Rationale and prospects for drugs that target nicotinic acetylcholine receptors

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Introduction

Nicotinic acetylcholine receptors (nAChRs) are implicated in a variety of disorders of the human central nervous system including addiction to nicotine, Alzheimer’s disease, anxiety, autism, depression, epilepsy, Parkinson’s disease, schizophrenia, and Tourette’s syndrome [1, 2]. Mechanisms of nAChR impairment in this disparate group of syndromes are poorly understood. Additionally, in healthy organisms nAChRs play a significant role in a number of cognitive processes including learning and memory [3, 4]. Because nAChRs are involved in normal cognitive processes as well as a complex range of central nervous system disorders, it is important to define the means by which these receptors exert their action in the brain and interact with disease-related neuropathology. It is also imperative to explore the prospects of therapeutic manipulations of nAChRs in human central nervous systems disorders.

The characteristic structure of nAChRs is a ring of five subunits arranged around a ligand-gated excitatory ion channel. Neuronal nAChR subunits are classified as α (α2 to α10) and β (β2 to β4) subunits. The two main neuronal categories that have been identified on the basis of function and pharmacology are: (1) heterologous pentamers, constructed from combinations of α- and β-subunits [5], and (2) homologous pentamers, constructed from one subunit type, α7, α8, and α9 [6]. Besides the α4β2 nAChRs that constitute the high affinity binding sites for nicotine [7], the α7 nAChRs being highly permeable for Ca²⁺ are expressed widely and abundantly in the mammalian brain [8, 9]. The α7 nAChRs produce multiple effects at the cellular level including presynaptic modulation of neurotransmitter release [10] and post-synaptic generation of depolarizing currents [11].

The various types of nAChRs have characteristic patterns of distribution in the brain, and they have several loci on neurons, including on terminals, soma, and dendrites [12]. Neuromodulation of communicative processes in the brain is one role for nAChRs [13]. The demonstrated involvement of nAChRs in
cognitive processes, the dramatic cognitive impairment apparent in Alzheimer’s disease (AD), and the mounting number of AD patients in the world are among the compelling rationales for investigating nAChRs in AD. The role of nAChRs in AD and the potential for drug therapies in this neurodegenerative disease is the focus of this chapter. Studies of nAChR subtypes in the brains of patients with AD indicate that nAChRs that participate in high-affinity nicotine binding (e.g., α4β2) are substantially reduced but those binding to α-bungarotoxin (e.g., α7) are better maintained (for a review, see [14]). However, neuropathology may interact with the nAChR subtypes that survive in the AD brain and render them functionally ineffective.

**Relationship of β-amyloid (Aβ) and nicotinic acetylcholine receptors (nAChRs)**

AD is neurochemically characterized by cholinergic dysfunction, in particular affecting the nAChRs, and neuropathologically by intracellular neurofibrillary tangles and the extracellular β-amyloid (Aβ) plaques (for a review, see [14]). However, finding the possible link between these markers has remained elusive. Although increasing numbers of reports suggest that Aβ can potentely inhibit various cholinergic transmitter functions independently of apparent Aβ neurotoxicity (for review cf. [15]), little attention has been paid to the cholinceptive site. Nowadays there are numerous indications that chronic exposure to nicotine may protect against Aβ pathology (cf. [16]). In addition, it was shown recently that nicotine not only dose-dependently inhibited fibril formation from Aβ1-40 and Aβ1-42 but also disrupted preformed fibrillary Aβ1-42 in vitro [17]. Although epidemiological studies of tobacco use and risk of developing AD are somewhat contradictory [18, 19], a significant lower plaque density has been observed in autopsy cortical tissue of smokers as compared to non-smokers [20, 21].

**In vitro** studies have also indicated protective effects of nicotine against Aβ toxicity ([22, 23], for review cf. [24, 25]). Initial evidence for an interaction of Aβ with nAChRs was provided by the finding that stimulation of the α7 and α4β2 nAChR subtype in cultured rat cortical neurons was able to inhibit Aβ cytotoxicity [22, 23]. Furthermore, Alzheimer patients carrying the Swedish amyloid precursor protein 670/671 mutation and early and excessive accumulation of Aβ plaques were shown to display nAChR deficits [26]. In the meantime, numerous studies have investigated the relationship of Aβ and nAChR expression and the mechanisms that may be involved in this interaction.

So far only few reports on human brain have addressed potential links between AD neuropathology and cholinergic dysfunction. Our own previous studies on autopic human cortex combining in situ hybridization and immunohistochemistry revealed that in the frontal cortex neurons expressed α4 as well as α7 mRNA even in the vicinity of Aβ-immunoreactive plaques [27]. The density of α4 and α7 transcript expressing neurons was not altered
in AD. This finding is confirmed by other studies [28, 29] and may point to a lack of the impact of Aβ plaques on nAChR expression. By contrast to the findings on the mRNA level there is a decrease of nAChR subunit proteins in AD. Several groups by using immunohistochemistry, Western blot, and binding studies reported on a massive reduction of the α4 subunit, whereas less consensus exists regarding the decrease of the α7 subunit protein (for review see [27, 30]).

