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Donepezil Treatment Restores the Ability of Estradiol to Enhance Cognitive Performance in Aged Rats: Evidence for the Cholinergic Basis of the Critical Period Hypothesis

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Abstract

Recent studies suggest that the ability of estradiol to enhance cognitive performance diminishes with age and/or time following loss of ovarian function. We hypothesize that this is due, in part, to a decrease in basal forebrain cholinergic function. This study tested whether donepezil, a cholinesterase inhibitor, could restore estradiol effects on cognitive performance in aged rats that had been ovariectomized as young adults. Rats were ovariectomized at 3 months of age, and then trained on a delayed matching to position (DMP) T-maze task, followed by a configural association (CA) operant condition task, beginning at 12–17 or 22–27 months of age. Three weeks prior to testing, rats started to receive either donepezil or vehicle. After one week, half of each group also began receiving estradiol. Acclimation and testing began seven days later and treatment continued throughout testing. Estradiol alone significantly enhanced DMP acquisition in middle-aged rats, but not in aged rats. Donepezil alone had no effect on DMP acquisition in either age group; however, donepezil treatment restored the ability of estradiol to enhance DMP acquisition in aged rats. This effect was due largely to a reduction in the predisposition to adopt a persistent turn strategy during acquisition. These same treatments did not affect acquisition of the CA task in middle-aged rats, but did have small but significant effects on response time in aged rats. The data are consistent with the idea that estrogen effects on cognitive performance are task specific, and that deficits in basal forebrain cholinergic function are responsible for the loss of estradiol effect on DMP acquisition in aged ovariectomized rats. In addition, the data suggest that enhancing cholinergic function pharmacologically can restore the ability of estradiol to enhance acquisition of the DMP task in very old rats following long periods of hormone deprivation. Whether donepezil has similar restorative effects on other estrogen-sensitive tasks needs to be explored.

Keywords

spatial learning; hormone therapy; cholinesterase inhibitor; surgical menopause; aging; critical period hypothesis

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INTRODUCTION

Basic research has demonstrated many beneficial effects of estrogen therapies (i.e., compounds that bind and signal via known estrogen receptors) on the brain. These include neuronal protection from ischemia and other trauma-related injuries (Suzuki, et al., 2006), beneficial effects on hippocampal connectivity and function (Spencer, et al., 2007; Woolley, 2007), reduced amyloid beta formation (Bhavnani, 2003; LeBlanc, 2002), enhanced cognitive performance (Daniel, 2006), and prevention of age-related cognitive decline (Gibbs, 2006; Rapp, et al., 2003). Results of human studies are less clear. Results of the Women's Health Initiative Memory Study (WHIMS), and of the Women's Health Initiative Study of Cognitive Aging (WHISCA), reported significant negative effects of hormone therapy (a daily oral regimen of conjugated equine estrogens and medroxyprogesterone acetate) on verbal memory and dementia in women aged 65 and older (Resnick, et al., 2006; Shumaker, et al., 2004). The cause of these negative findings has been a subject of much debate.

Several animal studies suggest that the beneficial effects of estrogens on brain and cognitive function can change with age, and may be reduced or lost following long-term hormone deprivation (reviewed in Frick, 2008). A number of human based studies agree (Sherwin, 2007). Based on these findings, a critical period hypothesis has been proposed which states that there is a window of opportunity following the loss of ovarian function during which estrogenic therapy must be initiated in order to be effective (Resnick and Henderson, 2002; Sherwin, 2007). Mechanism(s) that might account for a critical period currently are unknown.

We and others have demonstrated significant effects of estrogens on cholinergic neurons located in the medial septum (MS), diagonal band of Broca, and nucleus basalis magnocellularis (NBM) (Gibbs and Gabor, 2003). These neurons provide the majority of cholinergic input to the hippocampus and frontal cortex, and are well known to play an important role in learning and attentional processes (Baxter and Chiba, 1999; Everitt and Robbins, 1997). Aging and dementia are associated with significant decreases in cholinergic parameters in the brain. These include decreases in the number and size of cholinergic neurons (Altavista, et al., 1990; Fischer, et al., 1992; Fischer, et al., 1989; Mesulam, et al., 1987; Stroessner-Johnson, et al., 1992), decreases in high affinity choline uptake in the hippocampus and frontal cortex (Kristofiková, et al., 1992; Sherman and Friedman, 1990), as well as decreases in acetylcholine synthesis (Gibson, et al., 1981; Sherman, et al., 1981; Sims, et al., 1983), acetylcholine release (Araujo, et al., 1990; Takei, et al., 1989; Wu, et al., 1988), and cholinergic synaptic transmission (Taylor and Griffith, 1993). Alzheimer's disease (AD), the most common cause of dementia in elderly, is associated with a significant loss of cholinergic neurons in the MS and NBM (Bartus, 2000; Davies and Maloney, 1976; Perry, et al., 1977; Whitehouse, et al., 1982).

Studies show that estradiol treatment can enhance basal forebrain cholinergic function in rats as demonstrated by (a) increased levels of choline acetyltransferase (ChAT) mRNA and protein (Bohacek, et al., 2008; Gibbs, 1996; Gibbs, 1997; Gibbs, 2000a; Gibbs and Pfaff, 1992; Gibbs, et al., 1994; Luine, 1985; Singh, et al., 1994), (b) increases in high affinity choline uptake in the hippocampus and frontal cortex (Gibbs, 2000a; O'Malley, et al., 1987), and (c) increased density of cholinergic fibers in specific regions of prefrontal cortex (Kritzer and Kohama, 1999; Tinkler, et al., 2004). Estradiol also increases potassium-stimulated acetylcholine (ACh) release in the hippocampus of young ovariectomized rats (Gabor, et al., 2003; Gibbs, et al., 2004; Gibbs, et al., 1997; Marriott and Korol, 2003), and can reduce cognitive impairments caused by cholinergic antagonists in both animals (Fader, et al., 1998; Gibbs, 1999; Gibbs, et al., 1998; Packard and Teather, 1997) and in humans (Dumas, et al., 2006). Recently we showed that destruction of cholinergic neurons in the MS abolished the ability of estradiol to enhance DMP acquisition in rats (Gibbs, 2007). Similarly, blockade of M2 cholinergic receptors was

shown to block the ability of estradiol to enhance acquisition of a radial arm maze task in rats (Daniel, et al., 2005). These data demonstrate that estradiol has biologically significant effects on basal forebrain cholinergic function, and that these cholinergic projections are necessary for estradiol to enhance performance on specific cognitive tasks.

