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# Phosphodiesterase inhibition by sildenafil citrate attenuates the learning impairment induced by blockade of cholinergic muscarinic receptors in rats

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**Abstract**

We examined whether treatment with sildenafil citrate (the active compound of Viagra), a cyclic nucleotide phosphodiesterase type 5 inhibitor (PDE5), would reverse the learning impairment induced by cholinergic muscarinic (mACh) receptor blockade [0.75 mg/kg scopolamine HCl, intraperitoneal (i.p.) injections]. Rats were pretrained in a one-way active avoidance of foot shock in a straight runway and the next day received 15 training trials in a 14-unit T-maze. Performance in this maze paradigm requires accurate responding to avoid mild foot shock and has been shown to be sensitive to aging and to impairment in central cholinergic systems. Intraperitoneal (i.p.) injections of scopolamine or saline and sildenafil or vehicle were given 30 and 15 min before training, respectively. The combined treatment conditions were as follows: saline+vehicle (control), scopolamine (0.75 mg/kg)+vehicle, and scopolamine (0.75 mg/kg)+sildenafil (1.5, 3.0, or 4.5 mg/kg). Behavioral measures of performance included deviations from the correct pathway (errors), run time from start to goal, shock frequency, and duration. Statistical analysis revealed that scopolamine impaired maze performance and that sildenafil (3.0 mg/kg) significantly attenuated this impairment in a dose-dependent manner. These results suggest that sildenafil citrate may serve as a cognitive enhancer for therapeutic treatment of cholinergic dysfunction in age-related cognitive decline and Alzheimer's dementia (AD).


*Keywords:* Phosphodiesterase inhibition; Acetylcholine; Muscarinic receptors; Aging; Animal model; Alzheimer's disease; Learning and memory; Cognitive performance; Stone maze; Rats

## 1. Introduction

The cholinergic hypothesis of geriatric memory dysfunction (Bartus et al., 1982) has received considerable empirical support from animal and human studies over the past 2 decades (Bartus, 2000). Consistent with the hypothesis, an important neuropathological feature that appears as an early marker of Alzheimer's disease (AD) is a loss of cholinergic basal forebrain neurons that innervate the neocortex and hippocampus (Auld et al., 2002; Giacobini, 2003), structures essential to cognitive learning and memory (Petri and Mishkin, 1994). Drug development for the treatment of AD has consequently targeted the cholinergic system and produced novel compounds that increase acetylcholine (ACh) activity by inhibiting the enzyme that degrades ACh, acetylcholinesterase (AChE) (Greig et al., 2000; Grutzendler and Morris, 2001), hence improving learning and memory function (Ikari et al., 1995; Patel et al., 1998).

Neurobiological research on the long-term potentiation of synaptic transmission has suggested that glutamatergic NMDA receptors play an important role in synaptic plasticity and learning and memory (Bliss and Collingridge,

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1993; Collingridge and Bliss, 1995; Martin et al., 2000). Furthermore, it has been suggested that the excitatory amino acid system may be responsible for some of the clinical manifestations of AD (Greenamyre and Young, 1989). In support of this hypothesis, the uncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, memantine, was recently approved by the FDA for treatment of moderate to severe AD (Doraiswamy, 2003; Ferris, 2003). At high concentrations, memantine, like other NMDA receptor antagonists, can inhibit mechanisms of synaptic plasticity that are believed to underlie learning and memory. However, at clinically relevant lower concentrations, memantine may preserve or enhance memory in animal models of AD. For example, memantine has been shown to protect against the excitotoxic destruction of cholinergic neurons (Wenk et al., 1997) and may even confer disease-modifying activity by inhibiting the hypothesized excitotoxicity and progressive neuronal loss contributing to the evolving dementia (Rogawski and Wenk, 2003).

Another therapeutic approach to improving learning and memory targets NMDA receptor-dependent signal transduction (Ingram et al., 1994, 1996; Meyer et al., 1998). Activation of NMDA receptors stimulates an influx of calcium that in turn activates multiple cascades. One pathway involves the production of nitric oxide (NO) which is believed to function as a retrograde messenger that stimulates soluble guanylyl cyclase (sGC) in the presynaptic terminal (Garthwaite, 1991; Hawkins et al., 1998). The stimulation of sGC leads to the formation of the second messenger, guanosine 3′ 5′ cyclic monophosphate (cGMP). Increased cGMP levels stimulate further release of glutamate and hence may constitute a presynaptic mechanism contributing to the early phase of long-term potentiation of excitatory neurotransmission and perhaps even some forms of learning and memory (Selig et al., 1996; Bon and Garthwaite, 2003).

