Preclinical Experiments on Cognition Enhancement in Alzheimer’s Disease: Drugs Affecting Nicotinic Acetylcholine Receptors

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Abstract Nicotinic acetylcholine receptors (nAChRs) play a role in a variety of diseases of the central nervous system including Alzheimer’s disease (AD). There is currently great interest in evaluating AD-related nAChR changes, and pharmacological treatment of nAChR deficits is a promising therapy. In AD, α7 nAChRs remain relatively stable in contrast to α4β2 nAChRs that are lost in substantial numbers. However, α7 nAChRs may be functionally impaired in AD because β-amyloid, a major neuropathology in AD, blocks α4β2 and α7 nAChRs. Agonists selective to α7 or α4β2 nAChRs are neuroprotective against β-amyloid. A preclinical test of cognition-enhancing drugs affecting nAChRs is eyelink classical conditioning. This task is severely impaired in human probable AD patients and is impaired by antagonists to nAChRs. Three drugs with different mechanisms of action on nAChRs (partial α7 agonist [GTS-21], acetylcholinesterase inhibition and allosteric modulation [galantamine], nootropic activation [nefaranetam]) were tested in young and older rabbits using eyelink classical conditioning. All three drugs ameliorated learning and memory impairments in older rabbits and reversed an antagonist to nAChRs in young rabbits. Galantamine, with its allosteric modulatory action, was the only drug that facilitated learning in young rabbits. On the basis of efficacy of these drugs that affect nAChRs in preclinical studies and in Phase I (GTS-21), Phase II (nefaranetam), or Phase III (galantamine) clinical trials, exploration of nAChRs as targets for therapeutic intervention via a number of different pathways seems warranted. Drug Dev. Res. 56:335–346, 2002. © 2002 Wiley-Liss, Inc.

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INTRODUCTION

There is a long-standing association between memory impairment and disruption of brain acetylcholine (ACh) neurotransmission [e.g., Deutsch et al., 1966]. Severe memory impairment is the most prominent clinical symptom of Alzheimer’s disease (AD). With the demonstration that ACh levels were lower in the brains of AD patients [Davies and Maloney, 1976; Perry et al., 1977] and that lower ACh levels were associated with cognitive impairment in AD [Bowen et al., 1976], the cholinergic neurotransmitter system became a central focus for research.
on AD [Bartus et al., 1982; Coyle et al., 1983]. A research focus on brain cholinergic neurotransmission in AD continues to the present time [Bartus, 2000].

There are two broad classes of ACh receptors in the nervous system that differ in their primary agonists. Nicotinic acetylcholine receptors (nAChRs) are activated by nicotine that acts like ACh on the nAChR. MUScarinic acetylcholine receptors (mAChRs) respond to the agonist, muscarine. Whereas nAChRs are classical neurotransmitter-gated ion channels, muscarinic cholinergic receptors have G-protein-mediated second-messenger driven responses. Subgroups of receptor types are included within both nicotinic and muscarinic categories of receptors. The focus here is on nAChRs because some subgroups of this receptor subtype are lost or blocked by Aβ in AD, whereas nAChRs are preserved in AD.

nAChRs in the central nervous system are composed of five subunits arranged around a ligand-gated excitatory ion channel [Cooper et al., 1991] permeable to Na⁺, K⁺, and Ca²⁺ [Holliday et al., 1997; Lindstrom et al., 1995]. Many subtypes of nAChRs can be constructed from various combinations of the nine α subunits (α2 to α110) and three β subunits (β2 to β4). Two main neuronal categories have been identified on the basis of function and pharmacology: (1) the heterologous pentamers, constructed from combinations of α- and β-subunits [Conroy et al., 1992]; and (2) the homologous pentamers, constructed from one subunit type, α7, α8, and α9 [Lindstrom et al., 1991]. Of the homologous pentamers, only the α7 nAChR is expressed widely and abundantly in the mammalian brain [Couturier et al., 1990; Schoepfer et al., 1990]. The various types of nAChRs have characteristic patterns of distribution, and they have several loci on neurons, including on terminals, soma, and dendrites [Court and Perry, 1995; Lindstrom et al., 1995; McGhee and Role, 1995]. Considerable evidence indicates that nAChRs act as neuromodulators in communicative processes in the brain [Lindstrom, 1996] and that nAChRs are involved in cognitive and memory functions [Gould et al., 2001; Gould and Wehner, 1999; Levin, 1992; Newhouse and Kelton, 2000; Sahakian and Coull, 1994].