Cholinergic neurons in the MS and NBM also are adversely affected by ovariectomy and aging (Gibbs, 1998; Gibbs, 2003). For example, ovariectomy produces significant decreases in relative cellular levels of both ChAT and trkA mRNA in the MS and NBM beyond the effects of normal aging (Gibbs, 1998; Gibbs, 2003). This is consistent with reductions in ACh release that have been reported in aged rats (Moore, et al., 1996), and suggests that long-term loss of ovarian function contributes to an age-related decline in basal forebrain cholinergic function. Based on these findings, we hypothesize that the critical period for estrogenic effects on cognitive performance is defined, in part, by a progressive decline in basal forebrain cholinergic function that occurs in response to ovariectomy and aging. We refer to this as the *cholinergic basis of the critical period hypothesis*. If correct, we reasoned that it may be possible to reinstate the critical period and restore estrogenic effects on performance by treating aged rats with donepezil, a cholinesterase inhibitor commonly used in treating Alzheimer's-related dementia. This hypothesis is consistent with a report by Schneider et al. (1996) which showed that postmenopausal women with AD and who were also on estrogen therapy responded better to tacrine (another cholinesterase inhibitor used in the treatment of AD) than women not on estrogen therapy; however the hypothesis has not been tested in animals. In the present study, middle-aged and aged ovariectomized rats were treated with either donepezil, estradiol, or a combination of donepezil + estradiol prior to being trained on two tasks, a DMP spatial learning task, and a configural association operant conditioning task.

METHODS

All procedures were conducted in compliance with PHS guidelines on the care and use of laboratory animals, and with the approval of the University's Institutional Animal Care and Use Committee. Sprague-Dawley rats were ovariectomized at three months of age and maintained at Hilltop laboratories, Inc. Note that we previously showed that estradiol treatment administered within three months following ovariectomy in middle-aged rats significantly enhanced DMP acquisition (Gibbs, 2000b). In the present study we chose to ovariectomize rats at three months of age in part to assess whether estradiol treatment would enhance DMP acquisition in middle-aged rats after a longer period of hormone deprivation. Rats were delivered to the University of Pittsburgh at either 10 or 20 months of age and maintained on a 12h:12h light cycle with lights on at 0700. Deliveries were organized so that each shipment included cohorts of middle-aged and aged rats. Middle-aged rats were tested at 12–17 months of age. Aged rats were tested at 22–27 months of age. Three weeks prior to testing, rats in each age group began receiving daily injections of donepezil-HCl (Don, 3 mg/Kg) or sterile saline delivered i.p. Donepezil is a piperidine-based highly potent mixed, non-competitive reversible inhibitor of acetylcholinesterase, with an *in vitro* IC₅₀ of approximately 6.7 nM and an *in vivo* ID₅₀ of approximately 2.6 mg (6.8 μMol)/Kg brain tissue (Sugimoto, et al., 2002). The *in vivo* ID₅₀ is much higher because the majority of donepezil (98%) is bound by serum proteins. The dose was based on a study by Haug et al. (2005) which reported that daily administration of 3.0 mg/Kg donepezil in rats resulted in a stable decrease of 70 ± 3% in AChE activity in the brain when measured 4h after dosing, and an increase in cerebral acetylcholine concentration of 35%–37%. In the present study, injections were administered in the late afternoon past the time when all behavioral testing would be completed for the day. After one week of treatment, half of each group received a silastic capsule (4 mm length, 0.058" inner diameter, 0.077" outer diameter, Dow corning Corp., Midland, MI) containing 17-β-estradiol (E) implanted s.c. Controls received implants of empty capsules. As an additional control,

several rats from each age group received no daily injections to control for possible effects associated with the minor stress of daily injections.

Behavioral Testing

One week following implant surgery, rats were food restricted to 85% body weight and then trained on a delayed matching-to-position (DMP) T-maze task. This task involves both hippocampal and frontal cortex circuits and is sensitive to both estradiol treatment and to basal forebrain cholinergic lesions (Gibbs, 1999; Gibbs, 2007; Gibbs and Johnson, 2007; Johnson, et al., 2002). Rats were adapted to the maze as previously described (Gibbs, 1999) and trained to run to the ends of the goal arms by using a series of 6 forced “choices” per day for 4 days, each rewarded with 4 food pellets (Formula 5TUM 45 mg pellets from Test Diets, Inc.). Right and left arms were alternated in a random, balanced fashion to avoid the introduction of a side bias. Animals then began DMP training.

DMP training was performed in trial pairs as previously described (Gibbs, 1999). Each rat received 8 trial pairs/day. The first trial of each pair consisted of a forced “choice” in which one goal arm was blocked, forcing the animal to enter the unblocked arm to receive food reward (4 pellets). The rat was then immediately returned to the approach alley. All arms of the maze were quickly (<5 sec) cleaned with 70% ethanol in order to minimize information from intramaze odor cues, and all arms were opened for the second trial. A choice was defined as an animal placing both front legs, and at least part of both rear legs, into a goal arm. Returning to the same arm visited on the forced trial resulted in food reward (4 pellets; the rat remained in the arm for 10–20 seconds while eating the food). Entering the incorrect arm resulted in no food reward and confinement to the arm for 60 seconds. Forced choices were selected in a random, balanced fashion to avoid the introduction of a side bias. Rats were run in squads of 4–6. After each trial pair, an animal was returned to its cage for 5–10 minutes while training proceeded with the other animals. Rats continued to receive 8 trial pairs/day until they reached a criterion of 15/16 correct choices over two consecutive days or until they had received 30 days of training.

