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Nicotinic Cholinergic Modulation: Galantamine as a Prototype

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ABSTRACT

Nicotinic acetylcholine receptor pharmacology is becoming increasingly important in the clinical symptomatology of neurodegenerative diseases in general and of cognitive and behavioral aspects in particular. In addition, the concept of allosteric modulation of nicotinic acetylcholine receptors has become a research focus for the development of therapeutic agents. In this review the scientific evidence for changes in nicotinic acetylcholine receptors in Alzheimer's disease is described. Within this context, the pharmacology of galantamine, a recently approved drug for cognition enhancement in Alzheimer's disease, is reviewed along with preclinical studies of its efficacy on learning and memory. Galantamine modestly inhibits acetylcholinesterase and has an allosteric potentiating ligand effect at nicotinic receptors. The data collected in this review suggest that the unique combination of acetylcholinesterase inhibition and nicotinic acetylcholine receptor modulation offers potentially significant benefits over acetylcholinesterase inhibition alone in facilitating acetylcholine neurotransmission.

INTRODUCTION

Acetylcholine neurotransmission plays a crucial role in learning and memory and has been the focus of pharmacological therapy for Alzheimer's disease. The major aim of this review is to address a means of ameliorating impaired acetylcholine neurotransmission beyond acetylcholinesterase inhibition alone. This mechanism is called allosteric modu-

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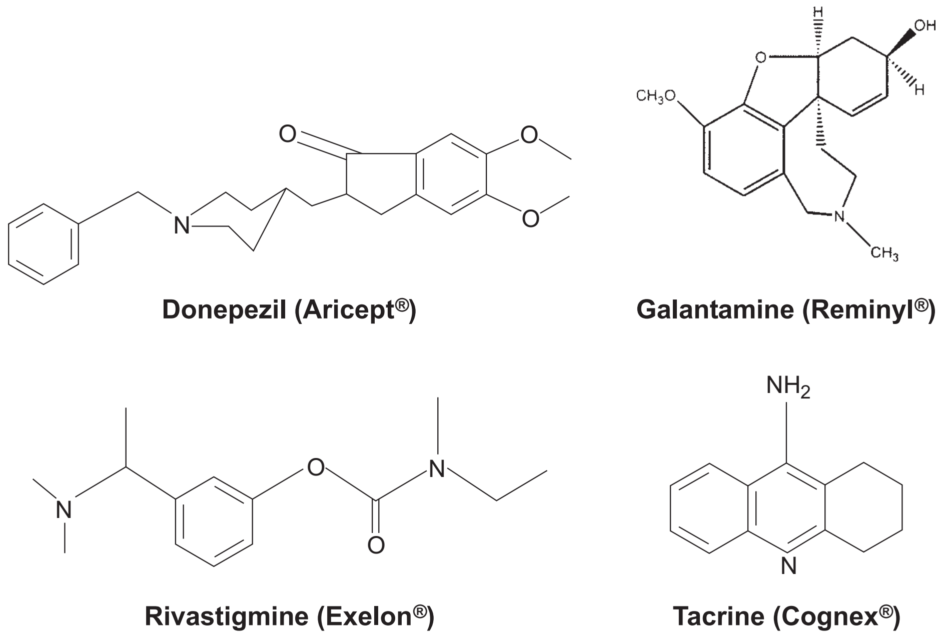

Fig. 1. Since 1993, four acetylcholinesterase AChE inhibitors have been approved by the FDA for the treatment of cognitive impairment in Alzheimer's disease. Shown here are the markedly different chemical structures of these drugs. Interestingly, the structural disparity of these drugs is reflected in their pharmacologic properties. Tacrine (a 4-aminopyridine derivative), donepezil (a benzylpiperidine derivative), and galantamine (a phenanthrene tertiary alkaloid) are reversible AChE inhibitors (i.e., their binding lasts just minutes), whereas rivastigmine (a carbamate derivative) is a "pseudo-irreversible" inhibitor, with an intermediate (i.e., ~10 h) duration of action.

lation of nicotinic acetylcholine receptors. The focus of the review is on preclinical studies and pharmacological analyses, with the main drug model for nicotinic allosteric modulation being galantamine. A review of clinical studies using galantamine appeared recently (34).

The brain acetylcholine neurotransmitter system is comprised of several distinct clusters of nuclei that have extensive projections to cortical and subcortical structures. The basal forebrain includes major groups of cholinergic cells in the medial septal nucleus, the nucleus of the diagonal band, and the nucleus basalis of Meynert. Projections from the basal forebrain contain cholinergic neurons innervating the hippocampus and amygdala as well as widespread regions of the cerebral cortex. Cells in this group are destroyed in Alzheimer's disease (AD), reducing acetylcholine levels in the brain.

Pharmacologic therapies to preserve the action of a dwindling acetylcholine pool in the AD brain have focused on prolonging its presence at the synapse. The acetylcholine molecule is inactivated in a single step. The enzyme acetylcholinesterase (AChE) breaks down acetylcholine into choline and acetic acid. Inhibition of AChE is equivalent to increasing the activity of acetylcholine. All currently approved drugs for mild-to-moderate AD work at least in part as AChE inhibitors (Fig. 1). To date, AChE inhibitors are the only drug class to have produced demonstrable — although modest — improvements in cognition for six months or longer in large-scale, double-blind, randomized controlled clinical trials. Moreover, these drugs are reasonably well tolerated by patients with AD.

There are two broad classes of acetylcholine receptors in the mammalian nervous system that respond to the natural alkaloids: nicotine or muscarine, to imitate the effects of acetylcholine as a neurotransmitter. Nicotinic acetylcholine receptors (nAChRs) are activated by nicotine, and muscarinic acetylcholine receptors respond to 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 ROLE OF nAChRS IN VARIOUS NEUROLOGICAL DISEASES

Evidence is accumulating that nAChRs play a role in a variety of disorders of the central nervous system including addiction to nicotine, Alzheimer's disease, anxiety, autism, depression, epilepsy, Parkinson's disease, schizophrenia, and Tourette's syndrome (51,82). This is not to imply that there is a common mechanism in these various neurological and psychiatric diseases. The mechanisms of nAChR impairment in this disparate group of syndromes are poorly understood. Since nAChRs are involved in a complex range of central nervous system disorders, it is important to define the means by which nAChRs exert their action in the brain.

Nicotinic acetylcholine receptors in the central nervous system are composed of five subunits arranged around a ligand-gated excitatory ion channel (9). The nAChR ion channel is permeable to Na^+, K^+, and Ca^{2+} (25,36). The nAChR subunits that have been isolated and cloned from mammalian or avian tissues to date are classified as α, β, γ, δ, and ε subunits. Neuronal subunits are limited to α and β. Many subtypes of nAChRs can be constructed from various combinations of the nine α subunits (α2 to α10) and three β subunits (β2 to β4), but two main neuronal categories have been identified on the basis of function and pharmacology. These two subtypes are the heterologous pentamers, constructed from combinations of α and β subunits (8) and the homologous pentamers, constructed from one subunit type, α7, α8, and α9 (37). Contrasted to the α8, and α9 homologous pentamers, only the α7 nAChR is expressed widely and abundantly in the mammalian brain (11,62). 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 (10,36,47). Considerable evidence indicates that nAChRs act as neuromodulators in communicative processes in the brain (35) and that nAChRs are involved in cognitive and memory functions (18,19,33,51,59).

