CHRONIC TREATMENT OF OLD RATS WITH DONEPEZIL OR GALANTAMINE: EFFECTS ON MEMORY, HIPPOCAMPAL PLASTICITY AND NICOTINIC RECEPTORS

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Abstract—The function of the cholinergic system is known to change during normal aging and in pathological conditions such as Alzheimer’s disease. The present study was designed to assess, within the same group of old animals, the behavioral, electrophysiological and neurochemical effects of chronic treatment with agents that increase the function of the cholinergic system through both muscarinic and nicotinic mechanisms. Doses were determined that produced 60% cholinesterase inhibition by donepezil and galantamine for the old rats. This was chosen to be analogous to therapeutic levels achieved for treatment of human Alzheimer’s disease patients with these agents. Because of the well-known age-related changes in spatial memory and hippocampal synaptic plasticity, spatial working memory in the radial eight-arm maze and hippocampal long-term potentiation induction and decay, as well as nicotinic receptor density and affinity, were measured in old rats implanted with minipumps that delivered donepezil, galantamine or saline. There was no effect of drug treatment on baseline synaptic transmission or on the threshold or magnitude of long-term potentiation induction. Both drug treatment groups, however, showed significantly extended long-term potentiation decay times at the perforant path–granule cell synapse over the saline control animals, as measured during the week following induction. Both drugs also elevated the number of nicotinic receptors within the hippocampus and neocortex.

This is the first demonstration of cholinergic modulation of synaptic plasticity over the time-course of days. Furthermore, the durability of long-term potentiation was significantly, positively correlated with nicotinic receptor binding in the hippocampus. Chronic treatment with donepezil or galantamine had no significant effect on a well-learned spatial working memory task on the radial maze.

These data suggest that the therapeutic doses of cholinesterase inhibitors used to treat patients with Alzheimer’s disease may have effects on neurophysiology and neurochemistry that are close to the threshold for producing detectable behavioral improvements. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: long-term potentiation, spatial memory, AChE inhibitors, aging.

During normal aging, and in Alzheimer’s disease (AD), the brain develops a complicated pattern of degenerative changes that include, but are not limited to, the degeneration, atrophy or dysfunction of specific cell groups that underlie the normal neural mechanisms associated with higher cognitive function.11,23,25 AD is associated with a profound alteration of neural mechanisms associated with normal aging and AD in humans.3,7,25,33,46

Recognition of the role of cholinergic dysfunction in the memory and attentional impairments associated with AD has led to the development of drugs that selectively enhance cholinergic function by inhibition of the cholinergic catabolic enzyme, acetylcholinesterase (AChE). Many drugs have been designed to enhance cognitive function in AD patients by targeting this enzyme, including, tetrahydroaminoacridine (Tacrine, Parke-Davis), donepezil (Aricept, previously E2020, Pfizer) and galantamine. Tacrine has been reported to provide only limited clinical benefit,15 while donepezil has produced significant cognitive improvements in some AD patients.3,51 Donepezil is a reversible, selective, non-competitive AChE inhibitor with a long plasma half-life of approximately 70 h.40 Unlike Tacrine,48 donepezil treatment has not been associated with hepatotoxicity.34 Galantamine (Inteligen, Janssen) is a recently developed AChE inhibitor17 that may offer some clinical benefit.10,45,50,51 The alkaloid galantamine was first isolated from the Caucasian snowdrop Galanthus woronowi32 and was subsequently determined to occur naturally in other plants of the Amaryllidaceae family.20 Galantamine is a selective competitive AChE inhibitor that is approximately 50 times more effective against human AChE than against human butyrylcholinesterase at therapeutic doses.16,18,44 This feature is advantageous, since there is no direct evidence that butyrylcholinesterase is involved in any cognitive function and the inhibition of butyrylcholinesterase may contribute to the peripheral toxicity of most AChE inhibitors.29

AChE inhibitors, such as galantamine and donepezil, may have a dual action at the cholinergic synapse. First, they would elevate the level of available acetylcholine to act upon postsynaptic muscarinic receptors. In animal studies, galantamine significantly increased cortical acetylcholine levels by 37%.43 Second, galantamine and other AChE inhibitors may act as agonists at nicotinic receptors2,9,36 and enhance the release of acetylcholine via a nicotinic mechanism, particularly under conditions of impaired cholinergic function.24 Consistent with this action, chronic treatment with AChE inhibitors such as donepezil or galantamine or nicotinic receptor agonists1,42 significantly elevated the level of [3H]nicotine binding sites in the cortex of mice.19,31