To investigate the possible impact of Aβ on nAChR protein expression we approached co-localization studies by two different ways. First, we combined silver staining techniques to visualize plaques with immunohistochemistry to show nAChR expressing cells. Second, we applied a double-immunohistochemical protocol. With both methods we were able to demonstrate the presence of nAChR-immunoreactive neurons in close proximity to and sometimes even within plaques. Therefore it seems to be rather unlikely that plaques interfere with nAChR expression. This result is confirmed by an investigation demonstrating no correlation of the plaque load in AD patients and nicotine binding, whereas an inverse correlation between [³H]epibatidine binding and Aβ₁₋₄₂ levels was found [21]. In addition, studies in transgenic mice [31–34] support the emerging view for a plaque-independent Aβ toxicity in the development of synaptic deficits in AD. The positive correlation between the decrease of synaptophysin immunoreactivity and [³H]epibatidine binding [35] complements the picture.

The increasing evidence that soluble Aβ plays a more important role in the development of AD and the general acceptance that Aβ₁₋₄₂ is the pathogenic Aβ peptide [36] led to the development of various in vitro model systems to study the impact of Aβ on nAChR expression under standardized conditions. Using primary hippocampal cultures grown under defined serum-free conditions it was shown that incubation for three days with 1 µM Aβ₁₋₄₂ caused severe morphological alterations: Neuronal cell bodies appeared to be shrunken and a remarkable retraction of dendrites as well as a loss of dendritic density was observed. The reduction of labelled processes was most prominent in the α4-immunoreactive neurons, whereas α7-expressing neurons seemed to be less affected (cf. [30]). Comparable morphological changes occurred after incubation with 0.5 µM Aβ₁₋₄₂. This different behaviour may indicate a subunit-dependent impact of Aβ₁₋₄₂ on nAChR expression.

Interaction of Aβ with nAChRs

The underlying mechanism of nicotinic transmission and Aβ interaction remained unclear until recently when investigations of Wang and co-workers shed light on this issue [37, 38]. They found that Aβ₁₋₄₂ binds specifically and with picomolar affinity to the neuronal α7 nAChRs. So far numerous studies provide evidence for a functional block of acetylcholine (ACh)-evoked current responses by Aβ₁₋₄₂ via the α7 nAChR-subtype in various types of cells and
in α7-transfected oocytes [39–41]. At complete variance with these data, 10 pM of non-fibrillar Aβ1–42 have been reported to activate rat α7 nAChRs expressed in Xenopus oocytes, although only upon the very first exposure, whereas a blockade of the α7 nAChR only occurred after application of 100 nM Aβ1–42 [42]. An activation of α7 nAChRs by Aβ1–42 was described for Xenopus oocytes transfected with mutant rat and human α7 cDNA carrying a point mutation within the pore-forming region (rat: L250T [42], human: L248T [41]). The latter finding is in good agreement with data obtained from studies with mutant α7 nAChR receptors carrying the particular threonine-forleucine substitution showing that several antagonists of chick and human wild-type α7 nAChRs behave like agonists (for review see [41]).

Consensus exists on the ability of Aβ1–42 to block α-bungarotoxin (α-Bgt) sensitive nAChR subtypes like the α7 nAChR [37, 39–43]. Its interaction with non-α-Bgt sensitive nAChR subtypes, however, is not clear yet. In the initial study it was demonstrated on hippocampal membranes from sporadic AD brains by immunoprecipitation and Western blot analysis that the nAChR α7-subunit co-immunoprecipitated with Aβ1–42, whereas the nAChR α1-, α3-, α4-, α5-, α8-, or β2-subunit failed to yield any detectable co-immunoprecipitate with anti-Aβ1–42 [37]. In functional studies some groups did not find any blockade of non-α-Bgt sensitive nAChR subtypes by Aβ1–42 [40, 41], whereas other researchers found a binding affinity for the α4β2 nAChR subtype in the range of 20–30 nM [38] or a partial blockade of non-α7 nAChRs at higher concentrations [39]. In one paper even a higher depression of α4β2 than of α7 nAChR currents upon Aβ1–42 incubation was reported [43]. In PC12 cells nanomolar concentrations of Aβ1–40 and Aβ25–35 significantly decreased [3H]epibatidine and [125I]α-Bgt binding sites as well as the mRNA and protein amounts of nAChR α3-, α7-, and β2-subunits [44]. A possible explanation of the inconsistencies in these findings may be the different Aβ1–42 concentrations used or the different cell types studied [40].

Another controversial issue is the nature of the interaction between Aβ1–42 and α7 nAChRs, as Aβ1–42 has been reported to compete with α-Bgt for binding to α7 nAChRs [37], whereas no competitive displacement of α-Bgt by Aβ1–42 was observed neither in intact mouse hippocampal neurons nor in neurons derived from chick ciliary ganglia [40]. Also Guan et al. [44] failed to displace [3H]epibatidine and [125I]α-Bgt by Aβ1–42, Aβ1–40, and Aβ25–35 in PC12 cells. Further functional characterization of the interaction of Aβ1–42 with α7 nAChRs revealed that the blockade of α7 nAChR function does not require the presence of an agonist. On the contrary, co-application of Aβ1–42 and ACh resulted in a failure to induce blockade [40].