The most abundant nAChR subtypes appear to be: (a) those that participate in high-affinity agonist binding associated with α4 and β2 subunits, and (b) those sensitive to blockade by α-bungarotoxin and containing α7 subunits. In addition to a high affinity for α-bungarotoxin, the α7 nAChR has a high relative permeability for calcium. This homologous pentamer, constructed from α7 subunits, produces multiple effects at the cellular level. Presynaptically, α7 nAChRs modulate neurotransmitter release (46). Postsynaptically, α7 nAChRs generate depolarizing currents (16). Additional effects of α7 nAChRs observed in cell culture include an influence on neurite outgrowth (58) and an activation of second messenger systems (79). The multiple functions of α7 nAChRs make them of special interest as therapeutic targets for diseases affecting the central nervous system. In AD, α7 nAChRs are therapeutic targets for their potential role in sensory processing and cytoprotection (52).

MECHANISM OF ALLOSTERIC MODULATION IN NICOTINIC ACETYLCHOLINE RECEPTORS

A novel approach to drug treatment in AD is the application of allosteric modulators of nAChRs (39,40). Allosteric modulators are drugs that interact with the receptor through binding sites that are distinct from those for acetylcholine and nicotinic agonists and antagonists (for an excellent review of the application of allosteric modulation in drug discovery, see ref. 7). Since these modulators are not directly involved in the neurotransmission process they affect, they typically do not induce compensatory processes that the agonists and antagonists induce. It is hypothesized that problems such as receptor desensitization and down-regulation of expression can be avoided with allosteric modulators (41).

A means to up-modulate or potentiate the channel activity of nAChRs in response to acetylcholine is to use allosterically potentiating ligands (APLs). Representative nicotinic APLs are the plant alkaloids physostigmine, galantamine, and codeine, and the neurotransmitter serotonin (41). Maelicke and his co-workers have argued that the structural properties of APLs are different from the structural properties of AChE inhibitors, the type of drugs currently approved to treat cognition impairment in AD.

Some investigators limit the category of APLs to physostigmine, galantamine, codeine, and serotonin on the basis of functional properties tested with nicotinic cholinergic agonists and antagonists (40,41). A basic functional property is the amplification of currents through the nAChRs triggered by the endogenous ligand. Later in this review during a discussion of galantamine as a nicotinic allosteric potentiating ligand, we will elaborate on these basic functional properties.

There is no complete agreement on which ligands have allosterically potentiating effects. Some investigators argued that many well-established AChE inhibitors such as donepezil, metrifonate, rivastigmine, and tacrine do not act as APLs (60). Nordberg and her colleagues reported evidence of binding to an allosteric site on the nicotinic cholinergic receptor by tacrine, donepezil, and NXX-066 (23,71,72). However, no functional APL effects were reported. In oocyte models, tacrine and physostigmine were identified as potentiating ligands; however, their functional mode of action could be described better in a 2-site competitive model rather than a pure allosteric model (95,96). Of those compounds, the only molecule approved as an APL in Europe is galantamine. In the United States galantamine is approved as an AChE inhibitor by the FDA and is marketed for the treatment of AD. In addition to being a prototypical APL, galantamine (Reminyl®) is considered as a first-line therapy for dementia (34). The remainder of the review article will describe the pharmacology of this unique compound.

GALANTAMINE

Galantamine hydrobromide is a phenanthrene alkaloid similar to codeine, which can be isolated from a variety of plant sources, including the European daffodil or common snowdrop, *Galanthus nivalis* (i.e., resembling snow) (57). It has also been found in a number of other sources, e.g., various species of *Narcissus*, *Lycoris*, and several South African Amaryllidaceae (see review 21). It has been observed that deer will eat the flowers, but not the bulbs, of these plants, attesting to the pharmacologic acumen of these animals. The flowers were probably introduced from the Mediterranean by the Romans. An old glossary of 1465, referring to it as *Leucis i viola alba*, classified the flower under the narcissi, its healing properties are stated to be "digestive, resolutive and consolidante." This early citation already suggests that the pharmacodynamic properties of galantamine were known in the medieval times. Further early descriptions include a quotation from Sir Thomas Hammer in *The Garden Book* (1659), "The early white (bulbous Violet) whoise pretty pure white bellflowers are tipt with a fine greene, and hang downe their heads." Russian scientists rediscovered galantamine after World War II (54).

A full synthetic manufacturing process was described in 2000 (Janssen, data on file). The drug has three chiral centers, leading to 8 different optical isomers. The first reference that can be traced in automated literature searches for galantamine appears in 1965. Following this report, a number of mainly Russian-based studies can be located. The primary literature (in Russian) is difficult to access and is largely unknown in the West. Galantamine has been prescribed in several European countries for a number of decades as an accepted treatment for a variety of neuromotor diseases. In Austria, it was approved for the treatment of AD under the name Nivalin® in 1994, European approval was in December 2000, and the FDA approved galantamine for the treatment of mild-to-moderate AD in May 2001.

Galantamine as an Acetylcholinesterase Inhibitor

A number of *in vitro* studies have shown that galantamine is a reversible, competitive inhibitor of acetylcholinesterase. The affinity for the enzyme is quite modest, and results for IC_{50} range from 800 nM *in vitro* to over 2 μM in dog skeletal muscle (27) to values of 2.4 μM *ex vivo* from human brain tissue (78). In the study by Thomsen and associates, the inhibition of acetylcholinesterase by galantamine was similar in postmortem brain and brain cortical biopsies from patients submitted to brain-tumor removal. This indicates that postmortem change up to 28 h after death probably did not influence the measurement of AChE inhibition. Whereas physostigmine and tacrine acted equally on AChE from different sources, galantamine was 10-fold less potent in inhibiting the enzyme activity from human brain than from human erythrocytes. The IC_{50} measured on erythrocyte enzyme is about 365 nM. Comparison with tissues from mice revealed that galantamine was selectively more potent in suppressing AChE in human erythrocytes. When using IC_{50} values, the degree of inhibition may depend upon the substrate concentration in the case of a competitive inhibitor.

In contrast to the above studies, other studies have reported K_i values rather than IC_{50} values. Higher affinities were observed with AChE from electrical eel tissue (K_i = 120 nM) or from human erythrocytes (K_i = 200 nM) (31). Whether the difference in measurements is due to the different effect of galantamine on different isoforms of the AChE (G2-form in erythrocytes, a mixture of G1/G4 form in human brain) is unknown but probably unlikely.

Crystallographic studies document the interaction between galantamine and the Torpedo AChE at 2.3 A resolution (3,20,56). Galantamine binds at the base of the active site gorge of AChE, interacting with both the choline-binding site (Trp-84) and the acyl-binding pocket (Phe-288, Phe-290). The tertiary amine group of galantamine does not interact closely with Trp-84; rather, the double bond of its cyclohexene ring stacks against the indole ring. The tertiary amine appears to make a non-conventional hydrogen bond, via its N-methyl group, to Asp-72, near the top of the gorge. The hydroxyl group of the inhibitor makes a strong hydrogen bond (2.7 A) with Glu-199. The relatively tight binding of galantamine to AChE appears to arise from a number of moderate to weak interactions with the protein, coupled to a low entropy cost for binding due to the rigid nature of the inhibitor.