NICOTINIC ACETYLCHOLINE RECEPTORS IN ALZHEIMER'S DISEASE

In patients with AD, a substantial reduction was observed in nAChR subtypes that participate in high-affinity agonist binding (e.g., α4β2). In an early study, frontal lobe autopsy tissue from AD patients and healthy older adults was compared, and a drastic reduction in the number of high-affinity binding sites was reported for AD brains [Nordberg and Winblad, 1996]. Subsequent studies replicated this result, demonstrating that neocortical and medial-temporal lobe structures such as the hippocampus have a significant reduction of high-affinity agonist binding nAChRs in AD as contrasted to normal aging [e.g., London et al., 1989; Schroder et al., 1991]. More recent studies have reported a consistent finding that there is extensive loss of α4 nAChR subunits in AD [Martin-Ruiz et al., 1999; Wevers et al., 2000].

The PET technique is useful in the visualization of nAChRs in AD [Nordberg, 2001]. Whereas examination of nAChRs in autopsy tissue can only occur at the end stage of the disease, PET examination of nAChRs can occur in living patients. It is extremely important to examine nAChRs in living patients because the nAChR deficits in AD brains probably represent an early phenomenon in the course of the disease [Nordberg et al., 1990]. nAChRs are impaired even in the early, pre-diagnostic phase of AD called Mild Cognitive Impairment (MCI). In many cases, MCI represents a transitional state between normal aging and very mild AD [Petersen et al., 2001]. Older adults with MCI have a diminished capacity to acquire new memories. These individuals are not demented, but they convert to dementia and AD at a substantially greater rate than normal age-matched individuals [e.g., Petersen et al., 1999]. Prediction of conversion to MCI may be improved with assessment of nAChRs.

RELATIONS BETWEEN NACHRS AND β-AMYLOID

Extracellular amyloid plaques comprised of β-amyloid peptides (Aβ), particularly the 42 amino acid form (Aβ42) in neuritic plaques [Selkoe, 1999] and intracellular neurofibrillary tangles comprised of hyperphosphorylated tau [Lee et al., 1991] are major forms of neuropathology found in the brains of AD patients. The potential role of Aβ as a neuromodulator has drawn attention to the possibility that Aβ may affect ACh neurotransmission via nAChRs [Auld et al., 1998]. Marutle and associates [1999] investigated the influence of Aβ on nAChRs in autopsy brain tissue from AD patients carrying the Swedish APP 670/671 mutation. The Swedish mutation is unique in that it is the only AD mutation that has been shown to alter amyloid precursor protein (APP) metabolism, resulting in an overexpression of the amyloid leading to plaque formation [Mullan et al., 1992]. Reductions in the number of nAChRs in the brain autopsy tissue from patients with the Swedish APP 670/671 mutation were dramatic. Reduction of nAChRs ranged between −73 and −87%, whereas in the brains of sporadic AD cases the nAChR reduction ranged between −37 and −57% [Marutle et al., 1999]. The two distributions of percentage loss of nAChRs were non-overlapping.
even though the Swedish mutation group died on average 15 years younger than the sporadic AD patients. The association between overexpression of amyloid and extensive loss of nAChRs points to a possible interaction between Aβ and nAChRs.