The formation of an α7 nAChR-Aβ1–42 complex can be efficiently suppressed by Aβ1–28, implying that the binding epitope for α7 nAChRs resides in the amino acid 12–28 sequence region of Aβ1–42 [37]. Further studies using chimeric α7/5HT3 receptors suggested that Aβ1–42 interacts with the extracellular N-terminal domain of the α7 nAChR [40]. However, little information exists on the physical state of active Aβ1–42 that binds to nAChRs. In brain
slices incubation with freshly prepared Aβ₁₋₄₂ led to a rapid onset of inhibition, which speaks in favour of a soluble, oligomeric rather than a fibrillar form of Aβ₁₋₄₂ [39].


nAChR mediated signal transduction in respect to Aβ

The question remains whether the observed Aβ₁₋₄₂-induced nAChR functional changes affect synaptic transmission. It has been shown that Aβ₁₋₄₂ affects IₐCh with an IC₅₀ around 100 nM (~ 450 ng ml⁻¹) [39, 41, 42], although an IC₅₀ of about 7.5 nM has been described for rat hippocampal neurons [40]. The concentration of Aβ peptides in brain tissue from AD patients (2–20 μM) is higher than that required for maximal α7 nAChR blockade [45], but most Aβ peptides are concentrated in the Aβ deposits, and their exchange with interstitial fluid and their proximity to receptors are difficult to estimate. It is known, however, that mice genetically engineered to express Aβ peptides can display synaptic toxicity that correlates with Aβ₁₋₄₂ level in the 10–100 nM range [31, 32], concentrations that are effective at blocking α7 nAChRs.

In a recent review it was summarized that Aβ peptide can be directly neurotoxic, induce oxidative stress, incite an inflammatory response, and alter calcium homeostasis. These events might be mediated by direct interaction of Aβ aggregates with cellular membranes, or by binding of Aβ to microglial and neuronal cellular receptors [46]. High levels of Aβ₁₋₄₂ in AD may promote interactions with cholinergic neurons that express α7 nAChRs. The high affinity binding of Aβ₁₋₄₂ to α7 nAChRs may result in the inhibition of ACh release and may alter Ca²⁺-homeostasis for the affected cholinergic neuron. These significant and chronic physiological perturbations may lead to stress and even neurodegeneration.

An important role for α7 nAChRs in facilitating the entry and intraneuronal accumulation of Aβ₁₋₄₂ via endocytosis has been described [47]. The rate and extent of Aβ₁₋₄₂ internalization was directly related to the amount of α7 nAChR protein and effectively blocked by α-Bgt and by the endocytosis inhibitor phenylarsine oxid. Furthermore, the α7 nAChR was co-localized with Aβ₁₋₄₂ in prominent intracellular aggregates [47]. Based on these data the authors [47] suggest that internalization of Aβ₁₋₄₂ occurs predominantly in neurons expressing the α7 nAChR and may be facilitated by the high-affinity binding of Aβ₁₋₄₂ to α7 nAChRs on neuronal cell surfaces followed by endocytosis of the resulting complex. According to the authors their data provide a plausible explanation for the selective vulnerability of neurons expressing the nAChR α7 subtype in AD brain. The data are also interpreted to suggest that Aβ₁₋₄₂ is the dominant Aβ peptide in intracellular accumulation and amyloid plaques [47]. However, the nAChR subtype affected in AD most severely is the α4β2-subtype providing the high-affinity nicotine binding sites.

Further evidence for the view that high affinity α7 nAChR-Aβ₁₋₄₂ interaction may be a critical step leading to AD pathology arises from the findings of
the nACHR-mediated protection against glutamate- and Aβ-induced neurotoxicity (for review see [24]). Stimulation of nACHRs with nicotine or epibatidine resulted in a prevention of Aβ-induced cytotoxicity [22, 23, 37]. Evidence for a nACHR-mediated protection against the Aβ-enhanced glutamate neurotoxicity was provided by the finding that α7-Bgt suppressed this protection which therefore seems to be mediated via the α7 nACHR subtype. It is supposed that nicotine activates α7 nACHRs to stimulate the Src gene family, which in turn activates phosphatidylinositol 3-kinase (PI-3-K). PI-3-K phosphorylates Akt, which causes upregulation of Bcl-2 and Bcl-x preventing cells from neuronal death induced by Aβ and glutamate [24, 48]. Further insights into the underlying mechanisms of the nACHR-mediated neuroprotection were given recently by the findings of Shaw et al. [49] in PC12 cells. The authors provide evidence that nicotine stimulation of the α7 nACHRs transduce signals to PI-3-K and Akt via Janus kinase 2 (JAK2) in a cascade that results in neuroprotection. Exposure to Aβ leads to an activation of the apoptotic enzyme caspase-3 and cleavage of the DNA-repairing enzyme poly-(ADP-ribose) polymerase. This apoptotic cascade is inhibited by nicotine through JAK2 activation. Pre-treatment of cells with angiotensin II was able to block the nicotine-induced activation of JAK2 via the AT2 receptor and completely prevented α7 nACHR-mediated neuroprotective effects [49]. These findings identify novel mechanisms of receptor interactions relevant to neuronal viability and suggest novel therapeutic strategies to optimise neuroprotection [49].

A possible link between Aβ1-42 binding to α7 nACHRs and cognitive impairment in AD was suggested by a paper showing that Aβ1-42 is able to promote MAP kinase activation by inducing Ca2+ influx through mutant L250T α7 nACHRs, thereby interfering with long-term potentiation processes [50]. Contrary to the wild-type receptor that is blocked by Aβ1-42, the mutant receptor is activated. Therefore, the significance of the interaction between Aβ1-42 and α7 nACHRs for the etiology or the pathogenesis of AD is unclear [41] and needs further elucidation.

