The cholinesterase inhibitor, phenserine, improves Morris water maze performance of scopolamine-treated rats

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Abstract

Male Fischer-344 rats (n = 38) at 5 months old were tested in a Morris water maze to determine if treatment with the cholinesterase inhibitor, phenserine (PHEN), would overcome a learning impairment induced by scopolamine (SCOP), a muscarinic cholinergic receptor antagonist. Each rat was randomly assigned to one of five groups to receive two intraperitoneal injections 60 and 30 min, prior to testing, respectively, as follows: (1) saline-saline (SAL); (2) saline-1.0 mg/kg (SCOP); (3) 2 mg/kg PHEN-SCOP (PHEN2); (4) 4 mg/kg PHEN-SCOP (PHEN4); and (5) 1 mg/kg PHEN-SAL (PHEN1). Maze testing occurred across 5 days with 4 days of acquisition trials (4 trials per day) and a fifth day consisting of a single 120 sec probe trial. PHEN1 and SAL were combined into one control (CON) group for purposes of statistical analysis for both acquisition and probe trials as comparison of the two groups revealed that they did not significantly differ on any measure. SCOP-treated rats were significantly impaired compared to CON in learning the location of the submerged platform as measured by latency to locate the platform and the distance traversed to find the platform across days of testing. The PHEN4 group had significantly lower latencies and traveled a shorter distance to reach the submerged platform when compared to SCOP on the fourth day of trials while the PHEN2 group traveled more directly to the submerged
platform but did not have shorter latencies than the SCOP group. For probe trials, CON rats swam closer to the target area (a measure of proximity to the removed platform) than did all other groups, and the PHEN4 group swam in an area more proximate to the target area than did the SCOP-treated group. These findings demonstrate the ability of this drug to improve learning when cholinergic function has been impaired in a spatial memory task.

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Introduction

Alzheimer’s disease (AD) is an age-related neurodegenerative disorder whose most notable symptom is progressive memory loss followed by general cognitive decline that is affecting an increasingly large number of older persons (Giacobini, 2002; Sano, 2002). AD results from neurodegeneration characterized by deposition of amyloid plaques, development of neurofibrillary tangles, inflammation, and neuronal loss in specific forebrain regions (Sambamurti et al., 2002). Although multiple neurotransmitter systems appear to be affected in AD, degeneration and functional impairment in the cholinergic system has received the greatest amount of research attention (Giacobini, 2003). Neuronal loss in the basal forebrain, particularly in septohippocamal acetylcholinergic (ACh) systems that are involved in learning and memory processes, constitute a pathological hallmark of AD.

To address this cholinergic dysfunction, a major therapeutic strategy for the treatment of AD has been to develop compounds that can increase ACh at the synapse. The development of cholinesterase inhibitors (ChEIs) has been the general focus of this strategy (Darvesl et al., 2003; Giacobini, 2002; Greig et al., 1995) with acetylcholinesterase inhibition (AchEI) receiving most attention. Recently, butyrylcholinesterase (BchEI) and its role in Alzheimer’s disease, particularly in the reduction of β-amyloid deposition has received attention (Giacobini, 2002; Greig et al., 2002). Currently ChEIs represent the only class of drugs approved for clinical use for AD in the U.S. (Grutzendler and Morris, 2001), with the recent exception of memantine, which is an NMDA glutamate receptor antagonist (Reisberg et al., 2003). A number of AChE inhibitors (AChEI) are presently in use, in clinical trials or under basic investigation. Cognex, also known as (aka) tacrine, is a non-selective acetyl-and butyrylcholinesterase inhibitor with a duration of action of approximately 2–3 hr. This drug has been minimally effective in alleviating the cognitive deficits associated with AD and has a high incidence of hepatotoxicity (Qizilbash et al., 1998; Davis et al., 1992). Newer generations of AChEI have been developed and several, such as eptastigmine (aka heptyl-physostigmine; Braida et al., 2000; Braida and Sala, 2001), galanthamine (Corey-Bloom, 2003) and ganstigmine (Jhee et al., 2003). However, eptastigmine and ganstigmine have been dropped from clinical trials. Galanthamine, in addition to its short course of action, causes nausea and vomiting in many patients. The FDA has approved the AChEI E2020, aka Aricept or donepezil, for use in AD patients (Nobili et al., 2002). All of the above-mentioned drugs have had effects on cognition of a similar magnitude.