Post-Criterion Testing—One day after reaching criterion, animals received a probe trial during which the T-maze was rotated 180° (relative to extramaze cues) between the forced and open trial. This was done to assess whether rats were using a place strategy or a response strategy to perform the task. A place strategy (also known as an allocentric strategy) is defined as a strategy that relies on external cues in order to succeed. A response strategy (also known as an egocentric strategy) is defined as a strategy that relies on internal/kinetic cues in order to succeed. Animals that use a place strategy are expected to enter the arm located in the same position of the room that was previously visited, even though doing so requires turning in the opposite direction from that used on the preceding trial. Animals that use a response strategy are expected to turn in the same direction as on the preceding trial and thereby enter the same physical arm of the maze, even though it occupies a different position in the room. For the purpose of analysis, selecting the arm located in the same position of the room was assigned a score of 0, whereas selecting the opposite arm was assigned a score of 1. Beginning one day after the probe trial, animals received four days of 8 trial pairs/day with successively increasing intertrial delays (day 1=minimal delay (same as training condition); day 2 = 30 seconds; day 3 = 60 seconds; day 4 = 90 seconds) to assess short-term spatial memory.

CA Training—At the completion of DMP testing, old capsules were removed and fresh capsules were implanted. Two weeks later, rats were trained on an operant configural association (CA) negative patterning task as previously described (Gibbs, 2005; Gibbs and Johnson, 2007). This is a non-spatial task that requires rats to distinguish between simple and configural stimuli and to inhibit responses to the configural stimulus. While early studies

suggested that the hippocampus plays an essential role in configural learning (Rudy and Sutherland, 1989), subsequent studies showed that the critical neural system for configural associations is in the frontal cortex, with hippocampal outputs providing an important modulatory role (Rudy and Sutherland, 1995). This task makes use of the same food reward as the DMP task, but in our hands is not significantly affected by gonadal hormones (Gibbs, 2005; Gibbs and Gabor, 2003), nor is particularly sensitive to basal forebrain cholinergic lesions (Gibbs and Johnson, 2007).

Training was performed in operant chambers (Med. Associates, Inc., Georgia, VT) connected to a computer running Med-PC software. Each operant chamber contained a dim red house light, a ventilation fan, a 6W stimulus panel light, a speaker calibrated to present a 1500 Hz tone, a pellet dispenser, and a recessed food cup located immediately below the panel light. Entry into the food cup was monitored by a photosensor.

Rats were adapted to the chamber by receiving one 60 minute session during which they received a total of 16 food pellets delivered at intervals ranging from 2–6 minutes. CA training began the following day. Each rat received one training session per day for a total of 24 days. Twenty-four days was selected based on pilot studies which showed that, in our hands, performance plateaus by 24 days and does not improve significantly with additional training (up to 40 days; Gibbs, unpublished observations). Each session lasted for a maximum of 110 minutes. During the session, rats received 30 presentations of a tone conditioning stimulus (CS), 30 presentations of a light CS, and 30 presentations during which the tone and the light were presented simultaneously. If an animal entered the food cup within 10 seconds of presentation of the tone or the light, the CS was discontinued and the animal received a food reward (one 45 mg pellet). If an animal entered the food cup when the light and the tone were presented simultaneously, the stimuli were discontinued, the house light was turned off for sixty seconds, and no food was delivered. The CS presentations were randomly distributed throughout the session and occurred at one of 30 randomly selected intertrial intervals ranging from 12 to 70 seconds. During each session, the measures that were recorded included (a) the number of responses (i.e., the number of times presentation of a stimulus resulted in the animal entering the food cup), and (b) response time (i.e., time between presentation of each CS and entry into the food cup).

Tissue collection

Following training, animals were anesthetized with pentobarbital (100mg/kg; IP) and trunk blood was collected for the determination of serum estradiol levels. Animals were then perfused with ice-cold saline. Brains were removed, and tissues from the hippocampus, frontal cortex (FR1, FR2 and FR3), and prefrontal cortex (Cg1, Cg2, Cg3) (according to plate 7–20 of Paxinos & Watson (1986)), were dissected from 2 mm coronal sections. Retrosplenial/entorhinal cortex also was dissected from the posterior region of the forebrain. Tissues were immediately frozen on dry ice and stored at –80°C until processed for choline acetyltransferase (ChAT) activity.

Estradiol assay

Serum from estradiol-treated rats was assayed for levels of estradiol by radioimmunoassay. Samples were assayed in duplicate by the Assay Core of the University's Center for Reproductive Physiology. The estradiol assay had a minimum detection limit of 1.2 pg/mL serum.

ChAT Assay

ChAT assays were performed as previously described (Johnson et al., 2002). Frozen tissues were thawed and dissociated by sonication in a medium containing EDTA (10mM) and Triton X-100 (0.5%) and diluted to a concentration of 10 mg tissue/mL. An aliquot of each sample

was used for the determination of total protein (Bradford, 1976). Three 5 µl aliquots of each sample were incubated for 30 min. at 37 °C in a medium containing [³H] acetyl-CoA (50,000–60,000 d.p.m./tube, final concentration 0.25 mM acetyl-CoA; Sigma Inc., St. Louis, MO), choline chloride (10.0 mM), physostigmine sulfate (0.2 mM), NaCl (300 mM), sodium phosphate buffer (pH 7.4, 50 mM), and EDTA (10 mM). The reaction was terminated with 4 mL sodium phosphate buffer (10 mM) followed by the addition of 1.6 mL of acetonitrile containing 5 mg/mL tetraphenylboron. The amount of [³H] acetylcholine produced was determined by adding 8 mL of EconoFluor scintillation cocktail (Packard Instruments, Meriden, CT) and counting total cpm in the organic phase using an LKB beta-counter. Background was determined using identical tubes to which no sample was added. For each sample, the three reaction tubes containing sample were averaged and the difference between total cpm and background cpm was used to estimate the total amount of ACh produced per sample. ChAT activity was then calculated for each sample as pmol ACh manufactured/hr/µg protein.

Data Analysis

Data were analyzed by ANOVA with drug (donepezil vs. saline) and hormone treatment (estradiol vs. blank capsule) as between factors. Interaction effects were further analyzed by one-way ANOVA followed by a Tukey test. All statistical tests were performed using JMP v7.0 software. Significance was defined as $p \leq 0.05$.

Analysis of DMP Performance

Days to criterion (DTC) was analyzed by ANOVA as described above. Performance during acquisition was blocked into ten 3-day blocks of training. Once an animal reached criterion, a value of 0.9375 (15/16) was recorded for performance on subsequent days. The blocked data were then analyzed by ANOVA with repeated measures on 'block'. The effects of rotating the maze 180° were analyzed by contingency table and Chi-square test. Performance during increased intertrial delays was analyzed by ANOVA with repeated measures on 'Delay'.