Current therapy strategies targeting Aβ in the brain

Besides AChE-inhibitor approaches the most intensive therapeutic efforts thus far have been directed toward a decrease of Aβ in the brain. Procedures inhibiting Aβ formation or modulating Aβ production, assembly and/or removal might be useful as treatments for preventing AD or having a meaningful impact on disease progression. Immunization with Aβ of aged transgenic PDAPP-over-expressing mice led to a reduction in Aβ deposits, whereas immunization of young mice prevented formation of Aβ deposits [51]. In addition, in TgCRND8 mice (K670N/M671L and V717F human βAPP695) a reduction of the cerebral fibrillar Aβ and of cognitive dysfunction was documented, without, however, altering the total level of Aβ in the brain after Aβ immunization [52]. Passive immunization of PDAPP transgenic mice with
m266, an antibody directed to the central part of the Aβ, can rapidly reverse memory deficits but without altering brain Aβ burden [53]. These promising results have led to the development of an Aβ1–42-compound (AN-1792, Elan Pharmaceuticals), as a potential vaccine to treat AD. AN-1792 was undergoing Phase Ila trials in 360 AD patients, as it had to be stopped in January 2002 due to meningio-encephalitic presentation in about 5% of the study group participants [46, 54]. Very recently after the death of the first patient participating in this study an analysis of human neuropathology became available [55]. Some of the findings strongly resemble the changes seen after Aβ-immunotherapy in mouse models of AD. First, there were extensive areas with a low density of Aβ plaques without plaque-associated dystrophic neurites and GFAP-immunoreactive astrocytes. Second, remaining Aβ-immunoreactivity was associated with microglia in areas devoid of plaques. Third, there was persistence of cerebrovascular amyloid. Additional features that were not predicted by the mouse models of Aβ-immunotherapy comprise (1) a CD4+ lymphocytic meningoencephalitis, (2) persistence of neurofibrillary tangles and neuropil threads in areas devoid of plaques, and (3) extensive macrophage infiltration of cerebral white matter [55].

A possible alternative for plaque clearance by immunization with Aβ may be the stimulation of nAChRs. Chronic treatment using high doses of nicotine in the diet of APPsw transgenic mice carrying the Swedish mutation of the human amyloid precursor protein [Tg(Hu.App695.K670N-M671L)2576] led to a selective reduction in Aβ1–42 positive plaques by more than 80%, but there were no changes in soluble Aβ1–40 or Aβ1–42 levels [56]. However, there was also significant weight loss in these animals. Their findings led the authors to conclude that chronic nicotine administration can be added to an increasing number of treatments that are able to reduce Aβ deposition in animal models and that nicotinic drugs directed towards select nAChR subtypes represent a feasible neuroprotective therapeutic approach in AD [56]. Whether the chronic nicotine treatment of APPsw mice has any effect on cognitive improvement, however, needs to be further elucidated.

Designing compounds that distinguish individual receptor subtypes is a highly desirable therapeutic strategy for redressing some of the degenerative effects associated with AD. To the extent that α7 nAChRs represent an early molecular casualty of the disease, they should be considered a high-priority target for drug design [40].

An early attempt to target α7 nAChRs used a compound designed from a naturally-occurring substance: A synthesized analog of the marine natural product anabaseine [57] called GTS-21 (3-(2,4-dimethoxybenzylidene) anabaseine) that preferentially interacts with α7 nAChRs. When GTS-21 was introduced to cultured rat cortical neurons, it protected neurons against Aβ-induced death [22]. These results suggest that α7 nAChR activation can play an important role in neuroprotection against Aβ neurotoxicity. Activation of α7 nAChRs may be able to protect neurons from degeneration induced by Aβ and may have effects that counter the progression of AD. In a subsequent study,
Kihara et al. [23] reported that nicotine neuroprotection could be blocked by an α4β2 nAChR antagonist, suggesting a neuroprotective effect for α4β2 nAChRs as well as α7 nAChRs.

**Current therapy strategies with nAChRs targeting learning and memory**

A role for nAChRs has been demonstrated in a number of forms of cognition including attention [58, 59], sensorimotor gating [60–62], and learning and memory [63–66]. These studies have been carried out in several mammalian species, including humans. Because of the central role that memory loss plays in AD, the focus here is on nAChR involvement in learning and memory. Among the novel approaches to cognition enhancement is the application of agonists to nAChRs, in particular the α7 nAChR, and allosteric modulation of nAChRs.

**Role for nAChRs in working memory**

Repeatedly it has been shown that working memory is facilitated by both acute injections of nicotine [67, 68] and chronic infusion of nicotine [69–72]. Nicotine’s facilitatory effect on working memory is blocked by the nAChR antagonist, mecamylamine [67, 68, 72, 73]. Mecamylamine is not selective to one specific nAChR subtype. It appears to inhibit preferentially first α3, then α4, and finally, α7 subunits and may likewise block β2 and β4 nAChR subunits [74].