There is evidence that cholinergic muscarinic (mACh) and glutamatergic NMDA neurotransmission may interact during complex maze learning (Meyer et al., 1998). Because both neurotransmitter systems may influence the NO/cGMP pathway (Garthwaite and Boulton, 1995) possibly by increasing intracellular calcium levels, targeting this later stage of signal transduction may prove to be a useful therapeutic intervention. Consistent with this idea, some findings show that increasing cGMP via inhibition of cyclic nucleotide phosphodiesterases (PDEs), in particular, PDE type 5 (an enzyme that specifically hydrolyzes cGMP) may have positive effects on attention and memory. In a double-blind balanced cross-over design examining auditory selective attention and verbal recognition memory (Schultheiss et al., 2001), orally administered sildenafil citrate (as the active compound in Viagra), a phosphodiesterase type 5 (PDE5) inhibitor used for the treatment of sexual dysfunction, significantly enhanced auditory-evoked potentials indicative of an increased ability to focus attention and to select relevant target stimuli.

Studies using animal models of learning and memory support the above clinical findings. Baratti and Boccia (1999) reported that sildenafil could enhance performance of mice in an inhibitory avoidance task. Mice given 3 mg/kg intraperitoneal (i.p.) injection of the drug immediately after receiving a mild foot shock in a darkened chamber showed greater avoidance (retention) of this chamber when provided the opportunity to reenter it 48 h later (or even 1 week or 1 month later). Mice receiving this treatment 180 min after the avoidance training did not show enhanced retention 48 h later.

In a more recent study, Prickaerts et al. (2002) demonstrated improved memory performance on an object recognition task in rats treated with sildenafil or another PDE5 inhibitor, vardenafil. The drugs were given orally immediately after exposure to two identical objects. Mnemonic performance was tested 24 h later by presenting two dissimilar objects, the familiar sample and a new object, and measuring object exploration times. All treatments with sildenafil improved object discrimination performance in a dose-dependent manner, with the most effective dose being 3 mg/kg. In the same study, in vitro experiments with rat hippocampal slices showed that levels of cGMP were increased after incubation with the highest concentration of vardenafil, although no changes in cGMP levels were detected after incubation with different concentrations of sildenafil.

Based on the above findings of cognitive improvement, we tested whether sildenafil would reverse a scopolamine-induced impairment of complex maze learning in rats. The use of a cholinergic muscarinic (mACh) receptor antagonist, such as scopolamine, provides a well-established animal model of the cognitive impairment observed in Alzheimer's disease (Bartus et al., 1982; Ingram et al., 1994, 1996; Meyer et al., 1998; Bartus, 2000), and behavioral assessment of learning performance in the 14-unit T-maze has been shown to be sensitive to both mACh antagonism and aging (Spangler et al., 1986, 1988, 1989).

## 2. Materials and methods

All experimental procedures included in the study comply with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and were approved by the Gerontology Research Center (GRC) Institutional Animal Care and Use Committee. Forty-three 3-month-old virgin male Fischer 344 rats weighing ~250–300 g were shipped to the GRC from the NIA colony of Harlan–Sprague–Dawley (Indianapolis, IN). The rats were housed in pairs in large suspended plastic cages located in a movable metal rack in a vivarium maintained at 21 °C and on a 12:12-h light–dark photocycle (lights on 07:00 h EST). Water was freely available via an automated water system, and food (NIH-07) was provided ad libitum. All rats were acclimatized to the vivarium for at least 1 week prior to the start of behavioral testing.

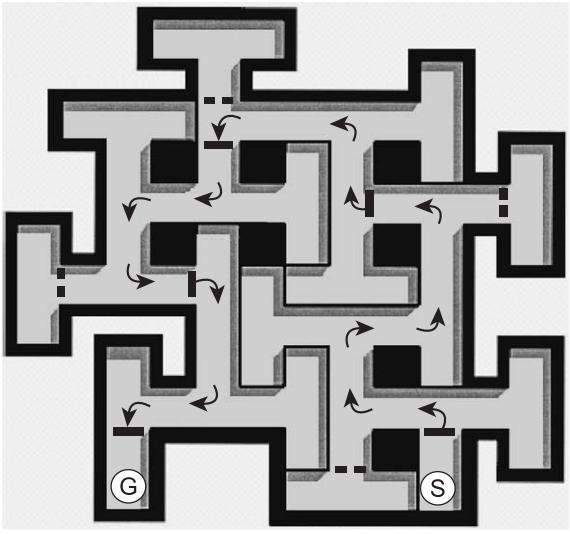


Fig. 1. Schematic diagram showing the configuration of the 14-unit T-maze. Arrows indicate the correct pathway. Errors are defined as any deviation from the correct pathway. S: start box, G: goal box, –: guillotine door, – –: false guillotine door.