Barnes et al. (2) documented the difference in the degree of AChE inhibition between donepezil and galantamine in an actual *in vivo* experiment. Using three month old rats and an Alzet minipump formulation, Lineweaver-Burke plots were determined for AChE inhibition. In a *post hoc* analysis, after careful analysis using the appropriate equations for non-competitive (donepezil) versus competitive (galantamine) inhibition, K_i values were determined as 0.77 mg/kg for donepezil and 2.99 mg/kg for galantamine. Under these particular *in vivo* conditions, donepezil is only 4 times more potent at inhibiting AChE than galantamine, compared to about 40-fold in *in vitro* experiments. This discrepancy between *in vitro* and *in vivo* results is probably due to the different *in vivo* pharmacokinetic profile of galantamine, which is much less subject to plasma protein binding and has a higher brain stability. Indeed, animal studies have shown that galantamine had an unusually high oral bioavailability of 77% and a brain penetration ratio of 1.4 to 1.8 with less than 5% metabolism and a long brain presence (50% reached after 8 h) (42). A similar study of oral donepezil suggests a lower oral bioavailability of 56% coupled with a brain penetration ratio of 3.3 but a much faster depletion to 50% of peak levels within 2 h (45).

Butyrylcholinesterase (BuChE) is another enzyme that hydrolyzes acetylcholine, but galantamine appears to be selective to AChE. Galantamine shows a high degree of selectivity (50 times) against the BuChE in human erythrocytes (77). The same degree of selectivity was observed when studying galantamine-treated patients. Furthermore, galantamine concentrations up to 10 μM did not affect choline acetyltransferase (ChAT) activity, [^{3}H]hemicholinium-3 (HCh-3) binding to the choline carrier, [^{3}H]quinuclidinylbenzilate (QNB) binding to muscarinic receptors, or [^{3}H]acetylcholine binding to nAChRs in cortical homogenates (74).

Galantamine as a Nicotinic Allosteric Potentiating Ligand

Patch-clamp electrophysiological methods were used to record single channel and whole-cell activity in response to acetylcholine in a variety of cell culture models (PC12 cells, rat embryonic hippocampal neurons and M10 cells). At concentrations between 1 and 10 μM galantamine was able to directly activate single channels, but failed to produce appreciable whole-cell currents. These effects of galantamine were not blocked by competitive nAChR antagonists, suggesting that distinct sites of the nAChR were involved (63).

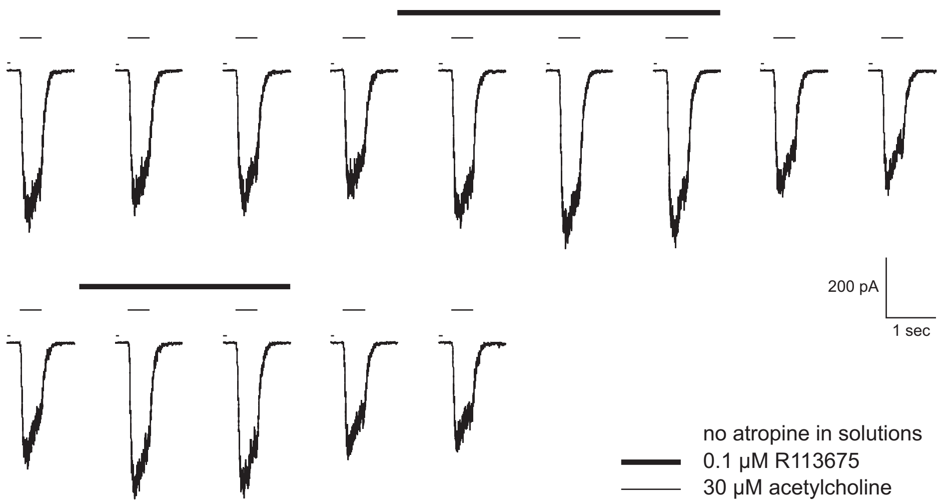

Fig. 2. Electrophysiological traces of acetylcholine-triggered α4β2 nAChR mediated currents in HEK-293 cells, expressing recombinant human α4β2 nAChR. Short pulses of 30 μM acetylcholine are given (short lines), in the absence and presence (long lines) of 100 nM R113675 or galantamine (applied twice). It is clear that the peak current instantaneously increases by about 20–30% when galantamine is applied. After wash-out the current immediately comes back to its normal basal value (data from ref. 43).

In the same study it was shown that galantamine, and its analogue N-methyl-galantamine were able to potentiate the acetylcholine-induced currents in PC12 cells. The effect was blocked by application of the monoclonal IgM antibody FK1, whereas this antibody was documented not to have any effect on the endogenous acetylcholine response. Taken together, these results suggest that the site of galantamine's effect on the receptor is different from the acetylcholine binding site, pointing towards an allosteric potentiating effect. However, it should be noted that IgM antibodies tend to have problems of specificity. The final proof of the allosteric effect awaits development of better and more specific antibodies against the putative allosteric site on the α-subtype of the nAChR (53).

The potency to act as an allosteric potentiating ligand was independent of galantamine's ability to block AChE, suggesting a second independent pharmacological mechanism (Fig. 2). In another experiment using recombinant HEK293 cells expressing the human α4β2 nAChR, galantamine (but not rivastigmine, donepezil, tacrine or metrifonate) was shown to dose-dependently and allosterically potentiate the acetylcholine response up to a maximal effect of 80% increase at a dose of 700 nM. At higher concentrations of galantamine the effect decreased. At a concentration higher than 10 μM, a clear inhibition of the current was seen for all AChE inhibitors used. Taken together, the data indicate that the net effect of galantamine is to shift the acetylcholine dose-response curve to the left (60).

Functionally unique features of APLs also include the ability to induce single-channel activity indistinguishable from the single-channel activity induced by acetylcholine. With allosteric potentiation, galantamine and related compounds induced single-channel activity in excised patches from various cells (53,54,70) that could not be blocked by established nicotinic antagonists. The fact, that the galantamine-induced single channel activity could not be blocked by nicotinic antagonists, was used as evidence that the activity was induced through a site different from that activated by acetylcholine or competitive ligands (41).

The effect of a chronic treatment with galantamine on nAChRs was studied in a permanently transfected fibroblast cell line (M10) expressing the major nAChR α4β2 subtype. Galantamine in a concentration range of 1–10 μM showed a dose-dependent increase in receptor binding of 25%, whereas at much higher doses (largely exceeding currently used therapeutic doses) a decrease was observed (72).

Using electrophysiological recordings it has been demonstrated that galantamine prolongs the action of neuronally released acetylcholine at neuromuscular junctions (22). Interestingly, those authors suggest that this action of galantamine is due to a potentiation of K^+ currents. This alternative explanation of increased efficacy of neuronal activity following administration of galantamine illustrates the difficulty of measuring a clear and unequivocal potentiating effect of an allosteric modulator.