Initial behavioral pharmacology research on the nicotinic cholinergic system used this broad-spectrum approach with agonists (nicotine) and antagonists (mecamylamine). More recent research has used a receptor-targeted approach. In the case of working memory as assessed by the 8- and 16-arm radial maze in rats, the approach using focal brain infusions and antagonizing the broad-spectrum agonist, nicotine with receptor-targeted antagonists has demonstrated that both α7 and α4β2 nAChRs in the ventral hippocampus are critical [71]. The work of Levin and associates demonstrates the utility of infusing drugs focally in the brain to identify sites of drug action. Experiments infusing antagonists specific to α7 nAChRs (methyllycaconitine) or to α4β2 nAChRs (dihydro-beta-erythroidine) into the ventral hippocampus demonstrated that both types of nAChRs are critical for working memory function measured by the radial arm maze in rats [69–71].

**Role of nAChRs in associative learning and memory**

Brain structures and systems of demonstrated involvement in eyeblink classical conditioning in rabbits and humans are compromised during the progres-
sion of AD. Patients diagnosed with probable AD are severely impaired in eyeblink conditioning beyond the impairment observed in normal aging [75–77]. For a decade, it has been our working hypothesis that disruption of the septohippocampal cholinergic system and selective loss of hippocampal pyramidal cells impair acquisition of eyeblink classical conditioning in AD beyond the impairment observed in normal aging. Additional evidence that septohippocampal lesions disrupt conditioning in humans comes from patients with a common cerebral aneurysm: aneurysm of the anterior communicating artery. The anterior communicating artery vascularizes the basal forebrain, and survivors of an aneurysm of this artery often display some degree of anterograde amnesia. Anterior communicating artery aneurysm survivors often sustain basal forebrain lesions that include lesions of the medial septum. Six patients with such lesions were tested in the delay eyeblink conditioning procedure and showed significant impairment [78].

In addition to septohippocampal disruption, AD-related injury to the cerebellum may play a role in impairing delay eyeblink classical conditioning in AD. Purkinje cell loss in the AD cerebellum is significantly greater than in age-matched control brains [79, 80], and there is exceptional vulnerability of the ACh system and ACh-receptive cells in AD that may be associated with nAChRs. With the mounting evidence for a role for ACh in the cerebellum as well as the hippocampus, the possibility exists that impaired eyeblink conditioning in AD occurs at least in part because of impairment in ACh modulation and nAChR function in the essential cerebellar circuitry.

Experiments with muscarinic ACh receptor agonists [81] and antagonists [82] and nicotinic ACh receptor agonists [83] and antagonists [84] have demonstrated a role for ACh in the model system of eyeblink classical conditioning in the rabbit. Eyeblink conditioning reveals natural age-related deficits in several non-human mammals, and the similarities between age differences in eyeblink conditioning in these animal species and humans are striking. Moreover, delay eyeblink conditioning is impaired profoundly in patients with AD, making the procedure relevant for preclinical studies of cognition-enhancing drugs. In addition to parallels with human behavior and neurobiology, the model system of eyeblink classical conditioning possesses a considerable advantage over the behavioral models commonly used preclinically: The essential neural circuitry in the cerebellum has been identified along with modulatory circuits in hippocampus and cortex.

The standard format for the presentation of stimuli in eyeblink classical conditioning is called the delay procedure. The subject is presented with a neutral stimulus such as a tone or light, called the conditioned stimulus (CS), for a short duration usually less than one second. Before the CS expires, the unconditioned stimulus (US) is presented concurrently, and the briefly coinciding CS and US co-terminate 50 to 100 ms later. The US, either a shock to the infra-orbital region of the eye or a corneal airpuff, always elicits from the organism an eyeblink or nictitating membrane (NM) unconditioned response (UR). With the repeated pairing of the CS and the US, the subject learns to
blink to the tone before the onset of the US. This learned response is called a conditioned response (CR).

The site essential for acquisition and retention of the classically conditioned eyeblink response in rabbits is the cerebellar interpositus nucleus ipsilateral to the eye receiving the US. In humans, this nucleus becomes two deep cerebellar nuclei (emboliform and globose). Cerebellar cortex ipsilateral to the US also contributes to the process of acquisition, such that an intact cerebellar cortex enables acquisition to occur at a faster rate. The hippocampus itself is normally involved during acquisition in the delay procedure, however, in a complex modulatory role. The role of the hippocampus during acquisition in delay eyeblink conditioning seems paradoxical in that conditioning proceeds normally in animals with bilateral removal of the hippocampus, but manipulation of hippocampal function (in an intact hippocampus) with drugs can facilitate or impair acquisition considerably. For example, the muscarinic cholinergic antagonist scopolamine impairs acquisition in the delay procedure only when the hippocampus is intact [85]. Likewise, the cognition-enhancing drug, nefiracetam ameliorates learning impairment in older rabbits in the delay procedure only when the hippocampus is intact [86]. This modulatory role for the hippocampus may be particularly significant in AD, since, in humans, AD appears to alter hippocampal neuronal function and cause a major disruption of the brain cholinergic system. Eyeblink conditioning impairment in AD may reflect medial-temporal lobe atrophy and central nervous system cholinergic dysfunction that occurs early in disease progression.

Identification of nAChRs as the cholinergic receptors impaired in AD led to a test of an antagonist to nAChRs in the animal model of eyeblink classical conditioning. Mecamylamine is a central nervous system nicotinic antagonist that binds to a site on the receptor other than the ACh recognition site. Papke et al. [74] demonstrated with electrophysiological recordings of nAChRs expressed in Xenopus oocytes that the residual inhibition produced by 10 µM mecamylamine was greatest for human β2-containing receptors and least for α7 nAChRs.