Evidence for a physiological role of Aβ1-42 in the inhibition of postsynaptic nAChRs was provided when Aβ1-42 blocked nAChR-mediated current and reduced the probability of open channels in rat hippocampal interneurons [Pettit et al., 2001]. Modulation by Aβ1-42 occurred rapidly, within milliseconds at single channels. Inhibition of nicotinic currents occurred at concentrations of Aβ1-42 as low as 100 nM. Experiments demonstrated that Aβ1-42 bound and inhibited multiple subtypes of nAChRs [Pettit et al., 2001; Wang et al., 2000]. Liu and associates [2001] demonstrated that β-amyloid peptides could block the function of α7 nAChRs both pre- and postsynaptically. The blockade of α7 nAChRs by Aβ1-42 is specific, noncompetitive, reversible, and has high affinity, exerted through the N-terminal extracellular portion of the receptor.

The pre- and postsynaptic blockade of α7 nAChRs by Aβ1-42 has major implications for cognitive impairment in AD. Somato-dendritic α7 nAChRs mediate synaptic currents [e.g., Frazier et al., 1998], and presynaptic α7 nAChRs modulate neurotransmitter release [McGehee et al., 1995]. β-Amyloid peptides are distributed widely in AD, and α7 nAChRs clearly play a role in cognition. The α7 nAChR, expressed widely and abundantly in the human brain, may be a significant molecular target of a major neuropathological feature of AD, β-amyloid peptides. Results demonstrating the inhibition of pre- and postsynaptic nAChRs by Aβ1-42 provide a possible mechanism to explain the early cognitive deficits seen in MCI and AD before extensive formation of β-amyloid plaques. Functionally, blockade of nAChR channels by Aβ1-42 may impair cognition even before the actual neurodegeneration characteristic of AD appears.

The possibility that Aβ1-42 might exert deleterious effects on cognition independently of plaque formation may explain a puzzling outcome of studies testing learning and memory in transgenic mice that overproduce APP. Results indicate that behavioral impairment occurs relatively early in adulthood in these mice before there is evidence of amyloid deposition in the brain [e.g., Holcomb et al., 1999; Moechars et al., 1999]. Soluble Aβ1-42 may exert an influence on behavior by blocking nAChRs before the fibrillar form of the Aβ1-42 develops. On the basis of their hippocampal slice experiments, Pettit and associates [2001] argued that the facts that the fibrillar form of Aβ1-42 would have very poor access to the extracellular space in brain slice tissue and that inhibition at single channels is extremely rapid (20 ms) are consistent with toxicity of the soluble form of Aβ1-42.

PROTECTION BY nAChRs AGAINST Aβ CYTOTOXICITY

Kihara and associates [1997] examined the protective effect against Aβ cytotoxicity by using agonists to nAChRs. When cultured rat cortical neurons were exposed to synthetic Aβ peptides, the number of viable neurons decreased dramatically. Administration of nicotine along with Aβ exposure markedly reduced the number of dead cells. When nicotinic antagonists were added, the neuroprotective effect of nicotine was blocked. This result suggested that the neuroprotective effect of nicotine was mediated by nAChRs. Introduction of α2-bungarotoxin (that selectively blocks α7 nAChRs) in the rat cortical cell culture also blocked the neuroprotective effect. This result suggested that the effect of nicotine was mediated by α7 nAChRs.

A synthesized analog of the marine natural product anabaseine [Kem et al., 1971] called GTS-21 (3-(2,4-dimethoxybenzylidene) anabaseine) has been found to preferentially interact with α7 nAChRs. When GTS-21 was introduced to cultured rat cortical neurons, it protected neurons against Aβ-induced death [Kihara et al., 1997]. 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. [1998] reported that nicotine neuroprotection could be blocked by an α4β2 nAChR antagonist, suggesting a neuroprotective effect for α4β2 nAChRs as well as α7 nAChRs.