Challenges in demonstrating allosteric potentiation

Apart from galantamine, there are published reports of other allosteric modulators of the nAChR. Classical allosteric modulators include codeine and serotonin (54) and N-methyl-galantamine (63). An unexpected finding was the modulatory effect of atropine, a classical muscarinic antagonist, on nAChRs expressed in oocytes. At concentrations in the 1–10 μM range, atropine clearly potentiated the acetylcholine and the nicotine induced current, while at higher concentrations there was a clear inhibition (95). These results underscore the need for a cautious interpretation of the results obtained in HEK293 cells, which have a high density of muscarinic receptors.

Interestingly, piracetam and aniracetam have been identified as potent allosteric modulators of nAChRs (92). These effects are not observed in recombinant cellular systems such as HEK293 cells expressing human nAChRs. Demonstration of allosteric modulation of nAChRs by piracetam and aniracetam requires the complete intracellular signaling of neuronal cells. These compounds are documented to interact with cAMP dependent phosphorylation, as forskolin and dibutryl cyclic AMP interfere with their action. A systematic study of the effect of n-alcohols on the nAChR demonstrated that there was a size-dependent allosteric modulatory effect (94).

Zwart et al. (95) characterized physostigmine and tacrine, previously reported to be allosteric modulators (71), more accurately as two-site competitive agonists. Evidence for this model of a two-site competitive agonist includes competition for the binding site of [^{3}H]epibatidine in oocytes, expressing α4β2 nAChRs.

This short overview illustrates the myriad of pharmacological actions previously described as "modulators" of the nAChRs. It also underscores the difficulty of reproducibility of some results and the complexity of the real mode of action.

Galantamine's Action in Brain Slices

Immunohistochemical studies have documented that in the rat hippocampus, α7 nAChRs are largely localized on presynaptic glutamatergic and GABA-ergic synapses (14). As a consequence, it is anticipated that the allosteric potentiating ligand effect of galantamine on α7 nAChRs could have functional consequences for GABAergic and glutamatergic synaptic transmission. In an elegant study using rat and human hippocampal slices, 1 μM of galantamine was found to enhance the effect of acetylcholine (30 μM) on

GABA release by about 20% (61). The same report documented the effect of galantamine on glutamatergic neurotransmission (measured by excitatory postsynaptic currents) evoked by field stimulation of Schaffer collaterals. An inverse U-shaped dose response curve was observed, with 1 μM as the concentration with the highest effect (20% potentiation). Galantamine amplified the currents triggered by both AMPA and NMDA receptors, suggesting that the APL effect was presynaptic. Also, galantamine had no effect on the membrane and action potential characteristics of glutamatergic neurons.

Interestingly the effect of galantamine was not blocked by a high concentration of an AChE inhibitor (metamidophos), but was sensitive to blocking of the α7 nAChR by methyllcaconitine. Again the galantamine effects could be blocked by the FK-1 monoclonal antibody, suggesting an allosteric mode of action.

In order to further confirm that the AChE activity of galantamine was not involved in facilitating GABA or glutamate release, pure AChE inhibitors such as rivastigmine and donepezil were tested. These AChE inhibitors were inactive in enhancing the glutamatergic and GABA-ergic currents. These data suggest that the APL activity of galantamine in complex models such as hippocampal slices can be extended to glutamatergic and GABA-ergic neurotransmission, in line with neuroanatomical observations of the localization of α7 nAChRs. As all these subsystems have been implicated in cognitive processing (48), the APL effect could be beneficial in the clinical setting of cognition enhancement in dementia.

Galantamine's Action in Animal Models

Rodent learning and memory

In vivo studies using nonlesioned animal models have shown that galantamine prolongs the activity of neuronally released acetylcholine and increases brain acetylcholine levels after systemic administration, consistent with the action of a cholinomimetic agent (31). Galantamine showed physiological cholinomimetic activity by causing hypothermia; and behavioral cholinomimetic activity by attenuating scopolamine-induced deficits in passive avoidance in mice. In addition, galantamine enhanced step-down passive avoidance, another measure of behavioral efficacy (6). In another study using scopolamine induced memory deficit, galantamine was tested in the T-maze (1.25, 2.5, or 5.0 mg/kg, i.p.) and in the Morris water maze (2.5 or 5.0 mg/kg, i.p.). Galantamine significantly attenuated scopolamine-induced deficits in both learning and memory models (15). In rats studied with active and passive avoidance tasks, galantamine at 1 mg/kg but not at 0.5 mg/kg significantly improved memory retention of a learned behavior (91).

Galantamine has also shown efficacy in animal models with brain lesions. In a study of localized damage to motor cortex of cats, where spontaneous recovery was documented to take place over 16 to 30 days, applying a nAChR antagonist reduced the recovery time to 10 to 16 days. Galantamine in combination with this nAChR antagonist was documented to further reduce the recovery time to about 5 to 10 days (66). In combination with a muscarinic antagonist however, galantamine was unable to reduce the recovery time. Additional studies along this line were not carried out. Hence, as this primary literature is relatively inaccessible it is difficult to judge the relevance. Nevertheless, these data suggest a different action of galantamine on muscarinic versus nicotinic receptors.

Working memory deficits caused by ibotenic acid-induced lesions of the nucleus basalis magnocellularis of mice trained in a Morris water maze were reduced by 70% when galantamine (5 mg/kg i.p.) was injected at 210 minutes before testing. In a subsequent study, using foot shock passive avoidance, it was shown that galantamine produced a dose-dependent improvement at doses between 2 and 3 mg/kg i.p. In this study, behavioral tolerance did not occur following repeated dosing over two weeks (75). Galantamine also improved performance in a water maze test using a strain of mice with deficiencies in learning abilities (73).

At 1 mg/kg i.p. galantamine also significantly enhanced the performance of scopolamine-treated mice in a conditioned aversion response mode (74). In a passive avoidance paradigm in mice with basal forebrain lesions, the optimal dose of galantamine ranged between 0.1 and 0.5 mg/kg i.p. (80). In nucleus basalis- lesioned rats, galantamine reversed the memory deficit in active as well as passive avoidance tests. Galantamine partially reversed scopolamine effects in the passive avoidance test, in a T-maze, and in a Morris water maze.

In a prolonged alcohol intake model of acetylcholine deficit in male Wistar rats, the effects of galantamine were examined (26). After 16 weeks of alcohol intake and a 2-week pause, rats administered galantamine (2.5 mg/kg/d i.p.) showed an improved speed of learning and short-term memory in the shuttle box test as compared to the saline-injected alcoholic group. Four weeks later, significant improvement in the passive avoidance memory of alcoholic galantamine-treated rats was noted in the eight-arm radial maze (14 day test duration) as compared to the saline-injected alcoholic group. Results showed that in rats under conditions of prolonged alcohol intake galantamine improved the speed of learning, short-term memory and spatial orientation.

As discussed previously, *in vitro* data identified donepezil as a more potent inhibitor of AChE activity than galantamine. Barnes et al. (2) determined doses of galantamine and donepezil with the intention to end up with equal levels of brain AChE inhibition in older rats. To this end they performed Lineweaver-Burke plots and determined these dosages to be 0.277 mg/day for galantamine and 0.695 mg/day for donepezil. These dosages seem at odds with the observed large differences in *in vitro* potency against the AChE enzyme (donepezil is about 40- to 100-fold more potent). Accordingly, when observing data presented in Figure 1 of the Barnes et al. (2) article, this order of potency is conserved. Consequently when calculating the AChE inhibition levels using the appropriate equations for competitive (galantamine) versus non-competitive (donepezil inhibition), it turns out that the 0.277 mg/day dose for galantamine corresponds to about 10% inhibition of the AChE in the brain, versus 60% inhibition for donepezil at 0.695 mg/day.