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Abbreviations: AChE, acetylcholinesterase; AD, Alzheimer’s disease; ANOVA, analysis of variance; EPSP, excitatory postsynaptic potential; LTP, long-term potentiation.
The increase in hippocampal nicotinic receptors following a 10-day chronic treatment with a nicotinic agonist was correlated with an improved learning ability in young rats.1

The response by nicotinic receptors to the presence of nicotinic agonists may represent either a desensitization37 or a reduction in receptor turnover.30 The change in receptor number probably occurs at the post-transcriptional stage of production, since the levels of mRNAs that encode the subunits of this receptor are unchanged after chronic treatment with nicotinic agonists.39 The actions of galantamine, tacrine and other AChE inhibitor drugs may be mediated by binding to the alpha subunits of the α2β4 nicotinic receptor.36,41 Serotonin may act as an endogenous ligand at this site in the brain to modulate acetylcholine release.5

The present study was designed to compare systematically the effects of chronic treatment with galantamine, donepezil or saline on spatial cognition, hippocampal plasticity and nicotinic receptor binding in old rats. It is well documented that old, spatial memory-impaired rats have altered long-term potentiation (LTP)8,22 compared with their young, memory-intact counterparts.5,12,13,26 In fact, the durability of LTP in the hippocampus and spatial memory on the circular platform4,5 and radial eight-arm maze42 tasks are significantly correlated in rodents. Furthermore, it is known that functional cholinergic transmission is diminished in each of the three hippocampal subfields of old rats.38 The hypothesis tested in the present study was that hippocampal plasticity and behaviors that are dependent on the hippocampus will be altered in old animals given chronic treatment with agents that facilitate the cholinergic system. The current study was divided into two phases. The first phase determined the individual daily doses of galantamine and donepezil that were necessary to produce a 60% decrease in AChE activity within the brains of old rats. This dose was chosen because it is the level of inhibition achieved in AD patients given therapeutic doses of AChE inhibitors.17 The second phase involved the study of the behavioral, electrophysiological and biochemical consequences of a 42-day infusion of each AChE inhibitor using the dose determined in the first phase.

EXPERIMENTAL PROCEDURES

Phase I: dose determination

The purpose of the first phase of these experiments was the determination of the dose of donepezil and galantamine that would produce a 60% inhibition of AChE activity in the aged rats. This dose was then used in the electrophysiological and behavioral studies. The general outline was as follows: 27 male F344 rats (22 months, Harlan Sprague–Dawley) were anaesthetized and implanted subcutaneously (lateral and dorsal to the spinal column and over the lower abdomen) with Alzet osmotic minipumps (model 2ML4, 2.5 μl/h X 32 days). Twelve minipumps were prefilled with galantamine (to deliver 0.1, 0.5, 1.0 or 2.0 mg/kg per day), 12 minipumps were prefilled with donepezil (to deliver 0.1, 0.5, 1.0 or 2.0 mg/kg per day) and three were prefilled with 0.9% saline. Donepezil and galantamine (Research Biochemicals, Inc., Natick, MA) were dissolved in saline. We determined that three rats per group was sufficient for this phase of the study due to the low variability in AChE inhibition produced by these two drugs in preliminary studies. Although we determined the level of AChE inhibition two weeks after pump implantation, our preliminary studies using young animals indicated that enzymatic inhibition reached maximal levels within three days and remained inhibited for at least 37 days (data not shown).

Two weeks after surgery, when steady-state levels of the drug had been achieved and maintained, the rats were killed and the brain of each rat was removed and prepared for neurochemical analysis of AChE activity according to the colorimetric method of Ellman et al.14 using a Beckman DU 640 spectrophotometer equipped with a Peltier temperature controller. The frontal cortex and hippocampus were homogenized in Hall’s buffer (pH 7.3). The incubation solution contained the butyrylcholinesterase inhibitor tetraisopropyl pyrophosphamide at a final concentration of 100 μM in order to measure AChE activity specifically. Lineweaver–Burk double reciprocal plots were produced by varying the concentration of the substrate acetylthiocholine from 0.03 to 0.5 mM (see Fig. 1). These plots were used to determine the V_max, K_m and K_i for donepezil, a selective, non-competitive inhibitor of AChE activity,16 and galantamine, a selective competitive inhibitor of AChE activity.16 The equation shown below was used to determine the IC50 for each drug. C_s is the concentration of the substrate used in the assay. The concentration to produce 60% inhibition of AChE activity, the level of inhibition produced by these drugs in AD patients when used at therapeutic doses,17 was determined by proportion from the IC50 value:

\[ K_i = \frac{IC_{50} + K_m}{K_m + C_s} \]

For the second phase of the experiment, Alzet osmotic minipumps containing the concentration producing 60% inhibition of either donepezil or galantamine were implanted subcutaneously during the same surgical procedure in which the electrodes were implanted for the electrophysiological recording studies (see below).