Phenserine, (−)–phenylcaramoyleseoline (L)-tartrate (phenserine), is a physostigmine derivative that has selective, long-acting and brain-directed AChEI activity (Greig et al., 1995). This compound has been effective in attenuating scopolamine-induced and age-related memory impairments in rats in a 14-unit T-maze (Ikari et al., 1995; Iijima et al., 1993). Phenserine is currently in multicenter phase 3 clinical trials, having demonstrated improvements with minimal side effects in cognitive performance in AD.
Preclinical work in the development of phenserine relied primarily upon one model of learning, the Stone 14-unit T-maze (Patel et al., 1998). This task requires the rat to learn a response pattern of left-right position discriminations to avoid the onset of a mild footshock. The objective of the current study was to investigate the efficacy of PHEN in another learning model that taxes spatial memory abilities, specifically the Morris water maze (Morris, 1984). The water maze paradigm has proven highly useful for assessing cholinergic dysfunction and has been used for assessing the efficacy of ChEIs (McNamara and Skelton, 1993). Similar to past demonstrations in the 14-unit T-maze, we examine the ability of phenserine to attenuate a learning impairment induced by scopolamine, a muscarinic cholinergic receptor antagonist.

Methods

Subjects

Thirty-eight male Fischer-344 rats 4 months old were shipped to the GRC from the NIA colony maintained by Harlan Sprague-Dawley (Indianapolis, IN). The rats were housed two per cage in a vivarium maintained at 21 °C. Food (NIH-07) was provided ad libitum and water was provided via an automated watering system. The vivarium was maintained on a 12-hour light-dark cycle with lights on at 6:00 am EST. Behavioral testing commenced when the rats were 5 mo old, and all testing occurred during the light cycle.

Drug administration

Each rat was randomly assigned to one of 5 groups to receive a set of two intraperitoneal injections 60 and 30 min prior to Morris water maze (MWM) testing on each of five testing days. Sixty minutes prior to MWM testing each rat received an injection of either physiological saline (SAL) or 1, 2, or 4 mg/kg dose of phenserine (PHEN1, PHEN2, or PHEN4) and 30 min later received an injection of either SAL or 1 mg/kg scopolamine (SCOP) to comprise the following groups: saline-saline (SAL; n = 8); saline-1 mg/kg SCOP (SCOP; n = 7); 2 mg/kg PHEN-1 mg/kg SCOP (PHEN2; n = 9); 4 mg/kg PHEN-1 mg/kg of SCOP (PHEN4; n = 9); and 1 mg/kg of PHEN-saline (PHEN1; n = 5). All drugs were prepared fresh daily, and physiological saline was used as a buffer. Volume of injection was 1ml/kg.

Apparatus and procedure

A MWM was utilized for all behavioral testing according to a procedure adapted from Frick et al. (1996). A white polyethylene tank 1.8 m in diameter and 0.6 m high, was filled with 35 cm of water. A clear plastic platform was submerged to 1.5 cm below the water surface. A white curtain surrounded the entire maze, and cues were hung on the curtain to facilitate spatial navigation. A camera suspended above the maze was wired to an automated tracking system (Accuscan, Columbus, OH) and was used to track movement and location of the animal in the maze. To facilitate tracking rats were dyed black with color-fast hair dye (Lady Clairol). Music was provided by four speakers located adjacent to the maze during testing to mask external noise.
On day 1 each rat received an acclimation trial in which it was placed onto the submerged platform for 15 sec. The centers of the three quadrants most distant from the submerged platform, were used as start points for each subject, and start points were pseudo-randomly rotated for each rat on each trial. Each day for four consecutive days, each rat was injected according to the schedule described above and then given four trials in the water maze. The position of the rat’s placement for starting each trial was changed daily so that each position was tested at least 4 times over the course of maze testing. The rat was allowed 120 sec to reach the platform on each trial. After 120 sec the rat was guided to the platform. The rat was allowed to remain on the platform for 30 sec followed by 30 sec in a holding cage prior to the next trial. On the fifth day the submerged platform was removed for a probe trial in which each rat was placed in the pool to swim for 120 seconds and search patterns were recorded.