As described in detail previously (Spangler et al., 1986), a straight runway 2 m long and constructed of clear plastic was used for pretraining in one-way active avoidance. The runway had a diagonally oriented grid floor comprised of stainless steel bars that were wired to receive scrambled shock from a Coulbourn Instruments (E13-08) grid floor shocker. Black plastic boxes with a guillotine door at the front of the box and a movable rear wall attached by a metal arm served interchangeably as start and goal boxes. A hand-held switch was wired to a clock that automatically initiated a mild foot shock (0.8 mA) once 10 s had elapsed. A hand-held timer was used to measure the duration of foot shock.

Training was conducted in a clear plastic 14-unit T-maze (Fig. 1) that has also been described previously (Spangler et al., 1986). The maze was separated into five sections by guillotine doors that prevented animals from backtracking into previous sections of the maze. Nonfunctional guillotine doors were placed at the entry to each cul-de-sac of the maze to prevent the actual doors from being used as cues to the correct pathway. A switchbox triggered a clock

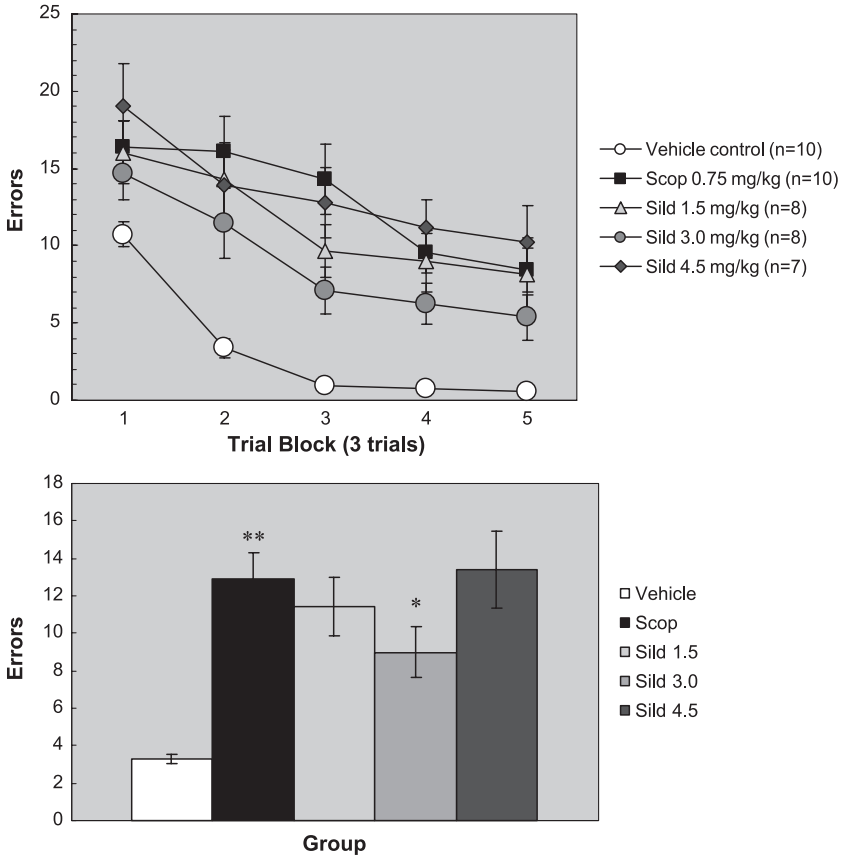


Fig. 2. Effects of scopolamine and sildenafil citrate on mean (±S.E.M.) number of errors during acquisition training in a 14-unit T-maze. Symbol-line graph (top) shows data plotted in blocks of three trials, and bar graph (bottom) shows data averaged across trial blocks. Scopolamine and sildenafil were administered 30 and 15 min prior to maze training, respectively. Rats received i.p. injections of either saline+vehicle ($n$=10), scopolamine 0.75 mg/kg+vehicle ($n$=10), scopolamine 0.75 mg/kg+sildenafil 1.5 mg/kg ($n$=8), scopolamine 0.75 mg/kg+sildenafil 3.0 mg/kg ($n$=8), or scopolamine 0.75 mg/kg+sildenafil 4.5 mg/kg ($n$=7). Symbols: **, significantly different from saline-vehicle group; *, significantly different from scopolamine+vehicle group. See text for description of statistical results. Scop: scopolamine; Sild: sildenafil.