Whereas Shimohama and Kihara [2001] viewed stimulation of α7 nAChRs as protective against Aβ, Dineley and associates [2001] provided indirect evidence that α7 nAChRs serve as receptors for Aβ1-42. These investigators used hippocampal slice preparations and demonstrated that Aβ1-42 coupled to the nitogen-activated protein kinase (MAPK) cascade via α7 nAChRs. On the basis of their results showing neuroprotection of nicotine and GTS-21, Shimohama and Kihara [2001] advocated the use of agonists to α7 nAChRs in AD, but Dineley et al. [2001] proposed that antagonists selective to α7 nAChRs would assuage the MAPK signaling derangement.

A ROLE FOR NICOTINIC ACETYLCHOLINE RECEPTORS IN EYEBLINK CONDITIONING

Identification of nAChRs as the receptors impaired in AD led us to test 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. It noncompetitively inhibits peripheral as well as central nAChRs [Banerjee et al., 1990]. Papke et al. [2001] 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.

At relatively low doses (such as 0.5 mg/kg): mecamylamine selectively impairs nAChR ion channel function [Levin, 1992]. Using a 0.5 mg/kg dose of mecamylamine, we demonstrated a role for nAChRs in eyeblink conditioning in young rabbits [Woodruff-Pak et al., 1994a]. 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 nAChR antagonist, scopolamine, suggest that nAChRs as well as nAChRs are involved in the modulation of eyeblink classical conditioning.

**Acetylcholine, Associative Learning, and Eyeblink Classical Conditioning**

Eyeblink classical conditioning reveals natural age-related deficits in several non-human mammals, and the similarities between age differences in eyeblink conditioning displayed in normal aging by these animal species and humans are striking [Green and Woodruff-Pak, 2000]. Brain imaging studies of eyeblink classical conditioning in young adults assessed with positron emission topography [Blaxton et al., 1996; Logan and Grafton, 1995; Molchan et al., 1994] or functional magnetic resonance imaging [Lemieux and Woodruff-Pak, 2000; Rammani et al., 2000] as well as studies with human patient populations with lesions in various brain structures, suggest that the neural circuitry for eyeblink conditioning in humans is similar to the circuitry demonstrated in mice, rats, cats, and rabbits. Moreover, delay eyeblink classical conditioning is a task in which patients diagnosed with probable AD are profoundly impaired, making the procedure relevant for preclinical trials of cognition-enhancing drugs [Woodruff-Pak, 1995]. In addition to its parallels with human behavior and neurobiology, the model system of eyeblink classical conditioning in non-human mammals possesses a considerable advantage over the behavioral models commonly used in preclinical trials: the neural circuitry is almost completely understood [Steinmetz, 1996; Thompson, 2000; Thompson and Krupa, 1994].

**Eyeblink Classical Conditioning Paradigm**

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 coterminate 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).

**Eyeblink Classical Conditioning in Alzheimer’s Disease**

Disruption of the brain cholinergic system in AD links this dementing disease to the model system of eyeblink conditioning in mammals, including humans [Woodruff-Pak et al., 1989]. Eyeblink conditioning impairment in AD may reflect medial-temporal lobe atrophy and central nervous system cholinergic dysfunction that occurs early in disease progression. The site essential for acquisition and retention of the classically conditioned NM/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. 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 [Solomon et al., 1985]. Likewise, the cognition-enhancing drug, nefiracetam, ameliorates learning impairment in older rabbits in the delay procedure only when the hippocampus is intact [Woodruff-Pak et al., 1997]. 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.