Using a 0.5 mg/kg dose of mecamylamine, a role for nAChRs in eyeblink conditioning in young rabbits was demonstrated [84]. The acquisition of conditioned eyeblink responses was severely disrupted so that young rabbits learned at a rate comparable to older rabbits. The deleterious effect of mecamylamine on eyeblink conditioning was not accompanied by a measurable change in brain nAChR concentration. These results in combination with studies using the muscarinic acetylcholine receptor (mAChR) antagonist, scopolamine, suggest that nAChRs as well as mAChRs are involved in the modulation of eyeblink classical conditioning.

*Partial agonism of α7 nAChRs*

Results with the nAChR antagonist, mecamylamine led to the prediction that nAChR agonists would facilitate eyeblink conditioning in older rabbits. The
effect of GTS-21, a selective nicotinic agonist acting primarily at the α7 nAChR subtype [87, 88], on learning was tested using the 750-ms delay eyeblink conditioning procedure [89]. There were 15 daily subcutaneous injections administered 15 minutes before behavioral training. At dosage levels of 0.5 and 1.0 mg/kg, GTS-21 acted as a cognition-enhancing agent in older rabbits, resulting in eyeblink conditioning performance comparable to young rabbits. The cognition-enhancing effect of GTS-21 upon eyeblink conditioning was not accompanied by a measurable change in brain nAChRs.

The effect of GTS-21 on acquisition, retention, and relearning was also examined with a focus on the duration of the effect of GTS-21 as assessed by retention and relearning [90]. First there were 15 sessions of acquisition training with injections of 0.5 mg/kg GTS-21 or vehicle. Then drug administration ended and older rabbits were tested for retention and relearning six weeks and 13 weeks after the beginning of the experiment. Acquisition of CRs was significantly better in GTS-21-treated rabbits. During the first tone-alone retention session in week six of the experiment, rabbits initially treated with GTS-21 produced significantly more CRs than vehicle-treated rabbits. There were no group differences in retention at the 13-week retest. Differences in relearning were numerically greater for GTS-21 treated older rabbits, but these effects did not attain statistical significance. Results indicated that treatment with GTS-21 ameliorated learning and memory beyond the period when the drug was actually administered.

Given the results outlined above confirming that nAChRs are involved in the modulation of acquisition and retention in eyeblink classical conditioning, reversal of the nAChR antagonist mecamylamine with nicotine or GTS-21 was explored [91]. Young rabbits were injected with 0.5 mg/kg mecamylamine in combination with nicotine or GTS-21 and compared to vehicle-treated rabbits. Control groups were tested in the explicitly unpaired condition. Both GTS-21 and nicotine reversed the deleterious effect of mecamylamine on acquisition of CRs enabling the rabbits treated with mecamylamine and the agonists to perform at the level of vehicle-treated control rabbits (Fig. 1). Combinations of GTS-21 or nicotine and mecamylamine did not cause sensitization or habituation in the explicitly unpaired condition. That fact that mecamylamine inhibits preferentially first α3, then α4, and finally α7 subunits and may likewise block β2 and β4 nAChR subunits [74] suggests that the reversal by GTS-21 and nicotine of mecamylamine’s antagonistic effect was via α7 nAChRs. There is some evidence that GTS-21 actually antagonizes α4β2 nAChRs in addition to serving as an agonist for α7 nAChRs [87]. If α7 nAChRs are of primary importance in eyeblink conditioning, then selective α7 antagonists such as methyllycaconitine should impair acquisition to a greater extent than selective α4β2 antagonists such as dihydro-beta-erythroidine. Demonstration that eyeblink conditioning relies more on α7 nAChRs than on α4β2 nAChRs would make this behavior a sensitive index of the drugs that are α7 agonists under development to treat AD.
effect of GTS-21, a selective nicotinic agonist acting primarily at the α7 nAChR subtype [87, 88], on learning was tested using the 750-ms delay eyeblink conditioning procedure [89]. There were 15 daily subcutaneous injections administered 15 minutes before behavioral training. At dosage levels of 0.5 and 1.0 mg/kg, GTS-21 acted as a cognition-enhancing agent in older rabbits, resulting in eyeblink conditioning performance comparable to young rabbits. The cognition-enhancing effect of GTS-21 upon eyeblink conditioning was not accompanied by a measurable change in brain nAChRs.

The effect of GTS-21 on acquisition, retention, and relearning was also examined with a focus on the duration of the effect of GTS-21 as assessed by retention and relearning [90]. First there were 15 sessions of acquisition training with injections of 0.5 mg/kg GTS-21 or vehicle. Then drug administration ended and older rabbits were tested for retention and relearning six weeks and 13 weeks after the beginning of the experiment. Acquisition of CRs was significantly better in GTS-21-treated rabbits. During the first tone-alone retention session in week six of the experiment, rabbits initially treated with GTS-21 produced significantly more CRs than vehicle-treated rabbits. There were no group differences in retention at the 13-week retest. Differences in relearning were numerically greater for GTS-21 treated older rabbits, but these effects did not attain statistical significance. Results indicated that treatment with GTS-21 ameliorated learning and memory beyond the period when the drug was actually administered.