**Statistical analysis**

To analyze learning, the 4 days of acquisition trials were subjected to a one-way ANOVA (drug treatment) with repeated measures (blocks of 4 daily trials) for the latency and distance measures. A separate ANOVA with repeated measures comparing performance of the SAL and PHEN1 groups revealed no significant differences on either measure, so these groups were combined for subsequent analyses resulting in the following groupings for the analyses of acquisition performance: CON (combined SAL and PHEN1), SCOP, PHEN2, and PHEN4.

Fischer’s LSD post-hoc tests were used to evaluate differences in mean scores for groups when main effects were observed. Significance was accepted as p < .05.

Analysis of the probe trial data were conducted on the first 30 sec of the probe trial only, because a bin analysis (Devan et al., 2003) at 30-sec intervals revealed that nearly all of the animals rapidly extinguished any preference for the quadrant where the platform had been located after 30 sec. As in the acquisition analyses, SAL and PHEN1 were combined into one group (CON) for purposes of statistical analysis, since comparisons of the two groups revealed no significant statistical difference.

For the probe trial a proximity analysis was conducted on the distance swim for each group. In this measure, the average distance from the center of the goal location (the removed platform) was measured for each sec of the initial 30 sec in the probe trial, similar to a measure developed by Gallagher et al. (1993), and automatically calculated by the tracking system (Accuscan; Columbus, OH).

A ONE-WAY ANOVA was conducted for the proximity measure. Fischer LSD post-hoc tests were then conducted to determine the locus of the differences observed.

**Results**

Figs. 1 and 2 show the mean distance each group swim to reach the submerged platform at each day, and the mean daily time to reach the submerged platform, respectively. The PHEN1 and SAL groups are shown separately to demonstrate that phenserine alone did not affect performance, but for purposes of statistical comparison the two groups were combined into one group, CON.

A two-way, one repeated measures ANOVA test of the acquisition phase of the experiment showed an overall significant Group effect for both distance swim to the platform $F(3, 34) = 34.98, p < 0.01$ and time it took for the subject to find the platform, $F(3, 34) = 27.25, p < 0.01$. The within subjects measure, Day, also achieved significance for both distance and time measures, $F$’s $(3, 32) = 4.56$ and 9.87,
respectively, indicating that learning had occurred across days of testing, as observed in Figs. 1 and 2. A significant Group by Day interaction was observed for the distance traversed measure, \( F(9, 102) = 2.72, p < 0.01 \), but not for the time required to find the submerged platform, \( F(9, 102) = 1.76, p > 0.05 \).

Post hoc Fischer LSD tests were conducted for the distance measure at each day due to the observed interaction between Day and Group. Results of these post-hoc analyses revealed that the CON group traveled a significantly (\( p < 0.05 \)) shorter mean distance (cm) than all of the other groups and thus swam a more direct path to the submerged platform during each day of trials. PHEN2 and PHEN4 traveled a smaller mean distance than the SCOP group to the submerged platform, but the differences between the PHEN groups and SCOP was significant only on the fourth day of trials (\( p < 0.05 \)).

Because no interaction was observed for the latency to locate the submerged platform measure, Fischer LSD post hoc tests were conducted on the overall mean latency per trial for each group to determine the locus of the difference between groups. These analyses revealed that the CON group differed from each of the other groups (\( p < 0.05 \)) such that the CON group required less time per trial to

![Distance Swam to Platform](image1)

**Fig. 1.** Mean total distance each group of rats swam (cm) to reach the platform across 4 daily blocks of 4 trials in the Morris water maze.

![Latency to Platform](image2)

**Fig. 2.** Mean time to locate the submerged platform for each treatment group across 4 daily blocks of 4 trials in the MWM.
locate the submerged platform. Phenserine did significantly lessen time to find the submerged platform but only in the PHEN4 group compared to the SCOP group ($p < 0.05$).

Analysis of the proximity to the target (location of the removed platform) during the probe trial revealed a significant main effect, $F(3,34) = 7.57$, $p < 0.001$. Basically, in this measure the smaller the number (cm), the more the rat swam in the vicinity of the previously placed, submerged platform. As shown in Fig. 3 the CON and PHEN4 groups swam significantly closer to the target area ($p < 0.05$). CON swam closer to the target area than did all other groups while the PHEN4 swam closer to the target area compared to the SCOP group ($p$’s $< 0.05$). Thus, phenserine did, at least in the PHEN4 group, improve memory for the previous location of the submerged platform, as measured by swimming in proximity to the removed platform during a probe trial, but it did not restore performance to CON levels.