Phase II: experimental subjects

F344 male, retired breeder rats were obtained from the National Institute on Aging’s colony at Harlan Sprague–Dawley at 22 months of age in batches of six rats (seven replicates, with the seventh batch containing four rats instead of six) for a total 40 rats. The batches of six rats were obtained separately and rats were randomly assigned to each treatment group at the time of arrival. All procedures were conducted in accordance with National Institutes of Health Guidelines for the Care and Use of Animals. Thirty-two rats (11 in each of the saline and galantamine treatment groups, 10 in the donepezil group) finished
the entire Phase II protocol (Table 1) and are included in the data presented here. This number of animals was used to provide adequate statistical power for the electrophysiological component of the entire experiment. Reasons for elimination of eight rats included illness or death (during either the behavior or physiology components), or no electrophysiological responses upon recovery from surgery. The experimenters conducting the behavioral, electrophysiological and neurochemical experiments were blind with respect to the composition of the treatment groups until the data were fully analysed.

Radial arm maze training

Rats were given one-week adaptation to the housing environment and handling after arrival. In the following week, rats were food restricted to achieve approximately 85% of their ad libitum weights, before radial maze training began. Rats were pre-trained to traverse the eight arms of a radial maze for food reward, with all eight arms in the “up” position (i.e. the arm was connected to the center platform and therefore available for entry). By the end of this week, all rats were able to complete several trials within an hour. During the first and second weeks of training, a different procedure was initiated. At the beginning of each trial, rats were given a randomly determined set of four arms from which to obtain reward. After the rat collected reward from the fourth arm, four additional arms were raised (with no delay). The rat completed the trial when food was retrieved from all eight arms. Repeated visits to the same arm were counted as errors. During the third week of training, a 30-s delay was imposed between the fourth and fifth arm choices. As before, the trial ended after the rat obtained food from all eight arms. Following drug treatment, and the week of LTP induction (see Table 1), this same 30-s delay procedure was again used on the old rats for two additional weeks.

Surgery

After the three weeks of radial arm maze training (see Table 1), rats were implanted bilaterally with recording electrodes in the hilus of the fascia dentata and stimulating electrodes in the angular bundle. Briefly, rats were anesthetized with 33 mg/kg Nembutal (supplemented with Metofane as necessary), the scalp was retracted and fascia dentata and stimulating electrodes in the angular bundle.6

Sections of the frontal cortex and hippocampus were homogenized and prepared for analysis according to the method of Nordberg et al.27 Membrane suspensions were incubated with [3H]nicotine (concentration ranged from 2.0 to 100 nM) in 50 mM NaKHPO4 (pH 7.4) buffer, with or without unlabeled nicotine to define specific binding. Data were analysed using the PHARM/PCS 4.2 program (MCS, Philadelphia, PA). Kd and Bmax values were determined following transformation of the data to fit the Rosenthal equation. The data were analysed using Kruskal–Wallis one-way analysis of variance

<table>
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<tr>
<th>Week</th>
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<tr>
<td>1</td>
<td>Radial eight-arm maze training (four-arm/eight-arm procedure, no delay)</td>
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<td>2</td>
<td>Radial eight-arm maze training (four-arm/eight-arm procedure, no delay)</td>
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<tr>
<td>3</td>
<td>Radial eight-arm maze training (30-s delay between choices 4 and 5)</td>
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<td>4</td>
<td>Surgery for LTP electrodes and Alzet pumps (galantamine, donepezil, saline), recovery</td>
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<tr>
<td>5</td>
<td>Baseline synaptic transmission recording in hippocampus and stimulus input/response output determination on day 5 of this week</td>
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<td>6</td>
<td>LTP induction and decay, and stimulus input/response output determination following drug induced</td>
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<td>7</td>
<td>Radial eight-arm maze retest at 30-s delay</td>
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<tr>
<td>8</td>
<td>Radial eight-arm maze retest at 30-s delay</td>
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<td>9</td>
<td>Killing for nicotinic receptor binding assays</td>
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Recording baseline synaptic transmission

In the week following surgery (Table 1), rats were attached to the electrophysiological recording system, while they were free to move about their home cage. Stimulus pulses (biphasic 100 μs) were delivered through the stimulating electrode while recording evoked responses on the recording electrode. The perforant path–granule cell evoked response was amplified 100 times, filtered between 3 Hz and 3 kHz (half-amplitude attenuation), and recorded for off-line analysis using an 80386 computer and Workbench software (DataWave Corp., Broomfield, CO). On the first four days of the week, rats were brought into the recording room and 30 responses were evoked (one every 10 s) at a stimulus intensity of 200 μA. On the fifth day, a stimulus input/response output series was conducted as follows. As the rat sat quietly in its home cage, five perforant path–granule cell evoked responses were recorded (10-s intervals), at the following stimulus intensities: 150, 200, 250, 300, 400, 450 and 500 μA. Averages of the response at each intensity were then calculated for each rat. This stimulus input/response output measurement was repeated at the 24-h time-point following LTP in the next week (Table 1).