Given the results outlined above confirming that nAChRs are involved in the modulation of acquisition and retention in eyeblink classical conditioning, reversal of the nAChR antagonist mecamylamine with nicotine or GTS-21 was explored [91]. Young rabbits were injected with 0.5 mg/kg mecamylamine in combination with nicotine or GTS-21 and compared to vehicle-treated rabbits. Control groups were tested in the explicitly unpaired condition. Both GTS-21 and nicotine reversed the deleterious effect of mecamylamine on acquisition of CRs enabling the rabbits treated with mecamylamine and the agonists to perform at the level of vehicle-treated control rabbits (Fig. 1). Combinations of GTS-21 or nicotine and mecamylamine did not cause sensitization or habituation in the explicitly unpaired condition. That fact that mecamylamine inhibits preferentially first α3, then α4, and finally α7 subunits and may likewise block β2 and β4 nAChR subunits [74] suggests that the reversal by GTS-21 and nicotine of mecamylamine’s antagonistic effect was via α7 nAChRs. There is some evidence that GTS-21 actually antagonizes α4β2 nAChRs in addition to serving as an agonist for α7 nAChRs [87]. If α7 nAChRs are of primary importance in eyeblink conditioning, then selective α7 antagonists such as methyllycaconitine should impair acquisition to a greater extent than selective α4β2 antagonists such as dihydro-beta-erythroidine. Demonstration that eyeblink conditioning relies more on α7 nAChRs than on α4β2 nAChRs would make this behavior a sensitive index of the drugs that are α7 agonists under development to treat AD.
Figure 1. Mean amplitude of conditioned responses (CRs) in 58 three-month-old rabbits for the various drug treatment groups for the first 10 daily training sessions of 90 trials/session. Each group consisted of a minimum of eight rabbits. Rabbits received daily subcutaneous injections of sterile saline vehicle, 0.5 mg/kg mecamylamine (Mec) alone, or 0.5 mg/kg mecamylamine in combination with 0.5 or 1.0 mg/kg GTS-21 or 0.25 or 0.5 mg/kg nicotine (Nic) 15 min before training. The group treated with 0.5 mg/kg Mec had significantly fewer CRs than the vehicle-treated group ($p = 0.005$). Early in training (Sessions 2–3) rabbits treated with mecamylamine and GTS-21 actually had numerically higher CR amplitudes than all other groups, but by Session 4 vehicle-treated animals began to show superior performance to the mecamylamine-alone group. Rabbits treated with agonist (GTS-21 or nicotine) antagonist (mecamylamine) combinations performed similarly to vehicle-treated rabbits and better than rabbits treated with the antagonist alone [91].

**Allosteric modulation of nAChRs**

Allosteric modulators, such as galantamine (Reminyl®; Inteligen, Janssen) and physostigmine employ a dual action at the cholinergic synapse. First, they act as acetylcholinesterase (AChE) inhibitors. Second, they also act as agonists at presynaptic nAChRs, enhancing the release of ACh. Functionally unique features of allosterically potentiating ligands include the ability as assessed with patch-clamp recordings to induce single-channel activity indistinguishable from the single-channel activity induced by ACh. With allosteric potentiation, galantamine and physostigmine induced single-channel activity in excised patches from various cells [92–94] that could not be blocked by established nAChR antagonists like mecamylamine.

Given that an α7 nAChR partial agonist, GTS-21 ameliorated learning deficits in older rabbits, the aim was to determine if nicotinic agonism using a different mechanism of action would be effective in the eyeblink classical con-
conditioning paradigm. With physostigmine, a wide range of doses (0.0005 to 2.0 mg/kg) were effective in ameliorating eyeblink conditioning deficits in older rabbits [95]. Control tests of rabbits in explicitly unpaired conditions demonstrated that non-associative factors could not account for the results. This allosteric modulator had dramatic effects on associative learning in older rabbits (see Fig. 2).

Figure 2. (A) Percentage of conditioned responses (CRs) of a total of 59 older rabbits (mean age = 28 mo., s.d. = 5.0 mo) treated with five doses of physostigmine (Phy) or vehicle over 15 daily training sessions in the 750 ms delay eyeblink classical conditioning procedure. There were a minimum of seven older rabbits in each of the Phy-treated groups and 12 rabbits in the vehicle-treated group. (B) The number of training trials to a learning criterion of eight CRs in nine consecutive trials in the 59 rabbits shown in the left panel. Physostigmine at a range of doses significantly improved learning in older rabbits [95].
Galantamine doses of 0.0, 1.0, 2.0, 3.0, and 4.0 mg/kg were tested in 10 daily sessions in old rabbits in the 750 ms delay eyeblink classical conditioning procedure [96]. A dose of 3.0 mg/kg galantamine was effective in improving conditioning in older rabbits, enabling them to achieve learning criterion rapidly and to produce a very high level of learning performance. Like phystostigmine, galantamine had no effects on sensitization, habituation, or motor aspects of the task.

Additional experiments with galantamine were carried out to compare the efficacy of the drug in young and older rabbits and to evaluate retention and relearning [83]. In one experiment, young and older rabbits were administered 3.0 mg/kg galantamine for 15 days during conditioning in the 750 ms delay procedure. Galantamine significantly improved acquisition in both young and older rabbits. AChE levels in the brain were reduced, and nAChR binding was increased. There was a statistically significant correlation between brain AChE levels and trials to learning criterion, \( r = 0.621, p = 0.007 \). Neither the correlation between trials to learning criterion and plasma AChE, nor the correlations between trials to learning criterion and \( B_{\text{max}} \) or \( K_p \) attained statistical significance.