Using osmotic mini-pump infusion for 35 days, galantamine resulted in a significant upregulation of nAChR binding sites (as assessed by [^{3}H]epibatidine) by 15% in the hippocampus and by 35% in the cortex. In comparison, treatment with donepezil for the same duration, at doses corresponding to a brain AChE inhibition of 60%, led to an upregulation of nAChR binding sites by 20% in the hippocampus and by 70% in the cortex. These results suggest that the potentiating ligand effect of galantamine is able to partially compensate for the large difference in brain AChE inhibition. Donepezil may have yielded this result due to an AChE inhibition mechanism, whereas galantamine operated as an allosteric modulator. The action of galantamine and structurally related drugs is allosteric rather than directly agonistic, and therefore, independent from the acetylcholine binding sites.

Classical eyeblink conditioning in rabbits and humans

The demonstrated role of acetylcholine in modulating the rate of learning in eyeblink classical conditioning in rabbits (4) makes this model system useful in preclinical investigations of cognition enhancing drugs (85). More is known about the neural structures and systems that are involved in eyeblink classical conditioning than in any other learning and memory task. Although the neural circuitry essential for acquisition and retention of the conditioned eyeblink response resides in the cerebellum (76), the hippocampus is engaged during delay eyeblink classical conditioning (5). In the delay procedure, a neutral stimulus such as a tone conditioned stimulus (CS) is presented half a second before the onset of a corneal airpuff eyeblink-eliciting unconditioned stimulus (US). The organism learns to blink to the tone CS before the onset of the airpuff US, and the learned response is called the conditioned response (CR). It is our working hypothesis that selective loss of hippocampal pyramidal cells (83) and disruption of the septo-hippocampal cholinergic system in AD (12) impairs acquisition of delayed eyeblink classical conditioning in AD beyond the impairment observed in normal aging. This hypothesis has been supported (86,87) and independently replicated (68).

Having demonstrated that the nicotinic cholinergic drug GTS-21 ameliorated learning deficits in older rabbits, the aim was to determine if the dual action of an APL would have even greater efficacy in the classical eyeblink conditioning model paradigm. A nicotinic APL, galantamine, was tested at doses of 0.0, 1.0, 2.0, 3.0, and 4.0 mg/kg (88). Forty older rabbits were tested in 10 daily sessions in the 750-ms delay conditioning paradigm. A dose of 3 mg/kg galantamine was extremely effective in improving conditioning in older rabbits, enabling them to achieve learning criterion rapidly and to produce a very high percentage of CRs. Trials to learning criterion, a measure that is larger when learning is poorer, revealed a classical U-shaped response curve with doses of 1.0 and 2.0 mg/kg s.c. galantamine producing non-significant effects over vehicle-treated rabbits. At 3.0 mg/kg s.c. galantamine reduced the number of trials to learning criterion to a mean significantly lower than vehicle-treated rabbits while 4.0 mg/kg galantamine produced a non-significant effect. Older rabbits treated with 3.0 mg/kg s.c. galantamine achieved learning criterion 40% faster than older rabbits tested with the optimal dose of GTS-21.

The results with a dose of 3.0 mg/kg s.c. galantamine were striking, but they were observed in a relatively small sample (88). Additional experiments were carried out to further explore the effect of 3.0 mg/kg s.c. galantamine on learning (89). In Experiment 1, 16 young and 16 older rabbits were administered subcutaneous injections of 3.0 mg/kg galantamine before training for 15 daily sessions of eyeblink classical conditioning. In Experiment 2, 53 retired breeder rabbits were tested over a 15-week period in four conditions. Groups of rabbits received 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. For these two experiments, there were three major aims. First, to examine behavioral and pharmacological effects of the 3.0 mg/kg dose of galantamine by testing the drug in young as well as older rabbits. Next, to compare behavioral and pharmacological effects of galantamine in larger groups of older rabbits at a dose that affected eyeblink conditioning in a 2-week experiment (3.0 mg/kg s.c.) and a dose that was not different in its behavioral effect from vehicle (1.0 mg/kg s.c.). Finally, to compare behavioral and pharmacological effects of short-term

(3 weeks of 5 daily injections/week) versus longer-term (15 weeks of 5 daily injections/week) administration of 3.0 mg/kg galantamine. The effects of galantamine in older rabbits were examined over a time period (15 weeks) that would simulate a human clinical trial, testing rabbits at monthly intervals for retention and relearning for three months after initial acquisition.

Galantamine at a dose of 3.0 mg/kg s.c. was effective in facilitating learning (Fig. 3). The 3.0 mg/kg dose of galantamine improved learning significantly in young as well as in older rabbits. Among the many cognition-enhancing drugs we have tested in 4-month-old rabbits (BMY-21502, donepezil, GTS-21, nefiracetam), galantamine is the only drug that has facilitated learning in young rabbits. Young animals acquire CRs at close to ceiling levels (around 400 training trials), making it more difficult to demonstrate a significant effect. With a dose of 3.0 mg/kg galantamine, young rabbits achieved learning criterion in 297 trials, whereas the mean trials to criterion for young vehicle-treated rabbits was 445 trials. Old rabbits treated with 3.0 mg/kg galantamine achieved criterion in 401 trials. At 3.0 mg/kg s.c. galantamine caused older rabbits to learn at the same rate as young vehicle-treated rabbits.

The 3.0 mg/kg dose of galantamine affected the rate of learning early in the acquisition process. Old rabbits treated with 3.0 mg/kg galantamine learned (on average) on training days 4 or 5. Old rabbits treated with 1.0 mg/kg galantamine learned (on average) on training day 6 or 7, and old rabbits treated with vehicle learned (on average) on training day 9 or 10. Since all rabbits were trained for 15 sessions, the groups were relatively equal at the end of acquisition. Although all the groups performed at about the same level at the end of acquisition, when they were retested for retention one month after acquisition was complete, the group continuously injected with 3.0 mg/kg galantamine performed significantly better. The significant retention effect did not occur in the group treated with 3.0 mg/kg s.c. galantamine only for the 15 days of acquisition training. Indeed, the group treated continuously with 1.0 mg/kg s.c. galantamine had a numerically higher retention score in the 1-month retest than did the group treated with 3.0 mg/kg galantamine for 15 days.

Data from some of the animal models might wrongly indicate that the dose-range of galantamine treatment is quite narrow. Direct extrapolation of the dose response curves of behavioral efficacy in animals to the human situation is not suggested since clinical practice in patients with AD suggests that a rather broad range of doses is therapeutic (16 to 32 mg/day). This can probably be explained by the observation that in AD the cholinergic deficit is much more amenable to AChE inhibition. As a consequence the linear dose dependent AChE-inhibition of galantamine significantly extends the inverted U-shape dose response profile of the allosteric potentiating ligand effect.

