neurofibrillary tangles containing phosphorylated tau in the hippocampus of old Irs2 knockout mice [69]. Insulin significantly reduces intracellular accumulation of Aβ by accelerating APP/Aβ trafficking to the plasma membrane from the trans-Golgi network, a major cellular site for Aβ processing [70]. IGF-I treatment of mice overexpressing mutant amyloid markedly reduces their brain Aβ burden [71]. Insulin degrading enzyme (IDE), an extracellular protease that degrades several small polypeptides, such as insulin, IGF-I, IGF-II, also degrades Aβ [72]. Transgenic overexpression of IDE in neurons significantly reduces brain Aβ levels, retards or completely prevents amyloid plaque formation [73]. On the other hand, IDE deficient mice show increased cerebral accumulation of endogenous Aβ, a hallmark of AD [74]. These results suggest that insulin dysregulation may contribute to AD pathology by several mechanisms, including decreased cortical glucose utilization, increased tau phosphorylation and neurofibrillary tangle formation, and increased β-amyloid aggregation through inhibition of IDE.

The diverse functions of insulin in neurons could affect cognition and memory on many levels. Converging evidence suggest that CNS insulin may also function as an important neuromodulator to influence cognition by regulating ion channels, neurotransmitter receptors and synaptic transmission [75-77]. The insulin-gated muscarinic regulation of $GABA_A$ receptors [56] (Fig. **1**) provides a framework for understanding how insulin signaling may interact with cholinergic functions and how this interaction might underlie their role in cognition and memory. As the insulin-mediated memory improvement happens very quickly (~30 min) [61], it is likely that this effect is through insulin receptor-activated downstream substrates that are directly involved in synaptic transmission and plasticity, rather than targets that take much longer time to modify (e.g. Aβ clearance). The acute effect of insulin in improving short-term memory provides the best entry point to study its role, in conjunction with other key factors, in the cellular and molecular mechanism of AD.

β-AMYLOID PEPTIDES INHIBIT CHOLINERGIC FUNCTIONS IN AD

Emerging evidence show that Aβ has very potent actions on the cholinergic system [78]. For example, at nanomolar concentration, freshly prepared $Aβ_{42}$, which is not neurotoxic, suppresses acetylcholine synthesis in primary cultures of basal forebrain neurons [79] and in a cell line derived from cholinergic neurons [80]. In another study, picomolar levels of Aβ peptides inhibits K^+-evoked acetylcholine release from hippocampal slices [81]. In cortical cultures, Aβ peptides disrupts muscarinic receptor – G proteins coupling as measured by GTPase activity and intracellular concentration of inositol phosphates and Ca^{2+} [82]. In rat hippocampal slices, $Aβ_{42}$ inhibits nicotinic receptor-mediated currents in interneurons at 100nM [83]. Moreover, transgenic mice carrying mutated APP and presenilin 1, which have extensive amyloidosis, demonstrate a prominent reduction in the density and size of cholinergic synapses in frontal cortex [84], suggesting that amyloid load could reorganize the cholinergic network. These pleiotropic effects of Aβ on cholinergic neurons, as well as on acetylcholine receptors in cortical and hippocampal neurons, suggest that low concentrations of Aβ peptides may impair normal cholinergic functions independently of concurrent neurotoxicity. It might mimic what is happening *in vivo* at the pre-symptomatic stage of AD, where the Aβ concentration is not high enough to be toxic.

The impact of Aβ on muscarinic regulation of GABA transmission has also been examined [85]. Activation of mAChRs significantly increases the amplitude of GABAergic spontaneous inhibitory postsynaptic current (sIPSC) in PFC pyramidal neurons from wild-type animals, but fails to increase the sIPSC amplitude in APP transgenic mice. Rat PFC slices pretreated with Aβ peptides give similar results. Inhibiting PKC blocks the mAChR enhancement of spontaneous IPSC amplitudes, implicating the PKC-dependence of the mAChR effect. In APP transgenic mice, application of mAChR agonists fails to activate PKC despite the apparently normal expression of mAChRs [85]. These results show that the muscarinic regulation of GABA transmission is impaired in the AD model, probably due to the Aβ-mediated interference of mAChR activation of PKC.