In another experiment, older rabbits were tested over a 15-week period in four conditions. Groups of rabbits received 0.0 (vehicle), 1.0, or 3.0 mg/kg galantamine for the entire 15-week period or 3.0 mg/kg galantamine for 15 days and vehicle for the remainder of the experiment. There were 15 daily conditioning sessions and subsequent retention and relearning assessments spaced at one-month intervals. The dose of 3.0 mg/kg galantamine ameliorated learning deficits significantly during acquisition and retention in the group receiving 3.0 mg/kg galantamine continuously. Nicotinic receptor binding was significantly increased in rabbits treated for 15 days with 3.0 mg/kg galantamine. All galantamine-treated rabbits had lower levels of brain AChE. The efficacy of galantamine in a learning paradigm severely impaired in AD was consistent with outcomes evaluating galantamine in clinical studies.

The single-channel activity of galantamine could not be blocked by antagonists to nAChRs suggesting by inference that the activity was induced through a separate allosteric site from the site for ACh and competitive ligands. Mecamylamine blocks many well-established AChE inhibitors tested with these electrophysiological techniques [92, 94]. Patch-clamp recordings of single ion channel activity demonstrated that donepezil, but not galantamine, could be blocked by mecamylamine. At the whole organism, behavioral level an experiment was carried out to determine whether galantamine, but not donepezil, could reverse mecamylamine-induced learning impairment. Young rabbits received delay eyeblink conditioning after one of six drug treatments: 0.5 mg/kg mecamylamine, 3.0 mg/kg galantamine, 3.0 mg/kg donepezil, 0.5 mg/kg mecamylamine plus 3.0 mg/kg galantamine, 0.5 mg/kg mecamylamine plus 3.0 mg/kg donepezil, or sterile saline vehicle [97]. Galantamine, but not donepezil, facilitated learning in young rabbits. However, both galantamine and donepezil reversed the deleterious effects of mecamylamine on
learning. Significant differences in plasma (but not brain) AChE levels were detected among the drug treatment groups. Fifteen daily injections of mecamylamine, galantamine, or donepezil, alone or in combination did not produce statistically significant changes in nAChR binding. One possible interpretation of these results is that donepezil affected nAChRs by raising the synaptic level of ACh and hence, the probability of receptor activation, whereas galantamine bound to distinct allosteric sites not blocked by mecamylamine. It may be possible to facilitate learning in young rabbits with allosteric modulation (galantamine), but not with AChE inhibition alone (donepezil).

Summary and conclusions

The widespread involvement of nAChRs in disorders of the human nervous system makes these receptors likely targets for drug therapy. In this chapter we focused on the involvement of nAChRs in AD and the relationships between Aβ and nAChRs. Our demonstration in brain autopsy tissue from humans with AD of the presence of nAChR-immunoreactive neurons in close proximity to and sometimes even within plaques makes it appear rather unlikely that plaques interfere with nAChR expression. This perspective receives confirmation from a number of additional studies investigating relationships between plaque load and nicotine binding in humans and transgenic mice.

Whereas Aβ plaques may not impair nAChRs, there is increasing evidence that soluble Aβ interacts with nAChRs to cause morphological alterations and impairment of function. Consensus exists on the ability of Aβ1-42 to block α-Bgt sensitive nAChR subtypes like α7 nAChR. The formation of an α7 nAChR-Aβ1-42 complex can be efficiently suppressed by Aβ12-28, implying that the binding epitope for α7 nAChR resides in the amino acid 12-28 sequence region of Aβ1-42. The interaction of soluble Aβ with non-α-Bgt sensitive nAChR subtypes, however, is not yet clear.

It is likely that the observed Aβ1-42-induced functional changes in nAChRs affect synaptic transmission. For example, the high affinity binding of Aβ1-42 to α7 nAChRs may result in the inhibition of ACh release and may alter Ca^{2+}-homeostasis for the affected cholinergic neuron. Chronic physiological perturbations such as inhibition of ACh release and altered Ca^{2+}-homeostatic stress neuronal function and even lead to neurodegeneration. High affinity α7 nAChR-Aβ1-42 interaction may be a critical step leading to AD pathology. In this regard, using a broad-spectrum agonist (nicotine) and an α7 receptor-targeted agonist (GTS-21) is neuroprotective against Aβ-enhanced glutamate neurotoxicity.

A possible alternative for plaque clearance by immunization with Aβ may be the stimulation of nAChRs. In particular, chronic stimulation of α7 nAChRs has significant therapeutic potential. In addition to its potential to affect AD neuropathology, chronic stimulation of α7 nAChRs has potential to reduce cogni-
tive impairment in AD. Learning and memory are among the cognitive processes affected early in AD. Animal studies demonstrated that both α7 and α4β2 nAChRs in the ventral hippocampus are critical for normal working memory. Functional blockade of α7 nAChRs and loss of α4β2 nAChRs likely contribute significantly to the documented impairment of attention and working memory in AD patients. Associative learning as assessed by eyeblink classical conditioning is severely impaired in AD, and preclinical experiments with a partial α7 agonist and with allosteric modulators of nAChRs demonstrate dramatic therapeutic effects. Targeting α7 nAChRs to treat AD has a range of potential benefits, as does the targeting of selected individual receptor subtypes affected in diseases such as addiction to nicotine, anxiety, autism, depression, epilepsy, Parkinson’s disease, schizophrenia, and Tourette’s syndrome.

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