Relevance of Galantamine to Neuropathology in Alzheimer's Disease

Nicotine, nAChRs and β-amyloid

Amyloid plaques comprised of β-amyloid 40- and 42-peptides ($A\beta_{1-40}$ and $A\beta_{1-42}$) in neuritic plaques (65) and intracellular neurofibrillary tangles comprised of hyperphosphorylated tau (32) are major forms of neuropathology found in the brains of AD patients. Although some research has been initiated relating nAChRs to tau protein levels (23,84), most investigations have focused on interactions between $A\beta_{1-40}$, $A\beta_{1-42}$, and nAChRs.

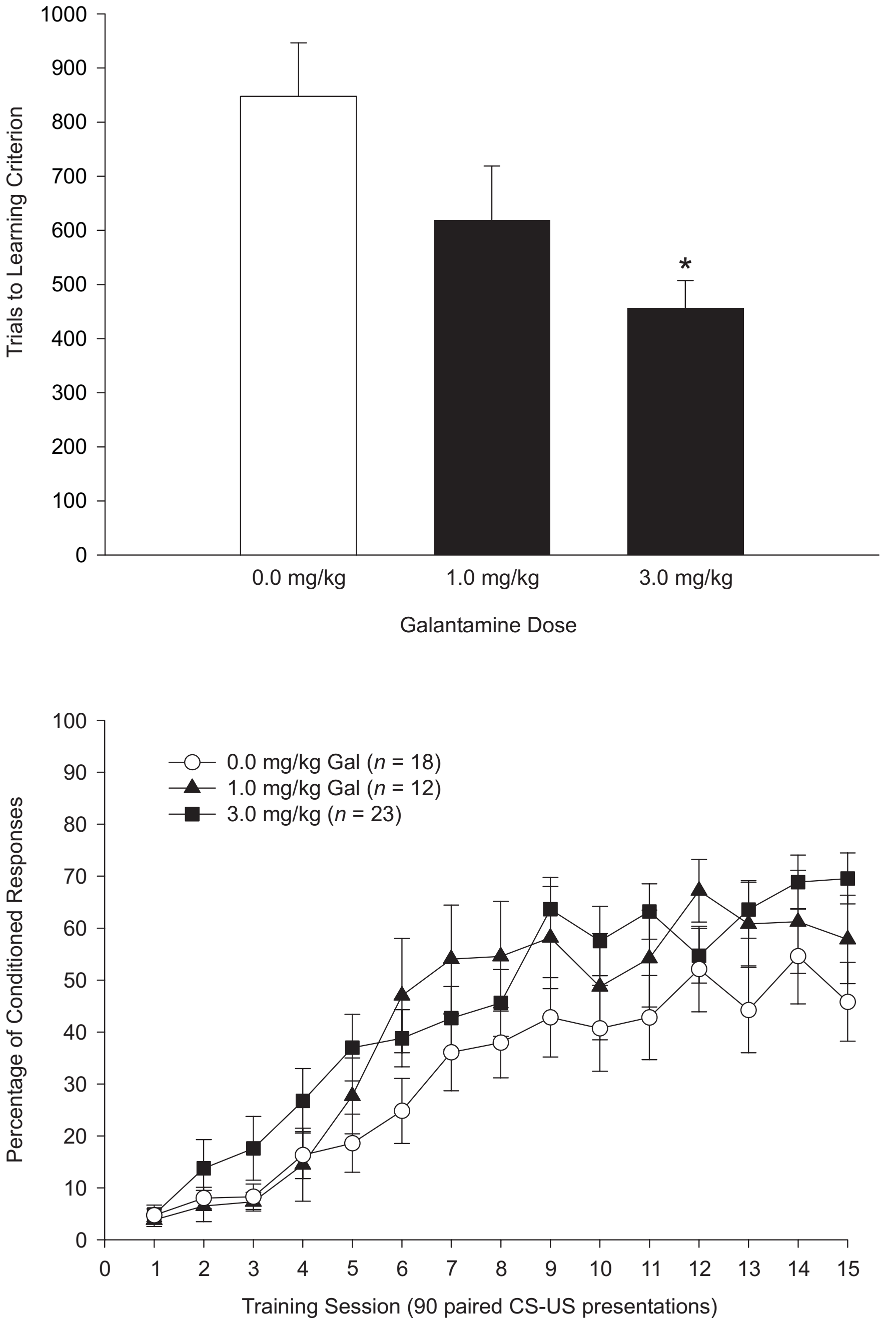

Fig. 3. (Top) Trials to a learning criterion of 8 conditioned responses (CRs) in 9 consecutive trials for older rabbits treated with 0.0 (sterile saline vehicle), 1.0, or 3.0 mg/kg galantamine and trained in the 750-ms delay eyeblink classical conditioning procedure for 15 daily sessions. Asterisk indicates a statistically significant difference ($p < 0.01$) between trials to criterion between groups treated with 0.0 and 3.0 mg/kg galantamine. (Bottom) Percentage of CRs over 15 daily training sessions in the same rabbits shown at the left. Percentage of CRs was significantly greater ($p < 0.01$) for rabbits in the 3.0 mg/kg galantamine group. Error bars are standard errors of the mean (data from ref. 89).

Galantamine increases the efficacy of nAChRs (in particular, the efficacy of α7 nAChRs) and may be neuroprotective against Aβ.

The potential role of Aβ as a neuromodulator in the brain has drawn attention to the possibility that Aβ may affect acetylcholine neurotransmission via nAChRs (1). Kihara et al. (30) provided the first evidence of an interaction between nAChRs and Aβ with the demonstration that stimulation of α4β2 nAChRs inhibited Aβ neurotoxicity. Marutle and associates (44) investigated the influence of Aβ on nAChRs in autopsy brain tissue from AD patients carrying the Swedish APP 670/671 mutation and in brain tissue from sporadic cases of AD. The mutation results in an overexpression of the amyloid leading to plaque formation (50). Reductions in the number of nAChRs in the Swedish APP 670/671 mutation were dramatic and statistically significant. In the Swedish APP 670/671 brains, nAChR reduction ranged between 73 and 87%, whereas in the brains of sporadic AD cases the nAChR reduction ranged between 37 and 57% (44). The two distributions in 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.

Wevers and associates (84) developed two experimental model systems using organotypic culture and primary hippocampal culture to test the impact of Aβ and hyperphosphorylation of the τ-protein on nAChRs. Preliminary results indicate that the α4 subunit exhibits lower tolerance to $A\beta_{1-42}$ than does the α7 subunit. Pettit, Shao, and Yakel (55) supported the greater tolerance of the α7 subunit for $A\beta_{1-42}$ in rat hippocampal slices when they determined that α7 subunit channel inhibition was 14%, whereas non-α7 subunit channel inhibition was 54%.

Evidence for a physiological role of $A\beta_{1-42}$ in the inhibition of postsynaptic nAChRs was provided when $A\beta_{1-42}$ blocked nAChR-mediated current and reduced the probability of open channels in rat hippocampal interneurons (55). Modulation by $A\beta_{1-42}$ occurred rapidly, within milliseconds at single channels, and inhibition of nicotinic currents occurred at concentrations of $A\beta_{1-42}$ as low as 100 nM. Experiments demonstrated that $A\beta_{1-42}$ bound and inhibited multiple subtypes of nAChRs (55, 81). Whether it is the fibrillar or the soluble form of $A\beta_{1-42}$ that is toxic remains unclear. For their hippocampal slice experiments, Pettit and associates (55) argued that the facts that the fibrillar form of $A\beta_{1-42}$ 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 $A\beta_{1-42}$.