Analysis of Response Patterns

We have observed that after several days of DMP training, some rats adopt a persistent turn whereby they consistently enter either the right or left arm of the maze. We quantified this as described previously (Gibbs and Johnson, 2007) by counting the total number of days during training that an animal chose the same arm of the maze 15 out of 16 times over a two-day period. Any animal that met this criterion was defined as having adopted a persistent turn. Chi-Square analysis was used to compare the number of animals that adopted a persistent turn between groups, and ANOVA was used to compare the number of days that this pattern persisted among the treatment groups. To evaluate the contribution that the number of days involved in a persistent turn made to effects on DTC, the duration of the persistent turn was subtracted from DTC for each animal and the results analyzed using ANOVA.

Analysis of CA Acquisition

Performance during acquisition of the CA task was blocked into eight 3-day blocks of training. Both the number of responses and response time to both simple and configural stimuli were analyzed by ANOVA with repeated measures on 'block'.

RESULTS

Animal Attrition and Controls

A total of 50 middle-aged and 75 aged rats were assigned to the study. Of these, 40 middle-aged and 43 aged rats completed the study. Many aged rats died prior to completion. Deaths

were mostly due to natural aging, the development of tumors, and other health-related issues commonly seen in aged rats. Several animals died due to complications with anesthesia during capsule implantation and several refused to perform the maze task.

Among the controls, those that received no injections and no estradiol did not differ significantly on any measure from those that received no estradiol and daily injections of sterile saline within their age cohort. Therefore these rats were consolidated into one control group within each age cohort. Final group sizes are indicated in Figure 1.

Serum levels of estradiol

Levels of estradiol in non-E-treated rats were undetectable. Mean serum levels of circulating estradiol in E-treated rats were 14.7 ± 2.4 pg/mL and 17.0 ± 2.4 pg/mL for E- and Don+E-treated middle-aged rats, and 12.8 ± 2.4 pg/mL and 18.5 ± 5.1 pg/mL for E- and Don+E-treated aged rats. These values did not differ significantly from each other.

DMP acquisition (days to criterion)

All but one rat (aged, Don) reached criterion on the task. No significant difference between middle-aged and aged control rats on the number of days required to reach criterion (DTC) was observed; middle-aged and aged controls required an average of 22.7 ± 1.4 and 21.4 ± 1.0 days to reach criterion. There did, however, appear to be significant effects of treatment that differed between middle-aged and aged rats.

In middle-aged rats, estradiol treatment, but not donepezil, was associated with a faster rate of acquisition. On average, each group of E-treated rats required less than 19 days to reach criterion whereas each group of non-E-treated rats required approximately 23 days to reach criterion (Fig. 1A). A two-way ANOVA of DTC revealed a significant effect of estradiol ($F[1,36]=10.6$, $p<0.003$), no significant effect of donepezil ($F[1,36]=0.01$, $p=0.92$), and no significant interaction between estradiol and donepezil ($F[1,36]=0.03$, $p=0.87$).

In aged rats, neither estradiol nor donepezil was associated with a reduction in DTC relative to controls. Aged rats treated with estradiol or donepezil required an average of 21.3 ± 1.1 and 23.0 ± 1.1 days to reach criterion (Fig. 1B). In contrast, rats treated with both donepezil and estradiol learned the task significantly faster, requiring only 17.1 days to reach criterion. The two-way ANOVA of DTC revealed a significant main effect of estradiol ($F[1,39]=8.2$, $p<0.007$), no significant effect of donepezil ($F[1,39]=1.5$, $p=0.22$), and a significant interaction between estradiol and donepezil ($F[1,39]=7.5$, $p=0.01$). Post-hoc analysis revealed that rats treated with the combination of donepezil and estradiol differed significantly from both donepezil-treated rats and saline-treated controls ($p<0.05$). Aged rats treated with the combination of donepezil and estradiol also learned the task significantly faster than non-E-treated, middle-aged, donepezil- and saline-treated groups ($p<0.05$). Including the age of the rats (in months) at the initiation of T-maze testing as a covariate in the analysis did not alter the significance of the effects.

DMP acquisition (learning curves)

Examination of the learning curves (Fig. 2) shows that all treatment groups performed worse than chance at the start of training and improved over time. In middle-aged rats, animals treated with estradiol improved at a faster rate than rats treated with donepezil or saline alone. In aged rats, animals treated with Don+E improved at a faster rate than all other groups. For middle-aged rats (Fig 2A), two-way ANOVA (drug $\times$ hormone) with repeated measures on block revealed a significant effect of estradiol ($F[1,36]=6.4$, $p<0.02$), no significant effect of donepezil ($F[1,36]=0.04$, $p=0.84$), a significant effect of block ($F[9,324]=243.7$, $p<0.0001$), and a significant interaction between estradiol $\times$ block ($F[9,324]=3.9$, $p<0.0002$). Post-hoc

analysis of the marginal means revealed that E-treated groups performed significantly better than non-E-treated groups on blocks 4–8, in part due to a slight negative effect of donepezil in non-E-treated rats. The separation of E-treated and non-E-treated groups is most apparent on blocks 6 & 7. In addition, Don+E-treated rats performed significantly better than Don-treated rats on blocks 3 & 4.

For aged rats (Fig 2B), two-way ANOVA revealed a significant effect of estradiol ($F[1,39] = 6.6, p < 0.02$), no significant effect of donepezil ($F[1,39] = 0.2, p = 0.66$), a significant effect of block ($F[9,351] = 195.7, p < 0.0001$), and a significant interaction between estradiol $\times$ block ($F[9,351] = 2.2, p < 0.03$). There also was a significant interaction between estradiol $\times$ donepezil ($F[1,39] = 4.4, p < 0.05$) in aged rats. Post-hoc analyses revealed that Don+E-treated rats performed significantly better than non-E-treated rats on blocks 3–6.