THE CHOLINERGIC SYSTEM AFFECTS β-AMYLOID SIGNALING

In addition to the pleiotropic roles of Aβ in the regulation of cholinergic functions, several lines of evidence indicate that the cholinergic system also exerts an important impact on Aβ signaling. *In vitro* studies have demonstrated that stimulation of muscarinic receptors can modify APP processing and inhibit amyloidogenic Aβ production [86, 87]. *In vivo* studies further show that muscarinic agonist treatment decreases APP levels not only in normal rats, but also in aged and cholinergic denervated rats that model Alzheimer's disease [88]. Moreover, cell culture studies have shown that AChE inhibitors, some of which are FDA approved drugs for the treatment of AD, affect the processing of APP and inhibit the secretion of Aβ [89, 90].

Interestingly, a recent study shows that muscarinic receptor activation can also reverse Aβ-induced biochemical and physiological changes [91]. Exposure of cortical slices to Aβ peptides induces a marked increase in the activation of PKC and Ca^{2+}/calmodulin-dependent kinase II (CaMKII) – two enzymes critically involved in a wide range of neuronal functions from synaptic plasticity and transmitter release to neurite outgrowth and cell survival [92, 93]. Activation of m1 muscarinic receptors, but not nicotinic receptors, significantly inhibits the Aβ activation of PKC and CaMKII.

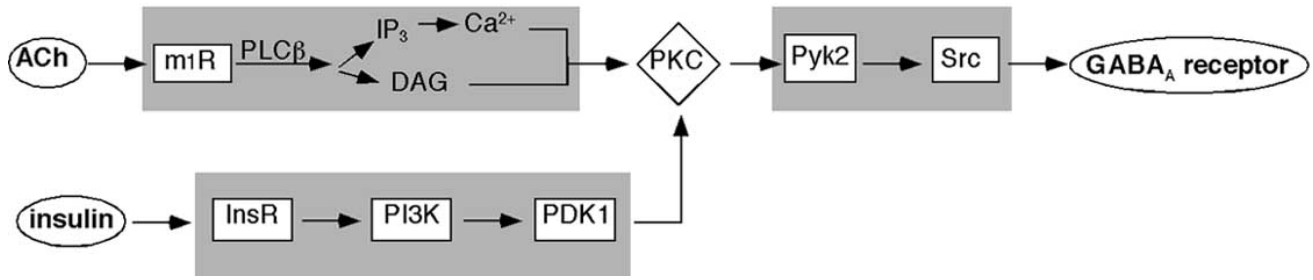

Fig. (1). A diagram illustrating the convergence of mACh and insulin signaling cascades on PKC and the downstream pathway leading to the potentiation of $GABA_A$ receptor currents. Muscarinic receptors enhance postsynaptic $GABA_A$ receptor functions in PFC pyramidal neurons through a PKC-dependent activation of protein tyrosine kinase Src signaling cascade. Furthermore, this cascade is gated by an insulin/PI3K/PDK1 pathway, which facilitates muscarinic activation of PKC and Pyk2. PI3K: phosphoinositide 3-kinase; PDK1: phosphoinositide-dependent kinase 1; Pyk2: proline-rich tyrosine kinase 2 (focal adhesion kinase). For details see ref [56].

Increasing inhibitory transmission mimics the m1 effect on Aβ, whereas blocking $GABA_A$ receptors eliminates the m1 action. Moreover, electrophysiological evidence shows that application of Aβ to cortical slices induces action potential firing and enhances excitatory postsynaptic currents, while muscarinic agonists potently increases inhibitory postsynaptic currents. These results suggest that Aβ activates PKC and CaMKII through enhancing excitatory activity in glutamatergic synaptic networks. Activation of m1 receptors inhibits Aβ signaling by enhancing the counteracting GABAergic inhibitory transmission [91]. This study indicates that muscarinic receptors can potently down-regulate Aβ actions by targeting the GABA system.

These aforementioned results suggest that the cholinergic deficiency in AD brains would lead to the loss of the negative regulation of Aβ generation and functioning by muscarinic receptors, which in turn could impair m1 signal transduction [82, 27, 85] and inhibit ACh synthesis and release [79, 81], aggravating further the cholinergic hypofunction. Since these "vicious cycles" may potentially be inhibited by m1 agonists, it suggests that enhancing m1 signaling is a promising point of pharmacological intervention in the treatment of AD [94, 95].

