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BRIEF COMMUNICATION

Lasting Impact on Memory of Midlife Exposure to Exogenous and Endogenous Estrogens

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We previously demonstrated that 40 days of prior midlife estradiol treatment results in enhanced spatial memory in aging ovariectomized rats long after termination of the estradiol treatment. Our current goal was to determine whether this benefit is due to lasting impacts on memory specifically of previous exogenous estradiol treatment or simply due to delaying cognitive deficits that occur following loss of ovarian hormones. Middle-aged rats were ovariectomized or underwent sham surgery. Ovariectomized rats received estradiol (Previous Estradiol) or vehicle (Previous Vehicle) implants. Rats undergoing sham surgery (Previous Intact) received vehicle implants. Forty days later, Previous Intact rats were ovariectomized, the other 2 groups underwent sham surgeries, and all implants were removed. Thus, no ovarian or exogenously administered hormones were present during behavior testing. Rats underwent 24 days of acquisition training on an 8-arm radial maze. Following acquisition and again 2 months later, rats were tested on delay trials, during which animals had to remember the location of food rewards across time delays inserted between fourth and fifth arm choices. During acquisition, rats that had previous extended exposure to exogenous estradiol (Previous Estradiol) and endogenous ovarian hormones (Previous Intact) significantly outperformed rats that did not experience extended hormone exposure (Previous Vehicle). However, during delays trials the Previous Estradiol group significantly outperformed both the Previous Vehicle and Previous Intact groups. Results demonstrate that whereas extended exposure to endogenous ovarian hormones may provide short-term cognitive benefits, midlife estradiol treatment following ovariectomy provides additional benefits that persist for months following termination of treatment.

Keywords: estradiol, memory, aging, hippocampus, ovariectomy

Decline in estradiol, the primary estrogen synthesized by the ovaries, is associated with impaired cognition and increased risk of Alzheimer's disease or age-related dementia (Henderson, Watt, & Buckwalter, 1996; Kawas et al., 1997; Tang et al., 1996). Furthermore, there is an increased risk of cognitive decline and dementias following premenopausal oophorectomy and early-onset menopause (Kurita et al., 2016). Women who received either uni- or bilateral oophorectomies and were otherwise healthy had poorer cognition later in life and were at risk for age-related dementias. The earlier in life surgery took place, the greater the risk for cognitive decline (Rocca et al., 2007). However, there is evidence that treatment with estrogens immediately following surgical or natural menopause is beneficial for cognition and can reduce risk and severity of Alzheimer's disease (Kawas et al., 1997; Paganini-Hill & Henderson, 1996; Tang et al., 1996).

Because of putative health risks associated with long-term chronic exposure to estrogens (Chen et al., 2006; Chen & Colditz, 2007), it is recommended that women use estrogen therapy to treat menopausal symptoms for the shortest time possible. Long-term implications of short-term hormone use are currently under investigation. Compared with women who never received hormone treatment, women who used hormone therapy previously in midlife for 2–3 years had decreased risk of cognitive impairment with age (Bagger et al., 2005). Similarly, women who used hormone therapy only in midlife had decreased risk of dementia compared with both never-users and those taking hormone therapy only in late life (Whitmer, Quesenberry, Zhou, & Yaffe, 2011).

In our laboratory, we use a rat model of menopause to investigate the long-term effects of short-term, midlife estradiol use. Ovariectomized, aged rats previously treated with 40 days of estradiol in midlife, which is roughly comparable with 3 years in humans, show enhanced memory on the hippocampal-dependent radial-arm maze task compared with ovariectomized controls when tested 1 (Witty, Gardella, Perez, & Daniel, 2013), 2.5 (Rodgers, Bohacek, & Daniel, 2010), and 7 (Rodgers et al., 2010) months after termination of estradiol exposure. Because aging rats previously treated in midlife

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with 40 days of estradiol rats are exposed to hormones for a longer period of time as compared with the ovariectomized controls, it is yet to be determined whether long-term enhanced cognition is a direct result of previous chronic, unopposed estradiol treatment or simply because of extended exposure to circulating estrogens. Thus, the aim of the present study was to test the hypothesis that previous midlife estradiol treatment uniquely enhances cognition long-term as compared to delayed ovariectomy.

Method

Subjects

Twenty-six middle-aged female Long-Evans hooded rats, retired breeders (~11 months of age), were purchased from Envigo, Inc. (Indianapolis, IN) Animal care was in accordance with guidelines set by the National Institute of Health *Guide for the Care and Use of Laboratory Animals* (1996), and the Institutional Animal Care, and the Use Committees of Tulane University approved all procedures. Rats were housed individually in a temperature-controlled vivarium under a 12-hr light, 12-hr dark cycle and had unrestricted access to food and water. See Figure 1a for an overview of experimental procedures.