which, when timed out, initiated a second clock to record the duration of shock (maximum of five shocks per trial). Infrared photocells were positioned throughout the maze and were wired in series to a microprocessor that recorded movement through the maze, time elapsed from start to goal, and time between photocell interruptions. Data collected from the photocells were analyzed by the microprocessor which calculated the number of errors (defined as any deviation from the correct pathway) and run time for each section of the maze. Data from the microprocessor were transferred to a personal computer for more detailed analysis as well as storage of raw data. The maze was surrounded by gray-painted walls to reduce extra maze visual cues. Speakers were located under the maze and provided music to mask auditory cues. The maze could be hoisted by motor-driven pulleys to clean the grid floor and reduce the presence of odor cues.

Prior to pretraining in the straight runway, the rats were moved to the maze room in their home cages and allowed to acclimatize for at least 30 min. After this time, the rat was removed from the home cage, placed into one of the black boxes, and the box was placed into the start area over the grid floor. The guillotine door was then raised, the rat was pushed gently forward onto the grid floor by using the metal arm attached to the box, and the door was then closed behind the rat. The manual switch was then initiated, and the rat had 10 s to avoid scrambled foot shock by moving down the straight runway to enter a black box placed over the grid floor at the opposite end of the runway. Failure to move down the runway to gain entry to the black goal box resulted in the initiation of foot shock that continued until either the rat entered the goal box or 120 s had elapsed. When the rat entered the goal box, foot shock was discontinued, and a guillotine door was closed behind the rat. The rat was then removed from the runway for an intertrial interval (ITI) of 90 s. Criteria for successful completion of straight runway pretraining was 13 out of 15 successful avoidances in 10 s or less per trial (maximum 30 trials) of foot shock in one daily session. All rats that successfully met the criterion were trained in the 14-unit T-maze the following day.

Prior to training in the 14-unit T-maze, rats were randomly assigned to one of five drug treatment groups. Control animals received i.p. injections of physiological saline (NaCl 0.9%). Each rat received two i.p. injections, one 30 min prior to maze training of either saline or scopolamine (0.75 mg/kg; Research Biochemicals International, MA) followed by vehicle (30 μl DMSO+90 μl