In a sample of 40 elderly adults, half of whom were diagnosed with probable AD, there were very significant differences in eyeblink conditioning between the patients (10.8% CRs in a 90-trial session) and age-matched, non-demented control subjects (31.5% CRs/session) [Woodruff-Pak et al., 1990]. In probable AD, there is very limited eyeblink conditioning in the first session of testing [Solomon et al., 1991; Woodruff-Pak et al., 1990]. However, when given a sufficient number of training trials (e.g., four or five days of 90-trial presentations), patients diagnosed with probable AD finally were able to produce about 30% CRs in a session [Solomon et al., 1995; Woodruff-Pak et al., 1996b]. This slowing of the rate of acquisition occurs in the animal model when antagonists to cholinergic neurotransmission are introduced [Moore et al., 1976; Woodruff-Pak and Hinchcliffe, 1997]. The site of interference of cholinergic antagonists in rabbits is in the septohippocampal system [Solomon and Gottfried, 1981; Solomon et al., 1983]. There is a clear parallel between the results that patients with AD eventually acquire CRs, albeit slowly, and the results in rabbits injected with ACh antagonists that they eventually acquire CRs, but at a slow rate. The results that probable AD patients eventually acquire CRs suggest that the cerebellar circuitry for CR acquisition is intact, but acquisition is disrupted in medial-temporal lobe structures.

Delay eyeblink classical conditioning may have utility in the differential diagnosis of neurodegenerative diseases. This procedure was shown to differentiate cerebrovascular dementia patients from patients with probable AD [Woodruff-Pak et al., 1996a], and eyeblink conditioning in adults over the age of 35 with Down's syndrome and AD neuropathology was similar to conditioning in probable AD patients [Woodruff-Pak et al., 1994c; Papka et al., 1994]. Conditioning in patients with Huntington's disease [Woodruff-Pak and Papka, 1996] and Parkinson's disease [Daum et al., 1996] is clearly differentiated from eyeblink conditioning in AD.

THERAPEUTIC FOCUS ON NACHRS FOR COGNITION ENHANCEMENT IN PRECLINICAL RESEARCH USING THE MODEL SYSTEM OF EYEBLINK CLASSICAL CONDITIONING

There are a number of advantages to using the eyeblink classical conditioning paradigm preclinically in the evaluation of cognition-enhancing drugs for AD. A major advantage is the fact that eyeblink conditioning is a task on which probable AD patients are significantly impaired, and this impairment occurs early in the disease process [Woodruff-Pak, 2001]. Blockade of nAChRs pre- and postsynaptically by Aβ1-42 may be a mechanism by which AD neuropathology disrupts the septo-hippocampal cholinergic system early in the course of the disease. Other advantages include the extremely well-characterized behavioral and neurobiological parameters of this paradigm in several mammalian species.

We have collected much of our data on aging rabbits using the 750-ms delay procedure, a CS-US interval in which extensive age-related deficits are apparent [Woodruff-Pak, 1995]. This procedure provides us the opportunity to demonstrate amelioration of the learning deficit in older rabbits and facilitation of learning in young rabbits. On average, with 90-trial daily sessions, younger rabbits (3–6 months old) attain a learning criterion of a minimum of 40% CRs for the session and 8 CRs in 9 consecutive trials on the 4th training day, in around 400 trials, whereas older rabbits (24 months and older) normally attain this learning criterion on the 9th or 10th training day, within approximately 1,000 trials.

After we demonstrated a modulatory role for nAChRs in eyeblink conditioning with the demonstration that mecamylamine severely impaired acquisition, we tested several cognition-enhancing drugs with various mechanisms of action affecting nAChRs. GTS-21 is a partial α7 nAChR agonist. Galantamine (Reminyl™, Janssen) is a weak AChE inhibitor and a nicotinic allosteric modulator. Allosteric modulators are drugs that interact with the receptor through binding sites that are distinct from those for ACh and nicotinic agonists and antagonists. Neuraceutan is called a nootropic drug, but recent experiments demonstrate that it acts on nAChRs to promote neurotransmitter release. All of these drugs ameliorate acquisition of conditioned eyeblink responses in older rabbits.

GTS-21: A Partial Agonist of α7 nAChRs

Results with the nAChR antagonist, mecamylamine, led us to predict that nAChR agonists would likely facilitate eyeblink conditioning in older rabbits. We tested the effect of the nicotinic agonist GTS-21 on learning, using 15 daily subcutaneous injections and the 750-ms delay eyeblink conditioning procedure [Woodruff-Pak et al., 1994b]. This selective nicotinic agonist acts primarily at the α7 nAChR subtype [De Fiebre et al., 1995; Kem et al., 1997], the subtype showing little numerical reduction in the brains of AD patients. 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.