Analysis of Response Patterns

We observed that after several days of training, many rats adopted a persistent turn whereby they consistently entered either the right or left arm of the maze. Notably, 75% of middle-aged controls and 91.7% of aged controls adopted a persistent turn at some point during DMP training. In middle-aged rats, treatments had no effect on the percentage of rats that adopted a persistent turn ($X^2[3] = 3.6, p = 0.31$). In contrast, treatments did affect the percentage of aged rats that adopted a persistent turn ($X^2[3] = 9.5, p < 0.05$). In this case, Don+E-treated rats were far less likely to adopt a persistent turn (33.3%) than either Don- (83.3%), E-treated (70.0%), or control (91.7%) rats. None of the rats in any group demonstrated a persistent turn at the very start of training, and there were no effects of age or treatment on the number of days that elapsed prior to adopting a persistent turn. In rats that adopted a persistent turn, the pattern typically persisted for several days (averaging 4.5–6.2 days in middle-aged rats, and 4.3–10.1 days in aged rats) and then ceased, suggesting a shift in strategy. In one aged control and one aged Don-treated rat, the pattern persisted for 24 and 25 days; however, there was no significant effect of age on the number of days that this pattern persisted. Among rats that adopted a persistent turn, no significant effect of treatment on the number of days that this pattern persisted was detected for either middle-aged ($F[3,30] = 0.6, p = 0.59$) or aged ($F[3,27] = 1.0, p = 0.40$) rats.

To test whether the effects of treatment on DTC were due predominantly to an effect on the predisposition to adopt a persistent turn, the number of days that an animal displayed a persistent turn was subtracted from the number of days required to reach criterion and these data were reanalyzed (Figures 1C and 1D). ANOVA revealed that in middle-aged rats, individual group differences were reduced slightly but the main effect of estradiol was unchanged; estradiol treatment was associated with a significant reduction in the number of days required to reach criterion relative to non-E-treated groups even after controlling for the number of days spent engaged in a persistent turn (Fig. 1C; $F[1,36] = 4.3, p < 0.05$). In contrast, the effect of Don+E treatment in aged rats was completely eliminated (Fig 1D; $F[1,39] = 0.05, p = 0.81$ for interaction effect). This suggests that the effect of Don+E on DMP acquisition in aged rats was due predominantly to a reduction in the predisposition to adopt a persistent turn.

Effects of the probe trial

The eighty-two rats that reached criterion on the DMP task underwent post-criterion testing. After reaching criterion, a probe trial was conducted in which the maze was rotated 180° between the forced and open trials in order to evaluate the use of place- vs. response-based performance strategy as described under ‘Methods’. In middle-aged rats, 50% of controls, 44.4% of E-treated rats, and 27.3% of Don+E-treated rats returned to the same physical arm of the maze on the open choice. These values did not differ significantly from chance, indicating that rotating the maze disrupted performance in these groups, consistent with the use of

extramaze cues to solve the task. In contrast, all eight (100%) of the Don-treated rats returned to the same physical arm of the maze, suggesting that rats in this group were not relying on extramaze cues. Chi-square analysis was significant ($X^2[3] = 13.5$, $p < 0.004$) and revealed a significant interaction between the effects of estradiol and donepezil ($X^2[1] = 7.3$, $p < 0.007$) on performance during the probe trial in middle-aged rats.

In aged rats, rotating the maze significantly disrupted performance in all four treatment groups, and no significant differences between treatment groups were detected ($X^2[3] = 3.6$, $p < 0.30$). In this case, 33.3% of controls, 27.3% of Don-treated, 44.4% of E-treated, and 66.7% of Don + E-treated rats returned to the same physical arm of the maze on the open choice, and none of these values differed significantly from chance. These data indicate that the performance of all four groups of aged rats and three of the four groups of middle-aged rats were disrupted by rotating the maze. This suggests that by the time these rats reached criterion, significant numbers of rats within each group were using extramaze cues to solve the task.

Effects of increasing the intertrial delay

One day after rotating the maze, rats received four days of testing with increasing intertrial delays (delay between the forced and open choice) of <10 (day 1), 30 (day 2), 60 (day 3), and 90 (day 4) seconds. Performance on day 1 (minimal delay) did not differ significantly for any group from the criterion performance achieved prior to the probe trial. In contrast, the performance of all groups of both age cohorts decreased significantly as a result of increasing the intertrial delay (Figure 3). ANOVA of drug $\times$ hormone with repeated measures on 'delay' revealed a significant effect of 'delay' for both middle-aged ($F[3,96] = 31.0$, $p < 0.0001$) and aged ($F[3,102] = 319.7$, $p < 0.0001$) rats; however, there were no significant effects of treatment, and no significant interactions between treatments and delay for either age group.

CA Acquisition

All treatment groups demonstrated acquisition of both simple and configural associations on the CA task. Acquisition of the simple associations (e.g., response to light, response to tone) occurred very rapidly and achieved threshold response (30/30 correct) from most animals within the first three blocks of training (Figures 4A–D). Acquisition of the negative patterning component took longer, and tended to stabilize at a group average of 19–22 incorrect responses for middle-aged rats, and 20–24 incorrect responses for aged rats out of thirty presentations after 24 training sessions (Figures 4E–F). For middle-aged rats, ANOVA revealed highly significant effects of block on all simple and configural associations ($p < 0.0001$ in each case), but no significant effects of treatment. In aged rats ANOVA likewise revealed highly significant effects of block on all associations ($p < 0.0001$ in each case), and no significant effects of treatment on responses to the configural association. Treatments did, however, produce small but significant improvements in acquisition of the simple associations in aged rats, in response to both the light stimulus (Don: $F[1,32] = 11.6$, $p < 0.002$; E: $F[1,32] = 9.6$, $p < 0.005$; Don+E: $F[1,32] = 4.9$, $p < 0.05$) and the tone stimulus (Don: $F[1,32] = 4.0$, $p = 0.05$; E: $F[1,32] = 5.0$, $p < 0.05$; Don+E: no effect $F[1,32] = 1.6$, $p = \text{NS}$).