**Discussion**

As in previously reported studies in the 14-unit T-maze (Patel et al., 1998), systemic administration of SCOP impairs learning performance in MWM in young rats. Whereas, a 0.75 mg/kg dose of SCOP consistently impairs performance in the 14-unit T-maze, in the present study, a slightly higher dose 1.0 mg/kg was required to observe consistent deficits in learning performance in the water maze. Systemic phenserine administration (i.p.) improved performance in SCOP-treated rats in the MWM, as hypothesized, more so at a 4 mg/kg than a 2 mg/kg i.p. dose. Moreover, the probe trial data provide additional evidence that phenserine may improve spatial memory, further supporting its utility in the treatment of cognitive decline and memory loss in human conditions such as AD.

With these results, phenserine treatment has now been demonstrated to improve learning performance in two complex learning paradigms, the MWM and the 14-unit T-maze, in the rat by overcoming the deficits induced by SCOP treatment, a muscarinic cholinergic antagonist that has
come to be accepted as a model of memory impairment in mammals. The 14-unit T-maze requires the rat to learn a fixed series of 14 position discriminations that are configured as sequences of left-right turns. Learning in this task does not require visual discriminations and it is likely that the rat simply learns the maze through vestibulo-motor patterns or an orientation of its body within the maze environment. The version of the MWM task used in the present study on the other hand, requires the rat to navigate to a hidden platform using visual cues located on the curtains surrounding the maze. Placement of the rat into the tank at different locations on each trial further increases the demands on the rat to attend visually to its environment, i.e., the location of the cues. Further, the two tasks differ in their motivational demands with 14-unit T-maze studies cited requiring the rat to avoid or escape a mild shock as it traverses the maze and in the MWM the rat must escape from water.

In the present study, the PHEN4 group failed to show strong spatial bias at the level demonstrated by the CON group, but did swim in closer proximity to the target area compared to the SCOP group. Gallagher et al. (1993) had previously introduced this variable indicating that it was a more spatially-selective alternative measure to other dependent variables used to assess age-related cognitive dysfunction in rodents. In the present study, it was anticipated that phenserine would significantly improve spatial learning performance in young animals treated with the muscarinic cholinergic antagonist, scopolamine. This finding confirmed our hypothesis that the phenserine treatment would not only improve acquisition, as observed in the fourth day of acquisition trials for both PHEN2 and PHEN4, but was also confirmed in probe trial performance to further demonstrate that learning had occurred as shown in the spatial bias for the quadrant where the submerged platform had previously been located in the PHEN 4 compared to the SCOP only group.

Brain extracellular acetylcholine levels are elevated following phenserine administration as assessed by in vivo microdialysis in the rat (Greig et al., 2000). Frey et al. (1985) using autoradiography demonstrated that $[^3H]$scopolamine binds to muscarinic receptors in the rat but that this binding in brain regions with documented high muscarinic receptor density, e.g., hippocampus, was slow to label. They interpreted these findings to mean that either tracer accessibility was limited or that endogenous levels of acetylcholine competed off the tracer at the receptor site. The most viable interpretation of the present data based upon the findings and observations in the Greig et al and Frey et al studies cited above would be that phenserine provides higher levels of endogenous acetylcholine through its potent, long-acting cholinesterase inhibition, and that this extracellular pool of acetylcholine then competes with scopolamine at muscarinic cholinergic receptor sites.

Phenserine is among a class of second generation cholinesterase inhibitors that are unlike first-generation natural products such as galanthamine and physostigmine. A group of anticholinesterases, including tacrine, galanthamine, and aricept have already been approved by the FDA in the United States for treatment of AD. The benefits of phenserine appear to be in its duration of action, high brain distribution, and its effectiveness in lessening cognitive effects of disease, without significant side effects including liver toxicity in preliminary clinical assessment. Whether or not these advantages culminate in a more optimal drug for AD treatment is the focus of ongoing phase 3 clinical trials.

The findings reported herein further confirm the ability of phenserine to overcome a scopolamine-induced memory impairment in another complex learning task, the Morris water maze. We have reported in several previous studies that phenserine could overcome a scopolamine-induced learning impairment in shock-motivated 14-unit T-maze, as well as improve the performance of aged rats in the 14-unit T-maze. The implications of this compound for human subjects with age-associated dementia such as AD
appear promising based on results of phase 2 clinical trials, but further confirmation must await the outcome of ongoing pivotal phase 3 trials.

References