Next we carried out an experiment to examine the effect of GTS-21 on acquisition, retention, and relearning [Woodruff-Pak et al., 2000]. We were interested in the duration of the effect of GTS-21 as assessed by retention and relearning. 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 6 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 6 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 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, we undertook an experiment to examine the possible reversal of the nAChR antagonist mecamylamine in young rabbits with nicotine or GTS-21. Mecamylamine at a low dose preferentially blocks β2 nAChRs. In this manner we were simulating an AD-related impairment, the loss of α4β2 nAChRs. 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. Combinations of GTS-21 or nicotine and mecamylamine did not cause sensitization or habituation in the explicitly unpaired condition. Because GTS-21 acts selectively at the α7 nAChR, and because mecamylamine only weakly inhibits α7 subunits, it is likely that the reversal of mecamylamine occurred via α7 nAChRs. Reversal by nicotine and GTS-21 of the nicotinic cholinergic antagonist mecamylamine suggests that α7 nicotinic cholinergic agonists may have efficacy in ameliorating deficits specific to AD.

Galantamine and Allosteric Modulation of nAChRs

Allosteric modulators, such as galantamine (Reminyl™; Integren, Janssen), employ a dual action at the cholinergic synapse. First, they act as AChE inhibitors. Second, they also act as agonists at presynaptic nAChRs, enhancing the release of ACh. Maelicke et al. [2001] limit the category of allosterically potentiating ligands on the basis of functional properties tested with nAChR agonists and antagonists. 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 induced single-channel activity in excised patches from various cells [Pereira et al., 1993, 1994; Storch et al., 1995] 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, we wanted to determine if nicotinic agonism using a different mechanism of action would be effective in the eyeblink classical conditioning paradigm. 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 [Woodruff-Pak and Santos, 2000]. 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 percentage of CRs. Control tests of rabbits in explicitly unpaired conditions demonstrated that non-associative factors could not account for the results.

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 [Woodruff-Pak et al., 2001]. 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 Bmax or KD 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 1-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.

Because the single-channel activity of galantamine could not be blocked by antagonists to nAChRs, Maelicke et al. [2000] asserted 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. Among the AChE inhibitors tested that were blocked by antagonists to nAChRs and that did not act as allosterically potentiating ligands were donepezil, metrifonate, rivastigmine, and tacrine.

Patch-clamp recordings of single ion channel activity demonstrated that donepezil, but not galantamine, could be blocked by mecamylamine. On the basis of these results, we carried out an experiment to examine at a whole organism, behavioral level whether galantamine, but not donepezil, could reverse mecamylamine-induced learning impairment. Young female rabbits received 15 sessions in the 750-ms delay eyeblink classical conditioning procedure after drug treatment with mecamylamine, galantamine, donepezil, or vehicle alone, or mecamylamine plus galantamine or donepezil. 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).

**Effects on nAChRs of the Nootropic Drug, Nefiracetam**

Our initial studies of nefiracetam in the early 1990s occurred before the effect of nefiracetam on nAChRs had been demonstrated. The impetus for the initial research was the known involvement of hippocampal CA1 pyramidal cells in eyeblink conditioning in rabbits and the demonstrated efficacy of nootropic drugs in modulating hippocampal synaptic transmission and long-term potentiation (LTP) in vitro [Olpe and Lynch, 1982; Satoh et al., 1986]. These results suggested that eyeblink conditioning would be relevant for the evaluation of nefiracetam. A cyclic derivative of GABA, N-(2,6-dimethyl-phenyl)-2-(2-oxo-1-pyrollidinyl)-acetamide, or nefiracetam (DM-9384), is classified as a nootropic drug. Most compounds developed as nootropic drugs are pyrrolidone derivatives (piracetam, oxiracetam, aniracetam, nefiracetam) and evidence indicates that these drugs activate brain neurotransmitter systems, in particular the cholinergic system [Spignoli and Pepen, 1987], but also the dopaminergic [Funk and Schmidt, 1984] glutamatergic [Marchi et al., 1990], and GABAergic [Nabeshima et al., 1990] systems.