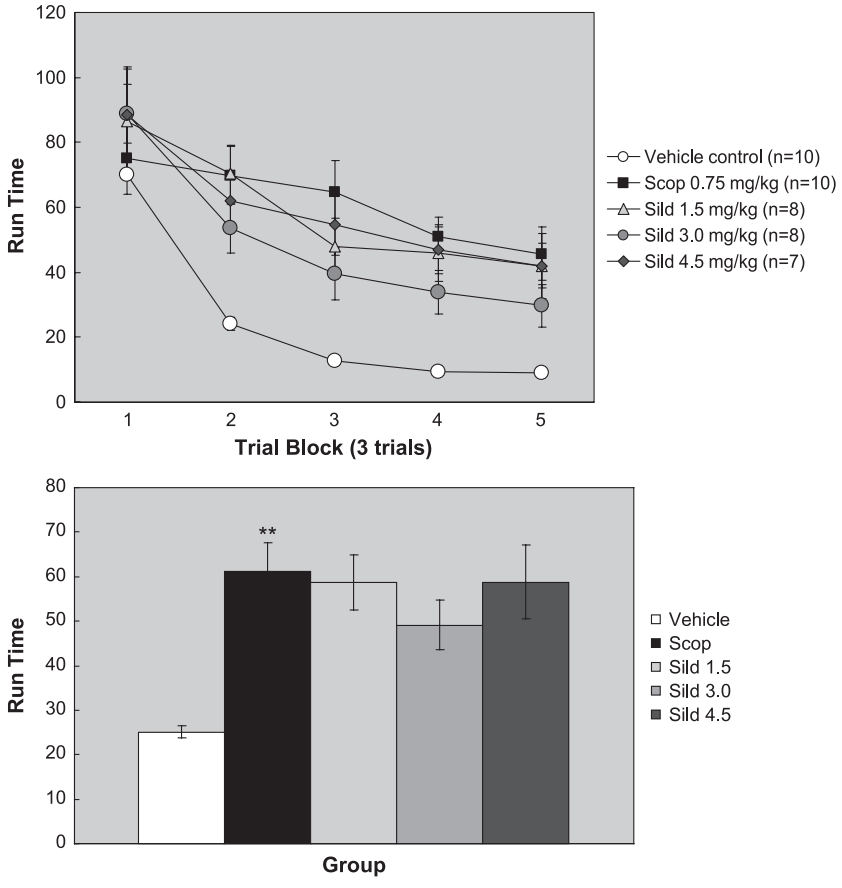


Fig. 3. Effects of scopolamine and sildenafil citrate on mean (±S.E.M.) run time (s) during acquisition training in a 14-unit T-maze. Symbol-line graph (top) shows data plotted in blocks of three trials, and bar graph (bottom) shows data averaged across trial blocks. Symbols: **, significantly different from saline-vehicle group. See text for description of statistical results. Scop: scopolamine; Sild: sildenafil.

Tween 80+0.78 ml of saline) or sildenafil (1.5, 3.0 or 4.5 mg/kg) 15 min prior to training (all drugs were prepared fresh in aqueous solution each day). Treatment groups were as follows: (1) saline+vehicle ($n$=10), (2) scopolamine+vehicle ($n$=10), (3) scopolamine+sildenafil 1.5 mg/kg ($n$=8), (4) scopolamine+sildenafil 3.0 mg/kg ($n$=8), and (5) scopolamine+sildenafil 4.5 mg/kg ($n$=7). Injection volume was ≤1 ml/kg body weight.

Consistent with pretraining, rats were brought into the maze room and allowed to acclimatize for at least 30 min before acquisition training in the 14-unit T-maze. The rat was then taken from its home cage and placed into a black start box. The box was inserted into the start position of the maze, the rat was pushed gently into the first section of the maze, and the guillotine door was closed. A switch was triggered manually which initiated a clock that controlled the shock contingency. The rat then had 10 s to navigate the first section of the maze and pass through the door into the second section. If the rat did not achieve this contingency, a scrambled foot shock (0.8 mA) was delivered through the grid floor until the rat escaped through the door. When the rat passed through the door into the second maze section, the guillotine door was lowered, and the shock contingency was reset. This contingency was reset each time the rat moved through the remaining three sections of the maze. A trial was terminated if a maximum of 300 s of shock was reached on any trial of acquisition (two terminated trials resulted in the discontinuation of testing). Upon entering the goal box, the door was closed behind the rat, and the box was placed in a holding area for an ITI of 90 s. During this time, the maze was hoisted, and the grid floor was cleaned with a 95% solution of ethanol. Each rat received a total of 15 massed trials. The 15 massed trials were collapsed into five blocks of three trials for purposes of statistical analysis and presentation of data. Data on maze errors, run time, shock duration, and shock frequency were analyzed using a two-factor Group×Trial Block (5×5) mixed analysis of variance (ANOVA), with repeated measures on the second factor. Planned comparisons between groups were based on the Fisher LSD method.

The specific a priori hypotheses tested in the experiment were the following: (h1) the group treated with scopolamine alone would perform significantly worse than the control group and (h2–4) groups treated with sildenafil (one or more of three doses) combined with scopolamine would perform significantly better than the impaired scopolamine alone group. The number of planned comparisons for each dependent measure was restricted ($k-1$) to test the four above hypotheses.