A WORKING MODEL

It is apparent that Alzheimer's disease is a multi-factorial disorder that may be too complex to be reduced to one simple model. However, several consistent features of this disease and the connections among these features prompt us to come up with a working model (Fig. **2**). This model aims to unify three separate lines of evidence into one coherent hypothesis. Previous studies have shown that: **(i)** Cholinergic functions, especially those mediated by muscarinic receptors, are critical for cognition and memory, and are impaired in AD. **(ii)** Aβ peptides strongly affect the normal functions of cortical neurons, which contributes to the pathogenesis of AD. **(iii)** Insulin improves memory deficits in AD patients through a fast, glucose-independent mechanism. Given the critical role of GABAergic inhibition in "working memory" [48], we propose that the GABA system is a key substrate of muscarinic actions in cognition and memory. Insulin pathway acts as a priming device that increases the strength of muscarinic signaling [56]. Aβ disrupts the muscarinic regulation of GABAergic transmission [85], which may be one of the mechanisms for Aβ to impair cortical functions, hence cognition and memory. On the other hand, mAChR activation inhibits the production and function of amyloidogenic Aβ [88, 91], and this negative regulation is likely to be impaired in AD due to the cholinergic deficiency.

In addition to Aβ accumulation, another early event correlating with cognitive impairment in AD is the accumulation of hyperphosphorylated tau into neurofibrillary tangles [2, 4]. It has been found that tau phosphorylation is reduced by insulin stimulation [68] or mAChR activation [96]. It suggests that the microtubule-associated protein tau is potentially another target of mAChR and insulin signaling involved in cognition and memory.

Many issues remain to be unsolved regarding the molecular and cellular mechanisms underlying the pathophysiology of AD. For example, how does Aβ impair the intracellular muscarinic signaling? Is it due to the disrupted insulin gating? Is the negative influence of Aβ specific for muscarinic receptors? Does Aβ alter other muscarinic functions, such as the mAChR regulation of glutamatergic excitatory transmission? What is the best way to reverse cholinergic and synaptic deficits in AD? By revealing functional rather than just structural synaptic changes on the earliest stages of AD, and correlating the electrophysiological results with both behavioral and biochemical measures, we should be able to assess the relative contributions of acetylcholine, Aβ, insulin and other related molecules to memory and cognitive impairments in AD. Knowledge gained from these studies would be helpful in improving the current therapy for AD and in providing novel targets for more precise pharmacological interventions.

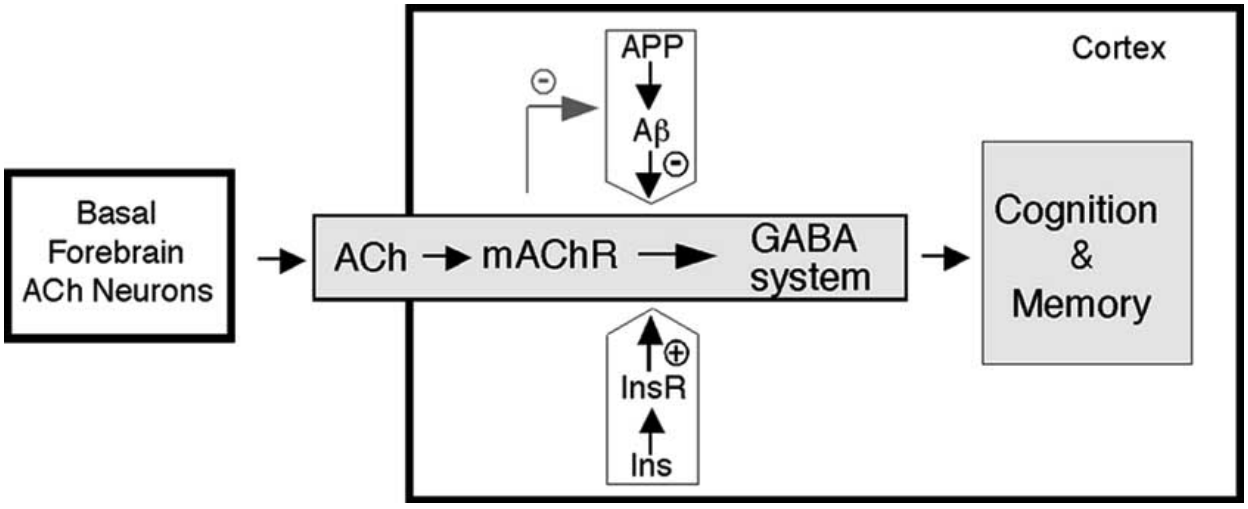

Fig. (2). A working model. Activation of mAChR signaling in cortex modulates the GABA system, which is critical for the regulation of cognition and memory. This cholinergic function is positively regulated by insulin signaling which acts as an enhancer, and negatively regulated by Aβ which acts as a suppressor. On the other hand, mAChR signaling also negatively regulates the generation and action of amyloidogenic Aβ. For details see ref [56, 85, 88, 91].

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