Comparing the effect of 6 doses of nefiracetam injected subcutaneously 15 min before testing in the 750-ms delay eyeblink conditioning paradigm, there was significantly better conditioning in rabbits treated with the 10 and 15 mg/kg doses. These doses did not cause sensitization, habituation or elevations in motor responding of the eyeblink [Woodruff-Pak and Li, 1994].

The 750-ms delay eyeblink conditioning procedure was used to investigate the magnitude and duration of the nootropic drug nefiracetam's effect on retention and relearning [Woodruff-Pak et al., in press]. After administering daily injections of 0 (vehicle), 5, 10, or 15 mg/kg nefiracetam to older rabbits during 15 days of acquisition, we tested retention and relearning 1, 5, and 12 weeks posttraining. Rabbits received no drug after the initial 15 daily injections. Significant relearning was observed in the 10 mg/kg nefiracetam group 1 and 5 weeks after initial acquisition. Differences in tone-alone retention did not achieve statistical significance, although responses were numerically greater in the 10 mg/kg nefiracetam group. The effect of nefiracetam upon the ability of older rabbits to relearn a previously learned task was apparent for intervals of 1 and 5 weeks after drug administration. Under normal conditions, a drug is administered continuously. In this experiment, nefiracetam had a significant effect long after drug administration had ceased. Prolonged administration of nefiracetam may have ameliorating effects greater than those observed in only 15 days of drug administration.

An experiment that addressed the possibility that nefiracetam affected nAChRs was carried out in the mid-1990s [Woodruff-Pak and Hinchcliffe, 1997]. We tested the effects of mecamylamine or scopolamine combined with nefiracetam in young rabbits. A dose of
10 mg/kg nefiracetam significantly reversed the effect of 0.5 mg/kg mecamylamine suggesting that nefiracetam acted via \( \alpha_7 \) nAChRs. The fact that nefiracetam reversed 1.5 mg/kg scopolamine suggested that nefiracetam acted on nAChRs when nAChRs were blocked.

Subsequent research supported our initial finding of nefiracetam’s efficacy on nAChRs. Nefiracetam persistently potentiated currents through \( \alpha_7 \) and \( \alpha_4\beta_2 \) neuronal nAChRs expressed in Xenopus oocytes and caused a marked increase in glutamate release from electrically stimulated hippocampal slices that was blocked by nAChR antagonists [Nishizaki et al., 2000]. There may be a specific action of nefiracetam on the presynaptic protein kinase C (PKC) isozymes that enhances the activity of presynaptic nAChRs [Yoshii et al., 2001].

Nefiracetam ameliorated the effect of 1.5 mg/kg scopolamine on eyeblink conditioning administered daily for 15 sessions in young rabbits [Woodruff-Pak and Hinchcliffe, 1997] and for 20 sessions in older rabbits [Pak et al., 2002]. Furthermore, nefiracetam is effective in older rabbits only when the hippocampus is intact [Woodruff-Pak et al., 1997]. The latter results support the position that nefiracetam ameliorates conditioning via the hippocampus.

**SUMMARY AND CONCLUSIONS**

AD involves alterations in nAChRs and affects ACh neurotransmission and the pre- and postsynaptic mechanisms in many neurotransmitters. The predominant subtype of mammalian nAChRs with high affinity for agonists is composed of \( \alpha_4 \) and \( \beta_2 \) subunits, and \( \alpha_4\beta_2 \) nAChRs are lost in AD in substantial numbers. The nAChRs that bind to \( \alpha_7 \)-bungarotoxin and contain \( \alpha_7 \) subunits remain intact in the AD brain, but they may be functionally impaired by Aβ.