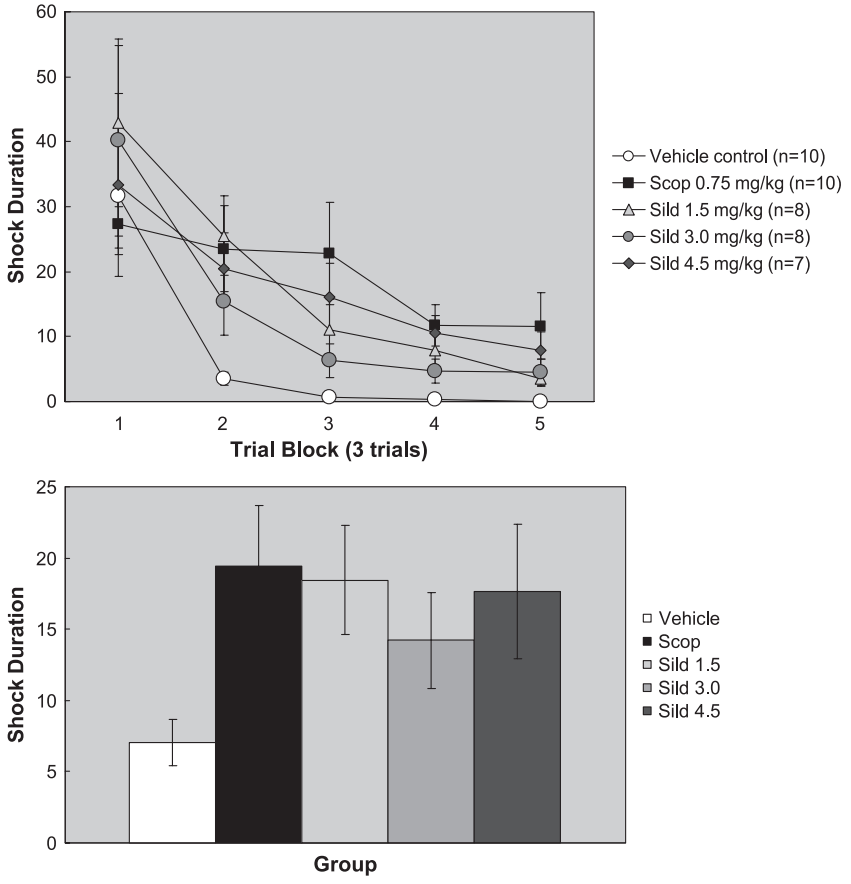


Fig. 4. Effects of scopolamine and sildenafil citrate on mean (±S.E.M.) shock duration (s) during acquisition training in a 14-unit T-maze. Symbol-line graph (top) shows data plotted in blocks of three trials, and bar graph (bottom) shows data averaged across trial blocks. See text for description of statistical results. Scop: scopolamine; Sild: sildenafil.

## 3. Results

Fig. 2 shows the number of errors for each group across blocks of three trials (top) and collapsed across trials (bottom). A 5×5 mixed ANOVA revealed a significant main effect of Group [$F(4,38)=9.92$, $p<0.0001$], a significant main effect of Trial Block [$F(4,152)=47.81$, $p<.0001$], but no significant interaction between these factors [$F(16,152)=1.45$, n.s.]. Planned comparisons using Fisher's LSD showed that the control group made significantly fewer total errors compared to the scopolamine+vehicle group ($p<0.01$). This finding shows that systemic administration of scopolamine results in a significant impairment of maze performance. Planned comparisons also revealed that the scopolamine+3.0 mg/kg sildenafil group made significantly fewer mean errors than the scopolamine+vehicle group ($p<0.05$); however, the other combined scopolamine+sildenafil groups (1.5 and 4.5 mg/kg sildenafil) did not differ from the scopolamine+vehicle group (n.s.). These results demonstrate a significant dose-dependent attenuation of the learning impairment produced by 0.75 mg/kg (i.p.) scopolamine.

Fig. 3 presents the mean run time (s) for each group across blocks of three trials (top) and collapsed across trials (bottom). A 5×5 mixed ANOVA yielded a significant effect of Group [$F(4,38)=7.30$ $p<.001$] and Trial Block [$F(4,152)=48.55$, $p<.001$] but no significant interaction between factors [$F(16,152)=1.58$, $p=0.078$, n.s.]. Planned comparison of marginal means revealed that the control group had a significantly faster overall mean run time compared to the scopolamine+vehicle group ($p<.01$). Other planned comparisons between the scopolamine+vehicle group and the combined Scopolamine+sildenafil groups did not reach statistical significance (n.s.).

Fig. 4 shows the mean shock duration for each group across blocks of three trials (top) and collapsed across trials (bottom). A 5×5 mixed ANOVA of shock duration yielded a significant effect of Trial Block [$F(4,152)=24.00$, $p<.001$] but a nonsignificant effect of Group [$F(4,38)=2.09$, n.s.] and a nonsignificant Group×Trial Block interaction [$F(16,152)=1.00$, n.s.]. Planned comparisons were not computed for the shock duration data given that the overall Group effect and the Group×Trial Block interaction did not reach significance.