Long-term potentiation induction and decay

On the first day of the week following baseline recording (Table 1), LTP was induced using the following procedure: at 60-min intervals LTP was induced at increasing stimulus intensity levels (200, 250, 300, 350, 400 and 450 μA). The high-frequency stimulation parameters used were 10 repetitions (at 10-s intervals) of 25-ms, 400-Hz stimulus bursts. This procedure was used to allow assessment of possible differences in LTP threshold between treatment groups. The changes in the evoked response were then monitored during the subsequent days of the week, to evaluate LTP decay rates in each group. In these sessions, 30 low-frequency (once per 10 s) test stimuli were delivered at 200 μA stimulus intensity. These low-frequency test stimuli (the same as used during baseline testing) do not, themselves, cause modification of synaptic transmission. The fractional change in the excitatory post-synaptic potential (EPSP) following LTP induction was calculated as follows: (EPSP amplitude after LTP – EPSP amplitude before LTP)/EPSP amplitude before LTP.

Nicotine receptor binding studies

Thirty-five days after the osmotic minipumps were implanted, each rat was lightly anesthetized with metofane gas and killed by decapitation. According to specifications provided by the manufacturers (Alza Corp., Palo Alto, CA), the 2ML4 minipump will contain approximately 2.3 ml and deliver 2.5 ml/h. Therefore, the rats were being administered drug until the day they were killed.

Table 1. Experimental protocol for Phase II (procedures were conducted in seven replicates)
Dose determination

The dose to produce 60% inhibition of AChE activity in brain tissue when galantamine and donepezil were administered by osmotic minipump to aged rats was determined to be 0.695 mg/day for donepezil and 0.277 mg/day for galantamine (see Fig. 1 for Lineweaver–Burk double reciprocal plots used for this determination). These were the precise doses that were contained in the osmotic minipumps, assuming that the pumps delivered 2.5 μl/h, that were implanted into the behaviorally tested rats. Although donepezil is a more potent inhibitor of AChE activity than galantamine, a higher dose was required in the present study because it achieves a lower plasma concentration at therapeutic doses and it has a much higher affinity for plasma proteins. These doses are smaller than those typically used in young rats because they have a greater inhibitory effect upon AChE activity in aged rats.

Baseline synaptic transmission

Repeated measures ANOVA showed a significant effect of Stimulus Intensity (F(2,203) = 90.33, P < 0.0001), but no significant effect of Drug Treatment (F(2,203) = 0.309, P > 0.05) on the amplitude of the EPSP at eight stimulus intensities (Fig. 2A) obtained before LTP induction; nor was there a significant difference in population spike size between treatment groups (P > 0.05). Thus, there was no effect of galantamine or donepezil on the amplitude of the perforant path–granule cell evoked response.

Long-term potentiation induction and decay

Repeated measures ANOVA showed a significant effect of LTP-inducing stimulation on EPSP amplitude 2 min following the high-frequency protocol at increasing intensities (F(5,145) = 6.225, P < 0.0001), but no significant effect of Drug Treatment (F(2,145) = 0.107, P > 0.05) on EPSP amplitude over the six stimulus intensities (Fig. 2B). This suggests that there is no drug treatment effect on the threshold or absolute magnitude of LTP immediately following induction protocols. When the amplitude of the EPSP was followed over hours and days following the LTP-inducing protocol, however, there was a significant effect of Drug Treatment (F(2,210) = 7.574, P < 0.001), but no effect of Time (F(6,210) = 1.909, P > 0.05; Fig. 3). Post hoc Student–Newman–Keuls tests revealed that the saline group decayed significantly faster than did the two drug treatment groups (P < 0.05), while the drug groups did not differ from each other (P > 0.05).