Although α7 subunit channel inhibition by $A\beta_{1-42}$ was substantially less than non-α7 subunit channel inhibition (55), α7 subunit channels in rat hippocampal slices were nevertheless impaired by $A\beta_{1-42}$. Liu, Kawai, and Berg (38) demonstrated that β-amyloid peptides could block the function of α7 nAChRs. The initial experiments using whole-cell patch-clamp recording were carried out in rat hippocampal neurons in dissociated cell culture. The results were replicated in chick ciliary ganglion neurons, which consistently yield high levels of α7 nAChRs. The blockade of α7 nAChRs by $A\beta_{1-42}$ is specific, noncompetitive, reversible, and has high affinity, exerted through the N-terminal extracellular portion of the receptor. The investigators concluded that the fact that α7 nAChRs on cell types as diverse as rat hippocampal neurons and chick ciliary ganglion neurons can be blocked by $A\beta_{1-42}$ suggests that the response to $A\beta_{1-42}$ may be a common feature of α7 nAChRs.

Whereas other laboratories had demonstrated the blockade of nAChRs by $A\beta_{1-42}$ to be a postsynaptic phenomenon (55,81), Liu and associates (38) demonstrated both a pre- and postsynaptic blockade in α7 nAChRs. The investigators tested the effects of $A\beta_{1-42}$ on presynaptic hippocampal α7 nAChRs by determining whether the peptide prevented a nicotine-induced increase in the frequency of spontaneously occurring responses unique to presynaptic α7 nAChRs. In all cases, the nicotine-induced increases in presynaptic responses were blocked by 100 nM $A\beta_{1-42}$.

The pre- and postsynaptic blockade of α7 nAChRs by $A\beta_{1-42}$ has major implications for cognitive impairment in AD. Somato-dendritic α7 nAChRs are thought to mediate synaptic currents (16) while presynaptic α7 nAChRs are thought to modulate neurotransmitter release (46). 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 the disease (i.e., β-amyloid peptides). Regardless of the causes of AD, the blockade of α7 nAChRs is a consequence that has long-term outcomes for the cognitive function of AD patients.

Results demonstrating the inhibition of pre- and postsynaptic nAChRs by $A\beta_{1-42}$ provide a possible mechanism to explain the early cognitive deficits seen in mild cognitive impairment (MCI) and AD before extensive formation of β-amyloid plaques. Functionally, blockade of postsynaptic nAChR channels by $A\beta_{1-42}$ may impair cognition even before the actual neurodegeneration characteristic of AD appears. The data suggest that $A\beta_{1-42}$ might exert deleterious effects on cognition independently of plaque formation. A similar explanation could be directed at the early cognitive effects reported in transgenic mice in which behavioral deficits precede amyloid deposition (24,49).

Protection of nAChRs against Aβ cytotoxicity

Kihara and associates (29) examined the protective effect of nicotinic receptor stimulation against Aβ cytotoxicity. They used the $A\beta_{25-35}$ peptide because of the reported neurotoxic effects of this fragment (90). Neurotoxicity induced by Aβ in cultured rat cortical neurons was dramatic. The number of viable neurons decreased significantly when cultures were exposed to synthetic Aβ peptides. Administration of nicotine along with Aβ exposure markedly reduced the number of dead cells. The nicotine-induced neuroprotection was dependent on the concentration of Aβ introduced into cell culture. When nicotinic antagonists were added, the neuroprotective effect of nicotine was blocked. This result suggested that the effect of nicotine was mediated by nAChRs. Introduction of α-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 anabasine (28) called GTS-21 [3-(2,4-dimethoxybenzylidene)anabaseine] has been found to preferentially interact with α7 nAChRs. When GTS-21 was introduced into the cell culture, it protected neurons against Aβ-induced death. These results suggest that α7 nAChR activation can play an important role in neuroprotection against Aβ neurotoxicity. Kihara et al. (29) concluded that α7 nAChR activation 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. (30) reported that nicotine neuroprotection could be blocked by an α4β2 nAChR antagonist, suggesting a neuroprotective effect for α4β2 nAChRs as well as α7 nAChRs.

Reviewing the programmatic research they have carried out on nAChR neuroprotection in cell culture, Shimohama and Kihara (67) developed a hypothesis for the mechanism of nAChR-mediated survival. Bcl-2 and Bcl-x are proteins of demonstrated involvement in neuroprotection. They prevent cell death induced by a variety of toxic attacks (93). Shimohama and Kihara (67) proposed that through a series of steps including activation of phosphatidylinositol 3-kinase to phosphorylate Akt, α7 nAChRs upregulate Bcl-2 and Bcl-x. Upregulation of Bcl-2 and Bcl-x prevents cells from neuronal death induced by Aβ and glutamate.

Whereas Shimohama and Kihara (67) view stimulation of α7 nAChRs as protective against Aβ, Dineley and associates (13) provided indirect evidence that α7 nAChRs serve as receptors for $A\beta_{1–42}$. These investigators used hippocampal slice preparations from APP transgenic mice and demonstrated that $A\beta_{1–42}$ is coupled to the mitogen-activated protein kinase (MAPK) cascade via α7 nAChRs. Interestingly, unlike brains of AD patients, those mice showed a significant upregulation (20-fold) of nAChR at an age of 20 months. This suggests that Aβ peptides *in vivo* chronically activate α7 nAChR. Whereas the target nAChR for therapy in AD is the α7 nAChR, Dineley et al. (13) proposed that antagonists selective to α7 nAChRs would assuage the MAPK signaling derangement.

MODELING THE COMPLEXITY OF PRECLINICAL DATA

From the pharmacology reported above, most of the results can be ascribed to a cholinomimetic effect of galantamine as a consequence of its action on AChE and modulation of nAChRs. However, especially for the *in vivo* experiments, it is very difficult to attribute the different pharmacology to either of the two modes of galantamine's action. As both the AChE and the nicotinic physiology are present, those two systems interact with each other. In addition, getting a quantitative sense of real interaction between these two modes of action is difficult. Indeed it is close to impossible for a scientist to keep track of all quantitative data on each of those subsystems so as to evaluate the relative contribution and possibly, the synergistic effect.

It is important to conceptualize the contribution of each of the AChE inhibition and allosteric modulation effects to the total pharmacology. Therefore, since the molecular interactions between galantamine and its various targets can be described by means of physico-chemical equations, a computer model was created using all available quantitative data (Fig. 4). This model uses anatomical, neurophysiological and neuropathological data to develop a model for the cholinergic synapse in a patient with AD. The model then introduces the known pharmacology of galantamine both towards the AChE enzyme and its interaction with the nAChR. The model is further based on a detailed description of all kinetic states of the α4β2 and the α7 nAChR (including the open, desensitized, and active states). Finally, the aspect of cholinergic action potential firing is introduced. Using this simulation, it has been shown that there is a small synergistic effect of the two modes of action with regard to the cholinergic neurotransmission (17). Such an approach can help explain in quantitative terms galantamine's unique combination of the two modes of action with their synergistic effects.