Similar treatment effects were observed for measures of response time. In each case, the time to respond to either a tone or a light stimulus decreased significantly during the first two to three blocks of training and then stabilized (Figures 5A–D). In contrast, response time to the configural stimulus decreased during the first 2–3 blocks of training and then increased significantly (Figures 5E–F). For middle-aged rats, ANOVA revealed highly significant effects of block on all simple and configural associations ($p < 0.0001$ in each case), but no significant effects of treatment. In aged rats ANOVA revealed highly significant effects of block ($p < 0.0001$ in each case), as well as significant effects of estradiol on response time to the light ($F[1,32] = 4.3$, $p < 0.05$) and tone ($F[1,32] = 4.3$, $p < 0.05$) stimuli, and a significant interaction

effect of Don+E treatment on response time to the configural stimulus ($F[1,32]=4.9$, $p<0.05$; Fig 5D). Further analysis revealed that treatment with Don, E, or Don+E improved (increased) response time to the configural stimulus relative to vehicle-treated controls ($p<0.05$ in each case; Fig 5D).

ChAT Activity

Mean ChAT activities are summarized in Table 1. Effects of treatment on ChAT activity were detected in the hippocampus ($F[3,29]=5.8$, $p<0.005$), frontal cortex ($F[3,29]=2.9$, $p=0.054$), and the prefrontal cortex ($F[3,29]=4.5$, $p=0.01$) of middle-aged rats. In middle-aged rats, a significant main effect of estradiol was detected in the frontal cortex, hippocampus and prefrontal cortex. In each of these cases, estradiol treatment was associated with *higher* ChAT activity. Post-hoc comparisons revealed significantly greater ChAT activity in E-treated rats vs. controls in the hippocampus and prefrontal cortex. This effect was mitigated to some degree in the hippocampus by donepezil treatment ($F[1,29]=7.8$, $p<0.01$ for interaction effect). In aged rats, a significant effect of treatment on ChAT activity was observed in the hippocampus ($F[3,24]=3.1$, $p<0.05$). In this case, donepezil treatment was associated with *lower* ChAT activity. No other effects of estradiol or donepezil on ChAT activity were detected in aged rats.

DISCUSSION

Evidence for the cholinergic basis of the critical period hypothesis

According to our hypothesis, beneficial effects of estrogen therapy on cognitive performance are lost with advanced age due to a significant decline in basal forebrain cholinergic function. Our data confirm that beneficial effects of estradiol on DMP acquisition were lost in aged rats, consistent with recent reports from other labs using different tasks (Gresack, et al., 2007; Savonenko and Markowska, 2003; Talboom, et al., 2008). Donepezil is a well characterized, highly effective cholinesterase inhibitor that acts predominantly in brain (Birks, 2006; Sugimoto et al., 2002). In addition to inhibiting acetylcholine degradation, donepezil has been shown to enhance cholinergic function by increasing nicotinic receptors in the hippocampus and neocortex (Barnes, et al., 2000; Kume, et al., 2005). The fact that donepezil restored the ability of estradiol to enhance the rate of DMP acquisition in aged rats supports the hypothesis that the loss of estrogen effect was due, at least in part, to a reduction in cholinergic function. Schneider et al. (1996) reported a similar effect in women with AD - women on estrogen therapy responded better to tacrine (another cholinesterase inhibitor used in the treatment of AD) than women not on estrogen therapy. This suggests that a similar interaction between estrogen effects on cognitive and cholinergic function may exist in postmenopausal women.

In the current study, the effect of Don+E in aged rats was due predominantly to a reduction in the predisposition to adopt a persistent turn during DMP acquisition. Studies show that cholinergic lesions in the MS and NBM of young rats reduce the rate of DMP acquisition primarily by increasing the adoption of a persistent turn (Gibbs and Johnson, 2007). Our data show that this same trend is observed as a function of age. The fact that Don+E reversed this trend in aged rats, and that this effect accounted for the effect of Don+E on the rate of DMP acquisition, further supports the hypothesis that the effect of Don+E on performance in aged rats was due to an effect on cholinergic function.

Other data, however, do not fully support the cholinergic hypothesis. For example, effects of Don+E on DMP acquisition did not correspond with effects on ChAT activity in any of the four brain regions examined. Estradiol (but not Don+E) significantly increased ChAT activity in the hippocampus and prefrontal cortex of middle-aged rats but not aged rats, and Don+E treatment did not restore this effect in aged rats. Hence effects on performance did not correlate with this cholinergic measure. Bohacek et al. (2008) demonstrated that the effects of estradiol

on ChAT levels in the hippocampus and prefrontal cortex of middle aged rats can vary markedly depending on whether treatment is initiated immediately or 5 months following ovariectomy. Whether these changes reflect differential effects on cholinergic function is not clear. ChAT activity is not a direct measure of cholinergic function. ChAT is necessary for the production of acetylcholine, and changes in ChAT activity have been correlated with changes in cholinergic function; however, the levels of ChAT within cholinergic neurons are generally in kinetic excess and are not the rate-limiting step in the production of acetylcholine (Rylett and Schmidt, 1993; Wu and Hersh, 1994). Hence, the lack of a correlation with ChAT activity is not sufficient to conclude that effects on cholinergic function do not underlie the effects on performance.

Nor is it clear why donepezil alone did not significantly affect DMP acquisition or the predisposition to adopt a persistent turn in middle-aged or aged rats. If these measures truly are sensitive to changes in cholinergic function, then donepezil alone should have had an effect. It is possible that the dose of donepezil was not optimal and that lower or higher doses would be more effective. Lower doses (≤ 1.0 mg/Kg/day) have been shown to enhance performance of young and aged rats on other spatial tasks including delayed visual matching to sample (Bennett, et al., 2006), Morris water maze (Cutuli, et al., 2008; Cutuli, et al., 2009; Hernandez, et al., 2006), object recognition (Prickaerts, et al., 2005), and radial arm maze (Cutuli et al., 2008; Cutuli et al., 2009) tasks. Donepezil at >1.0 mg/Kg has been shown to reduce impairments on a Morris water maze task in young rats treated with scopolamine (Takahata, et al., 2005), and in a rat model of encephalomyelitis (D'Intino, et al., 2005); however, not all studies have observed beneficial effects of donepezil on cognitive performance (Barnes et al., 2000). Most of these studies have been done in male rats, and it is possible that females respond differently.