Radial arm maze

Repeated measures ANOVA showed a significant effect of Days of Training (F(2,406) = 4.781, P < 0.0001), but no significant effect of Drug Treatment (F(14,406) = 0.476, P > 0.05) on percentage errors of total choices made during the three-week training period on the radial eight-arm maze (Fig. 4A). This indicates that there were no behavioral differences between groups before drug treatment was initiated. The fractional change in errors between the last day of pre-drug training on the 30-s delay condition and the 10 days of 30-s delay testing following drug administration (post-drug – pre-drug/pre-drug) is shown in Fig. 4B. Repeated measures ANOVA showed a significant effect of Days of Training (F(9,261) = 6.566, P < 0.0001), but no significant effect of Drug Treatment (F(2,261) = 0.833, P > 0.05) on fractional change in errors.

Nicotine receptor binding

The B_max values determined from the saturation experiments using frontal cortex and hippocampal membranes from control and drug-treated rats are shown in Fig. 5. Chronic therapy with both drugs significantly elevated the
**DISCUSSION**

Donepezil and galantamine significantly elevated the density of nicotine binding sites in both the hippocampus and prefrontal cortex of the treated rats, with the effect of donepezil being particularly pronounced (Fig. 5). In the present study, LTP induction threshold and final magnitude was not modified between treatment and control groups. Probably the most striking finding of the current study, however, was that LTP decay was extended in both the donepezil and galantamine groups, as compared with the saline control group (Fig. 3). Furthermore, there was a significant correlation between nicotinic receptor number in the hippocampus and maintenance of LTP, i.e. the greater the residual LTP remaining five days after induction, the greater the number of nicotinic receptor binding sites. These data are consistent with the interpretation that increased functionality of the hippocampal nicotinic cholinergic system positively modulates the durability of LTP in this structure.

The elevation in nicotinic receptor density was not as large as has been reported previously when using donepezil or galantamine.19,31 In the current study, we found only a 47% increase in B max, compared with about a 65% increase after a four-week treatment with donepezil in another study.31 The smaller increase in nicotine binding site density might be because we used aged animals in the present study, while previous studies used young animals.1,31,41 In addition, the smaller increase in the number of nicotinic sites might also be related to the fact that these rats had a lower level of AChE inhibition within their brains as compared to these previous studies. The rationale for using a lower drug dose in the present study was to reproduce the level of AChE inhibition achieved by standard

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**Fig. 3.** Mean ± S.E.M. fractional change in EPSP amplitude after the six sessions of LTP-inducing stimulation (measured at 200 μA stimulus intensity). There was no difference in the magnitude of LTP induction between treatment groups (P > 0.05). The 2-min time-point is the same as shown for the 200-μA stimulus intensity in Fig. 2B; however, the LTP magnitude in the donepezil and galantamine treatment groups decayed more slowly over five days as compared to the saline-treated old rats (P < 0.05). Numbers of rats: saline, n = 11; donepezil, n = 10; galantamine, n = 11.

**Correlations**

Because there were no significant treatment effects on behavior, we could not continue with the planned correlation analysis between the physiology or neurochemistry and behavior. The correlation between the magnitude of the EPSP after drug treatment (before LTP) with B max, nicotinic receptor binding did not reveal a significant relationship; however, there was a statistically significant positive relationship between the durability of LTP and nicotinic receptor binding (P < 0.05; Fig. 6).
pharmacotherapy in AD patients. The results of the current study suggest that the level of AChE inhibition, i.e. about 60%, that is produced by standard drug protocols for AD therapy is certainly enough to increase nicotinic receptor number and hippocampal plasticity mechanisms, but may still be below the threshold for producing striking behavioral improvements.

In previous studies, the change in nicotine binding sites correlated with improved performance in the Morris swim task. Although one may have predicted that radial maze performance would have been affected similarly, it is possible that the spatial working memory behavior measured in this task may not be as sensitive to nicotinic receptor changes as is the standard reference memory problem in the swim task. In contrast, the relationship between nicotinic receptor binding and behavior in the swim task was found using young rats. The present study is the first examination of this relationship in old rats. Thus, it is possible that the reduced effect on nicotinic receptor binding observed in our old rats was responsible for the non-significant effect on spatial behavioral performance in the present study. In addition, the drug-induced increase in nicotinic receptor number was greater in the cortex than in the hippocampus. Therefore, the absence of an effect of drug therapy upon performance in the hippocampal-dependent radial eight-arm maze task might be explained by relatively minor changes in the hippocampus compared with the cortex. If true, then the performance might have been enhanced significantly in tasks that are more sensitive to manipulation of neocortical cholinergic function, such as attention tasks. Taken together, the electrophysiological, biochemical and behavioral results of this study suggest that the 60% inhibition of AChE targeted in human patients with AD may be very close to the threshold for producing a significant cognitive benefit. Thus, future drugs that can both increase nicotinic receptor number throughout the old brain, but are associated with fewer peripheral side effects, should produce a more reliable therapeutic benefit.

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