Fig. 5 shows the mean number of shocks received by each group across blocks of three trials (top) and collapsed across trials (bottom). A 5×5 mixed ANOVA for shock frequency yielded a significant effect of Trial Block [$F(4,152)=73.93$, $p<.001$] and Group [$F(4,38)=7.09$, $p<.001$] and a significant Group×Trial block interaction

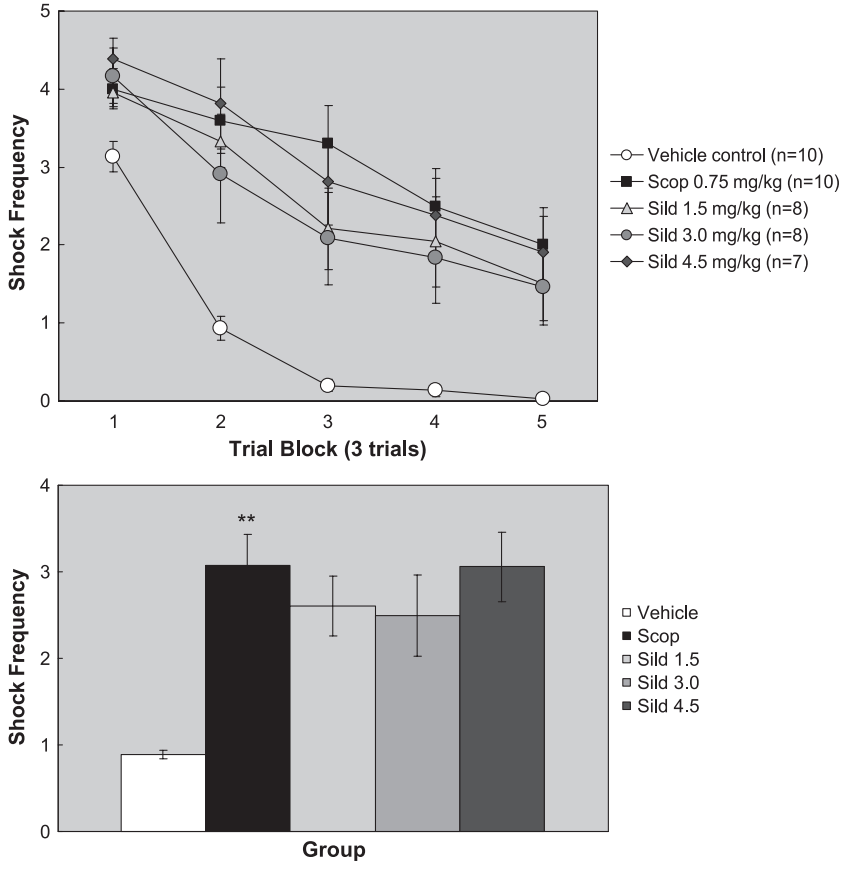


Fig. 5. Effects of scopolamine and sildenafil citrate on mean (±S.E.M.) shock frequency during acquisition training in a 14-unit T-maze. Symbol-line graph (top) shows data plotted in blocks of three trials, and bar graph (bottom) shows data averaged across trial blocks. Symbols: **, significantly different from saline-vehicle group. See text for description of statistical results. Scop: scopolamine; Sild: sildenafil.

[$F(16,152)=1.87$, $p<.05$]. Planned comparisons of marginal means revealed that the control group received significantly fewer shocks overall compared to the scopolamine+vehicle group ($p<.01$). It should be noted that, although the frequency of shock was increased for the scopolamine+vehicle group, the shock duration was significantly reduced across trials (see above), indicating that there was not a general failure to escape shock (e.g., learned helplessness). Other planned comparisons between the scopolamine+vehicle group and the combined scopolamine+sildenafil groups did not reach statistical significance (n.s.).