It is extremely important to examine nAChRs in living patients because the nAChR deficits in AD brains probably represent an early phenomenon in MCI, the pre-diagnostic phase of AD. Prediction of conversion from MCI to AD may be improved with assessment of nAChRs in living patients with PET. In this early stage of disease development, \( \alpha_4\beta_2 \) nAChRs may be lost and perhaps also functionally blocked by Aβ\(_{1-42}\). Blockage of \( \alpha_7 \) nAChRs pre- and postsynaptically may also occur before or during MCI. PET imaging can reveal loss of nAChRs, but current techniques are not able to demonstrate functional blockade by Aβ in living patients. Results demonstrating the inhibition of pre- and postsynaptic nAChRs by Aβ\(_{1-42}\) provide a possible mechanism to explain the early cognitive deficits seen in MCI and AD before extensive formation of β-amyloid plaques. Functionally, blockade of pre- and postsynaptic nAChR channels by Aβ\(_{1-42}\) may impair cognition even before the actual neurodegeneration characteristic of AD appears.

Interaction between Aβ and nAChRs has been demonstrated in a number of ways. Aβ inhibits pre- and postsynaptic nAChR function. Nicotine and GTS-21 reduced Aβ neurotoxicity. Excessive overexpression of Aβ in patients carrying the Swedish APP 670/671 mutation doubled the reduction of nAChRs over sporadic AD cases, β-amyloid peptides are distributed widely in AD, and \( \alpha_7 \) nAChRs clearly play a role in cognition. The \( \alpha_7 \) nAChR, expressed widely and abundantly in the human brain, may be a significant molecular target β-amyloid peptides.

To the extent that nAChRs are molecular targets for β-amyloid peptides in AD, nAChRs should be considered a high-priority target for drug design. In this regard, a partial \( \alpha_7 \) nAChR agonist, GTS-21, provided neuroprotection against Aβ peptides in rat cortical cell culture, ameliorated learning, and memory impairment in older rabbits tested with eyeblink conditioning, and reversed learning impairment in young rabbits produced by the nAChR antagonist, mecamylamine.

A means to up-modulate or potentiate the channel activity of nAChRs in response to ACh is to use allosterically potentiating ligands such as galantamine. Galantamine was particularly effective in ameliorating learning and memory impairment in old rabbits in the model system of eyeblink classical conditioning. Among all the cognition-enhancing drugs we have tested, galantamine is the only drug to facilitate learning in young rabbits. It may be that many of the mechanisms of action that are effective in ameliorating learning deficits in old rabbits (e.g., AChE inhibition, \( \alpha_7 \) nAChR agonism) are ineffective in young rabbits who are performing near ceiling levels. Allosteric modulation may be one of the limited means available to facilitate learning in young rabbits.

Nefiracetam, a nootropic cognition-enhancing drug, ameliorates learning and memory deficits in older rabbits and reverses both the effects of nAChR and mAChR antagonism in young rabbits. A specific action of nefiracetam on the presynaptic PKC isozymes may occur that enhances the activity of presynaptic nAChRs.

Novel \( \alpha_7 \) agonists are under development in a number of laboratories to explore further this direct mechanism of action on nAChRs. Tolerance and desensitization of nAChRs are potential limitations of this approach, although we did not observe these effects in our work with GTS-21, which was limited to 15 days of drug administration. Galantamine (Reminyl\textsuperscript{®}) is the first allosteric modulator to receive FDA approval for the treatment of memory impairment in AD. In the rabbit model system, this drug was
extremely efficacious and was the only drug to facilitate learning in young rabbits. The effects of nepracetam on dementia caused by AD are currently being studied in Phase II clinical trials. The complex nature of nepracetam’s neurobiological efficacy upon several brain structures and systems including nAChRs make it an interesting drug candidate for cognition-enhancement in AD. Further exploration of nAChRs as targets for therapeutic intervention via a number of different pathways seem warranted. In particular, treatment to affect Aβ and z7 nAChR interactions may prove beneficial in AD.

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