It also is unclear why Don+E did not affect the predisposition to adopt a persistent turn in middle-aged rats. Perhaps cholinergic function must decline below a certain level before adding donepezil treatment has an effect on this task. Another possibility is that donepezil and estradiol may have non-cholinergic effects that change with age. Studies show that at high concentrations, donepezil can block voltage gated sodium, potassium, and calcium channels (Solntseva, et al., 2007; Yu and Hu, 2005), or influence glutamatergic synapses either directly or indirectly (Narahashi, et al., 2004), independent of its effect on cholinergic transmission. Estradiol also has many non-cholinergic effects which may underlie some effects on cognitive performance and interact with effects of donepezil. For example, estradiol has been shown to potentiate neuronal L-type calcium channels (Sarkar, et al., 2008) and to enhance glutamate transmission, NMDA responses, and long term potentiation in hippocampal neurons (Smith and Woolley, 2004). Some of these effects, e.g., effects of estradiol on the expression of specific NMDA receptor subunits, change with age (Adams, et al., 2001). It is possible that non-cholinergic effects of donepezil increase with age. This could account for a significant effect of Don+E in aged rats, in the absence of an effect in middle-aged rats

Specificity of the Effects on Performance

Our studies also show that the effects of E and Don+E on the rate of DMP acquisition were not due to effects on short-term spatial memory, or on ultimate strategy selection. Nor did E or Don+E affect configural learning in middle-aged rats. To our knowledge, this study is the first to evaluate the performance of ovariectomized middle-aged and aged rats on acquisition of the CA task. Comparisons with young rats (Gibbs, 2005; Gibbs and Gabor, 2003; Gibbs and Johnson, 2007) suggest very little deficit on this task as a function of aging. Both E and Don+E improved (increased) response time to the configural stimulus in aged rats; however, we suspect this is due to a generalized increase in response time rather than to an effect on configural learning. These data demonstrate that the effects of E and Don+E on cognitive

performance are task specific. We hypothesize that this specificity reflects differences in the sensitivity of the tasks to changes in basal forebrain cholinergic function. For example, measures that were insensitive to E and Don+E (e.g., delay-dependent memory performance, CA acquisition) are relatively insensitive to selective lesions of cholinergic neurons in the MS (Baxter, et al., 1995; Chappell, et al., 1998; Dornan, et al., 1996; Gibbs and Johnson, 2007; McMahan, et al., 1997). In contrast, measures that were improved by E and Don+E (e.g., rate of DMP acquisition, predisposition to adopt a persistent turn) are significantly affected by cholinergic lesions in both the MS and NBM (Fitz, et al., 2008; Gibbs and Johnson, 2007). Other tasks that are adversely affected by selective cholinergic impairment and that are improved by estradiol treatment include acquisition of a radial arm maze (Daniel and Dohanich, 2001; Fader, et al., 1999), object and place recognition (Fernandez, et al., 2008; Hunsaker, et al., 2007; Luine, et al., 2003), inhibitory avoidance (Packard, 1998), and reinforced alternation (Fader et al., 1998). These data suggest that cognitive abilities which decline with age due to deficits in basal forebrain cholinergic function are the abilities most likely to benefit from estrogen therapy.

Further insights into the critical period hypothesis

Both human (Sherwin, 2007; Sherwin and Henry, 2008) and animal (Frick, 2009) studies support the idea that beneficial effects of estrogen therapy on cognitive performance decline with age and time following the loss of ovarian function. The fact that estradiol treatment significantly increased the rate of DMP acquisition in middle-aged rats demonstrates that estradiol can be effective on this task even after a very long period of hormone deprivation (8–13 months), provided rats are ovariectomized at a young age. Previously we showed that when rats are ovariectomized at 13 months of age and treatment is delayed 10 months prior to training, the ability to enhance the rate of DMP acquisition is decreased (Gibbs, 2000b). This suggests a critical period of less than 10 months for eliciting beneficial effects of estrogen on DMP acquisition when rats are ovariectomized at middle-age. Markowska and Savonenko (2002) likewise showed that aggressive estradiol treatment initiated in middle-aged and aged rats within 6 months following ovariectomy significantly enhanced performance on a delayed non-matching to sample task, whereas treatment initiated nine months following ovariectomy did not. This suggests a critical period greater than 6 months and less than 9 months for eliciting beneficial effects on this task. More recently, Daniel et al. (2006) showed that the ability of estradiol to enhance radial arm maze performance in 17 month old rats was lost when rats were ovariectomized at 12 months of age and treatment was delayed 5 months prior to testing. In contrast, estradiol significantly enhanced performance when rats were treated immediately after ovariectomy at 17 months of age. This suggests a critical period of less than 5 months for eliciting beneficial effects of estradiol on a radial arm maze task when rats are ovariectomized in middle-age. These studies demonstrate that when loss of ovarian function occurs in middle-age, 3–10 months of hormone deprivation can abolish the ability of estradiol to enhance cognitive performance, and that the length of the critical period may vary depending on the task.

One possible explanation for why the critical period was longer in the present study than in previous studies is that rats were ovariectomized at a younger age. In other words, the length of the critical period may be related not only to time following ovariectomy, but to an interaction between loss of ovarian function and age. This could be explained by a model in which cholinergic function declines steadily with age, and in which the neurons are further impaired by the loss of ovarian function (Fig. 6), both of which are supported by experimental evidence. If we assume that loss of estrogen effect occurs when basal forebrain cholinergic function drops below a critical threshold, then the model predicts that ovariectomy earlier in life would be associated with a longer critical period, as well as a greater longitudinal risk for cognitive decline and dementia. This is because the time it takes for cholinergic function to reach

threshold is greater following ovariectomy at a young age than following ovariectomy in middle-age (Fig. 6). Consistent with this model, McLay et al. (2003) reported that early menopause in women, whether due to surgery or to natural menopause, is associated with greater declines in cognitive function later in life. Rocca et al. (2007) reported that ovariectomy prior to menopause is associated with increased risk for cognitive decline and dementia, and that the risk increases as the age at ovariectomy decreases. These findings are consistent with our model, and with the idea that the length of the critical period is determined both by age and time following loss of ovarian function. This may have important implications for the potential benefits of estrogen therapy in women who experience oophorectomy prior to natural menopause.