## 4. Discussion

The present findings demonstrate that systemically administered sildenafil citrate can attenuate the learning impairment produced by blockade of cholinergic muscarinic receptors. Although the central mechanism for this effect is not known, it may involve retrograde signal transduction by NO/cGMP and enhanced activation of glutamate receptors within the hippocampus (Hawkins et al., 1998). In support of this possibility, hippocampal incubation with different PDE inhibitors in the presence and/or absence of sodium nitroprusside resulted in pronounced differences in the extent and regional localization of the cGMP response, suggesting that PDE activity in the hippocampus is high and diverse in nature (van Staveren et al., 2001). Despite this prevalence, it is not clear whether incubation specifically with sildenafil leads to an increase in hippocampal cGMP levels (Prickaerts et al., 2002). This latter finding is consistent with the recently reported mRNA expression patterns of different PDEs in the rat brain (van Staveren et al., 2003). Furthermore, in contrast to the above retrograde NO/cGMP signaling model, recent findings indicate that stimulation of hippocampal NMDA receptors results in accumulation of cAMP and cGMP that is hydrolyzed by PDE4 and PDE2, respectively (Suvarna and O'Donnell, 2002). Hence, if the effect of sildenafil reported here is based on specific PDE5 inhibition, then the site of action of the drug may not be downstream of hippocampal NMDA neurotransmission. Two alternative explanations are possible and will be considered below. Sildenafil may inhibit PDE5 within other brain structures and/or may inhibit other PDE isozymes (e.g., the recently cloned PDE10A enzyme).

In accordance with the first alternative, the sildenafil improvement of learning performance following cholinergic blockade may be due to actions within brain regions other than the hippocampus. Immunohistochemical localization of the PDE5 isozyme reveals specific labeling within cerebellar Purkinje neurons (Kotera et al., 2000; Shimizu-Albergine et al., 2003). Given the role of the cerebellum in motor learning (Ohyama et al., 2003), it is possible that this structure may contribute to the learning performance of the 14-unit T-maze task. Although cholinergic projections are rather sparse in the cerebellum compared to other parts of the brain, acetylcholine activation of muscarinic receptors can increase the firing frequency of granule cells, which in turn can increase the frequency of excitatory postsynaptic currents in Purkinje cells in the vestibulocerebellum (Takayasu et al., 2003).

Another neural system that may contribute specifically to the automatization of motor sequence learning is the dorsal striatum (Doyon et al., 2003). Stimulation of the NO/cGMP pathway activates protein kinase G and excites striatal cholinergic interneurons (Centonze et al., 2001) by regulating directly resting ion conductances in the somatodendritic region of these cells. The drug zaprinast, once thought to be a selective PDE5 inhibitor but now known to also inhibit PDE6, PDE8, PDE10, and PDE11, can mimic the effects of increased intracellular cGMP levels in cholinergic interneurons (Centonze et al., 2001). Hence, if the dorsal striatum is involved in motor sequence learning and contributes to performance of the 14-unit T-maze task, then it is possible that the ability of sildenafil to attenuate the learning impairment produced by systemic scopolamine may be based on modulation of cholinergic striatal function (Calabresi et al., 2000). Although PDE5 is not abundant in dorsal striatum, levels of PDE10A are highest in this area (Seeger et al., 2003), and sildenafil may inhibit this isozyme (e.g., Watling, 2001). Further research with direct intracerebral drug infusions are required to determine the site(s) of drug action within different brain regions contributing to the behavioral performance of learning/memory tasks.

The NO/cGMP pathway has been implicated in neuroprotection (Chiueh, 1999), and treatment with sildenafil has been shown to induce neurogenesis and promote functional recovery after stroke in rats (Zhang et al., 2002). Therefore, in addition to the temporally limited effect on learning/memory performance reported here and in other studies (Baratti and Boccia, 1999; Prickaerts et al., 2002), sildenafil may have more long-term effects on oxidative stress, excitotoxicity, and other neurodegenerative processes. Such effects may be relatively specific to inhibitors of PDE5 as inhibition of other PDE isoforms (PDE1–4) do not appear to offer such neuroprotection (Nakamizo et al., 2003).

Other drugs, such as the uncompetitive NMDA antagonist, memantine, which was recently approved by the FDA for treatment of moderate to severe AD, may also reduce such neurodegenerative processes. However, some reports indicate that memantine may actually impair learning and memory performance in healthy human subjects (Schugens et al., 1997; Rammsayer, 2001) or control animals (Creeley et al., 2003). Therefore, inhibition of certain PDE isoforms may offer a preferred drug target to influence both the immediate neurobiological changes associated with synaptic plasticity and learning/memory and attenuation of more chronic processes believed to contribute to the sustained dysfunction characteristic of neurodegenerative disease. Given that sildenafil citrate is already in widespread use for the treatment of sexual dysfunction and has been shown to have beneficial effects on learning, memory, and neuroprotection, further

research should be conducted to consider the full range of effects that it may offer to the growing elder population.

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