When extending the computer model to include the interaction between the cholinergic neurotransmission and other neurotransmitter systems, such as the dopaminergic system, the effects of galantamine on other neurotransmitter levels can be assessed. As dopamine

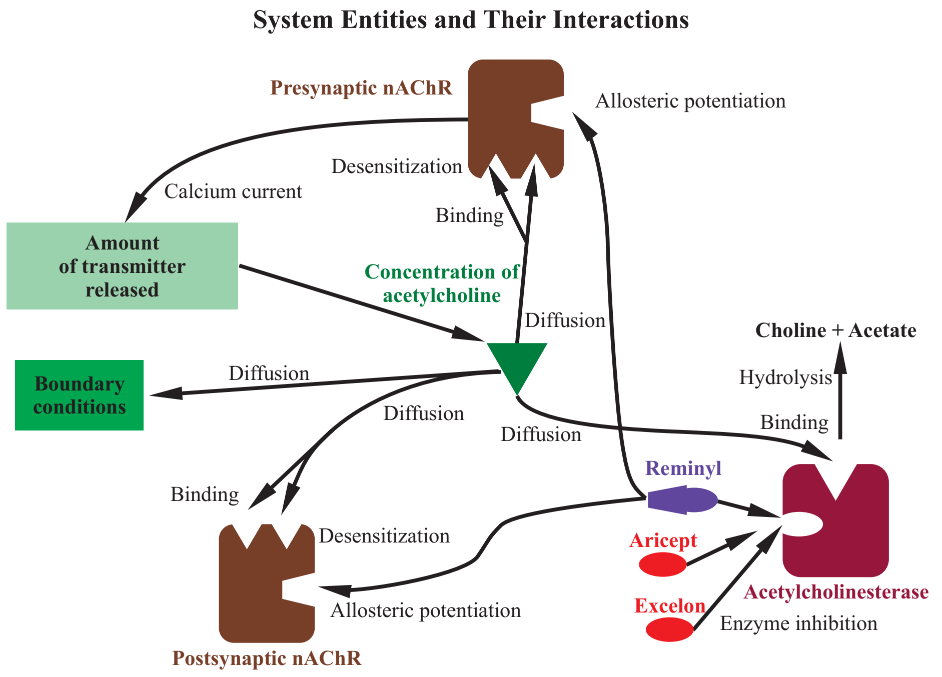

Fig. 4. Flow chart of the Virtual Cleft, illustrating the major entities and interactions taking place in the cholinergic synapse of a living AD patient. Major entities are presynaptic α7 nAChR, postsynaptic α4β2 nAChR and the acetylcholinesterase enzyme AChE. Interactions include binding and activation by the endogenous agonist acetylcholine (ACh) and binding and potentiation by Reminyl® of both pre- and postsynaptic nAChRs; diffusion, binding and subsequent hydrolysis of ACh by AChE; and pharmacological inhibition of AChE by Reminyl®, Aricept®, or Exelon®. Furthermore, a very detailed transition kinetic scheme at the level of the α4β2 nAChR and α7 nAChR has been developed to document the transitions from resting to open and desensitized states and the effects of agonists and potentiating ligands on these transitions (data from ref. 17).

is involved in aspects of concentration, depression and anxiety, this then helps explain some of the positive beneficial effects of galantamine on non-cognitive scales in human patients (68). The predictions of the computer model can be tested in *in vitro* slices.

Building a model *in silico* also formalizes thinking about the pharmacology and often identifies key knowledge gaps. As a consequence the model can be used to guide new experiments with much more relevant outcomes.

In the particular case of the allosteric modulation by galantamine, a key issue described by the model is the application speed of the endogenous ligand acetylcholine in many experimental systems, such as oocytes, neuronal *in vitro* systems and recombinant cell lines such as HEK293 cells. Unlike in realistic *in vivo* situations, no AChE enzyme is present to hydrolyze the endogenous acetylcholine. Current state of the art technology provides application speed of about 100 to 200 ms, much longer than *in vivo* situations where the acetylcholine is present for only about 1 ms, due to the hydrolysis by powerful AChE. nAChRs are very sensitive to desensitization (often arising with time constants in the few milliseconds range). As a consequence, allosteric modulatory effects can often be masked by desensitization and resensitization processes during the relatively long application times. In addition, using the full transition scheme for all states of the receptor, the model predicts a different outcome for the allosteric effect when using a co-application approach vs. the more clinically relevant pretreatment approach. This again emphasizes the difficulty of comparing different experimental setups. Using a computer simulation to address the complex interaction between different subsystems is the only approach that can keep track of all the subsystems and their interactions in a quantitative way.

SUMMARY AND CONCLUSIONS

Allosteric modulators of nicotinic acetylcholine receptors are drugs that interact with the receptors through binding sites that are distinct from those for acetylcholine and nicotinic agonists and antagonists. Because they are not directly involved in the neurotransmission process they affect, allosteric modulators typically do not induce compensatory processes that the agonists and antagonists induce. Of the compounds that have been identified as allosteric modulators, the only molecule approved as an APL (in Europe) is galantamine (Reminyl®). As a prototypical APL approved to treat AD, the focus of this review has been on pharmacological and preclinical studies of galantamine.

Galantamine hydrobromide is a phenanthrene alkaloid similar to codeine, which can be isolated from a variety of plant sources, including the European daffodil or common snowdrop. The functional pharmacodynamic properties of galantamine were known in the medieval times in terms of their effect on healing. In the twentieth century, a number of *in vitro* studies have shown that galantamine is a reversible, competitive inhibitor of AChE. However, the affinity for the AChE is quite modest. The potency to act as an allosteric potentiating ligand was independent of galantamine's ability to block AChE, suggesting a second independent pharmacological mechanism. The site of galantamine's effect on the nAChR is different from the acetylcholine binding site, pointing towards an allosteric potentiating effect. However, the final proof of the allosteric effect awaits development of better and more specific antibodies against the putative allosteric site on the α-subtype of the nAChR.

In vivo studies using non-lesioned animal models have shown that galantamine prolongs the activity of neuronally released acetylcholine and increases brain acetylcholine levels after systemic administration. The effect of galantamine in facilitating learning and memory in young and older rabbits is dramatic using eyeblink classical conditioning — a form of associative learning that is severely impaired in human AD. Galantamine has also ameliorated behavioral deficits induced by brain lesions in animal models.

Galantamine increases the efficacy of nAChRs, in particular the efficacy of α7 nAChRs. This feature of galantamine indicates that it has the potential to be neuroprotective against Aβ. Data demonstrating the neuroprotective effect of galantamine has not been yet published. However, experiments with nicotine and with an α7 nicotinic agonist (GTS-21) have demonstrated a neuroprotective effect against Aβ (67). By virtue of its ability to increase the efficacy of α7 nAChRs, galantamine is likely to have neuroprotective effects. A competing perspective is that antagonists to α7 nAChRs would be more likely to protect against Aβ (13). Additional research is needed to determine galantamine's role in neuroprotection.

The fact that competing data yield exactly opposite predictions for the efficacy of galantamine as a neuroprotective drug emphasizes the need for computer simulations and models of drug-neurotransmitter and drug-neuropathology interactions. The complexity of the systems addressed is so great that *in silico* models are useful in systematic calculations of interactions. We envision utility for these models in studying the mechanism of action of allosteric modulators, such as galantamine. We also envision their potential usefulness in the treatment of AD.

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