Summary

This study shows that donepezil treatment was able to restore the ability of estradiol to increase the rate of acquisition of a DMP spatial learning task by aged ovariectomized rats. These findings lend support to the cholinergic basis of the critical period hypothesis, which states that loss of estrogen effect with age is due primarily to a significant decline in basal forebrain cholinergic function. Effects of Don+E were task specific, and in aged rats were due primarily to an effect on the predisposition to adopt a persistent turn early on during training. These findings suggest that enhancing cholinergic function pharmacologically can re-open the 'window of opportunity' and reinstate the ability of estradiol to enhance performance on specific tasks. A similar effect in humans would suggest that cholinesterase inhibitors could reinstate beneficial effects of estrogen therapy on cognitive performance in postmenopausal women, even in older women who have not used estrogen therapy for many years.

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Figure 1.

Bar graphs showing the effects of treatments on Days to Criterion on the DMP task for middle-aged (A & C) and aged (B & D) rats. Panels A & B summarize days to criterion (DTC). Panels C & D summarize DTC minus the number of days that rats engaged in a persistent turn. Bars represent the mean $\pm$ s.e.m. Numbers in parentheses indicate group sizes. Note that after subtracting the number of days engaged in a persistent turn strategy, there was still a significant main effect of estradiol treatment on performance in middle-aged rats, but the effect of Don +E in aged rats was completely eliminated. ^aIndicates a significant main effect of E-treatment ($p < 0.05$); * $p < 0.05$ relative to controls and to Don-treated rats. [†] $p < 0.08$ relative to controls.

Figure 2.

Plot showing the effects of the treatments on the DMP learning curves for middle-aged (A) and aged (B) rats. Points represent the mean percent correct for each group $\pm$ s.e.m. Group sizes are indicated in Figure 1. *Indicates a significant main effect of E treatment, $p < 0.05$; †Don-treated rats differ significantly from Don+E-treated rats, $p < 0.05$. §Don+E-treated rats differ significantly from both controls and Don-treated rats, $p < 0.05$.

Figure 3.

Bar graphs summarizing the effects of increasing the intertrial delay on the performance of each treatment group for middle-aged (A) and aged (B) rats. Bars represent mean percent correct $\pm$ s.e.m. Group sizes are the same as in Figure 1 except that the number of Don-treated aged rats is reduced by '1', since one rat did not reach criterion. Note that increasing the delay decreased performance in all treatment groups. No significant effects of treatment were detected in middle-aged or aged rats. * $p < 0.05$ relative to controls.

Figure 4.

Plots summarizing the effects of treatments on the number of responses (maximum of 30) to the simple and configural stimuli during acquisition of the CA task by middle aged (A, C, E) and aged (B, D, F) rats. Each data point represents a group mean $\pm$ s.e.m. Both middle-aged and aged rats acquired the simple associations very quickly as demonstrated by the high number of responses (panels A–D). In response to the configural stimulus, rats initially increased their responses and then reduced responses as they learned to distinguish between the lack of reward associated with responding to the configural stimulus versus the positive reward associated with responding to the simple stimuli (panels E & F). Treatments did not significantly affect the number of responses in middle-aged rats. Treatments did produce small but significant

improvements in acquisition of the simple stimuli by aged rats. ^{aa}Don-, E-, and Don+E treated rats differ significantly from controls, $p < 0.05$; ^{bb}Don- and E-treated rats differ significantly from controls, $p < 0.05$. The legend in panel C applies to all panels.

Figure 5.

Plots summarizing the effects of treatments on response time to the simple and configural stimuli during acquisition of the CA task by middle aged (A, C, E) and aged (B, D, F) rats. Each data point represents a group mean $\pm$ s.e.m. Both middle-aged and aged rats quickly learned to associate light or tone with food reward, as indicated by the rapid decrease in response time (panels A–D). In response to the configural stimulus, rats initially decreased their response time, but then increased their response time as they learned to distinguish between the lack of reward associated with the configural stimulus versus the positive reward associated with the simple stimuli (panels E & F). Treatments did not significantly affect response time in middle-aged rats, but did increase response time to both simple and configural stimuli in aged rats. ^{cc}Indicates a significant main effect of estradiol treatment, $p < 0.05$; ^{dd}Don-, E-, and Don+E treatments differ significantly from controls, $p < 0.05$.

Figure 6.

Hypothetical model in which the critical period for attaining beneficial effects of estrogen therapy on cognitive performance is determined by a decline in basal forebrain cholinergic function. The model assumes that cholinergic function declines with age, and that the rate of decline is accelerated by loss of ovarian function. Threshold refers to the minimum level of cholinergic function necessary to confer beneficial effects of estrogen therapy on a cognitive task. The critical period is defined as the time between the loss of ovarian function and the decline in cholinergic function to threshold. Based on this model, loss of ovarian function at middle-age (open arrow) is associated with a shorter critical period (C2) than is loss of ovarian function at a younger age (filled arrow; C1).

Table 1

Effects of Treatments on ChAT Activity in Different Regions of the Brain

	ChAT (Middle-Aged)	ChAT (Aged)
FCX	**	
Controls	36.0 ± 1.6	38.3 ± 2.4
Don	33.0 ± 1.9	33.2 ± 2.0
E	37.3 ± 1.5	34.4 ± 1.6
Don+E	39.6 ± 1.6 ^b	35.0 ± 2.2
HPC	**	**
Controls	25.3 ± 1.3	30.8 ± 1.4
Don	29.5 ± 1.2	27.5 ± 1.5
E	33.9 ± 1.9 ^a	33.3 ± 1.2 ^b
Don+E	30.1 ± 1.2	28.4 ± 1.3
PFCX	**	
Controls	41.4 ± 2.1	46.9 ± 2.2
Don	43.0 ± 0.9	42.8 ± 3.0
E	51.9 ± 2.7 ^{a,b}	45.1 ± 6.3
Don+E	46.2 ± 1.9	43.9 ± 2.9
RS/E		
Controls	36.9 ± 1.3	37.0 ± 1.8
Don	33.9 ± 2.0	37.8 ± 2.2
E	36.1 ± 2.2	39.1 ± 5.3
Don+E	37.8 ± 1.5	39.7 ± 3.3

FCX=frontal cortex, HPC=hippocampus; PFCX=prefrontal cortex;

RS/E=retrosplenial/entorhinal cortex; Values represent group means ± s.e.m expressed as pmol ACh produced/hr/μg protein

** Indicates a significant main effect of treatment (p<0.05)

^a differs significantly from Controls;^b differs significantly from Don