Surgeries and Hormone Treatments

Rats were ovariectomized or sham ovariectomized while under anesthesia induced by injection of ketamine (100 mg/kg ip; Bristol Laboratories, Syracuse, NY) and xylazine (7 mg/kg ip; Miles Laboratories, Shawnee, KS) and implanted with 5-mm SILASTIC brand capsules (0.058 in. inner diameter and 0.077 in. outer diameter; Dow Corning, Midland, MI) on the dorsal aspect of their necks. Rats that were ovariectomized received capsules that contained 25% 17β-estradiol (Previous Estradiol; Sigma-Aldrich, St.

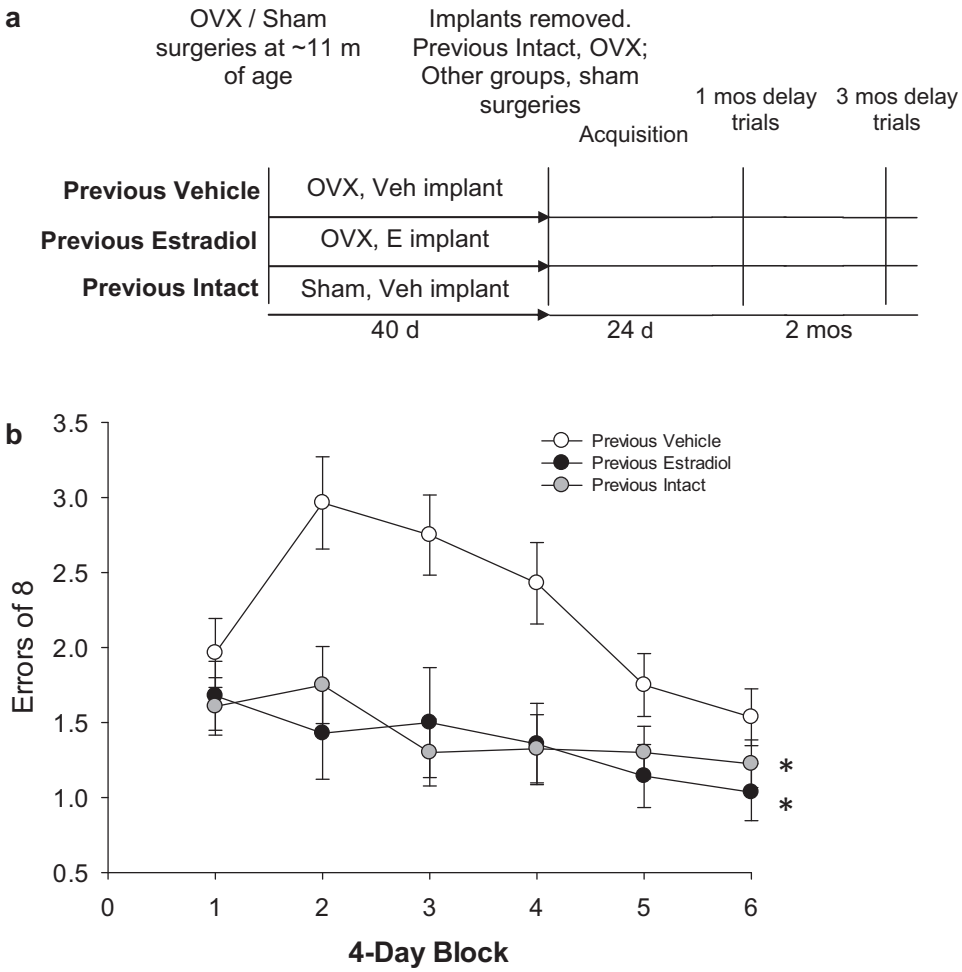

Figure 1. Experimental timeline and radial-maze acquisition. (a) Overview of experimental manipulations and behavioral testing. OVX, ovariectomy; Veh, cholesterol vehicle; E, estradiol. (b) Effects on acquisition of a radial-arm maze task in middle-aged ovariectomized rats of previous exposure to 40 days of exogenous estradiol treatment (Previous Estradiol), previous exposure to the same period of endogenous ovarian hormones (Previous Intact), or previous exposure to vehicle treatment (Previous Vehicle). Mean (±*SEM*) in first eight arm choices presented in 4-day blocks across 24 days of acquisition. * $p < .05$ versus Previous Vehicle.

Louis, MO) diluted with cholesterol or 100% cholesterol vehicle (Previous Vehicle). Rats that were sham ovariectomized received 100% cholesterol vehicle capsules (Previous Intact). Implants maintain blood plasma estradiol levels at approximately 37 pg/mL (Bohacek & Daniel, 2010), which falls in the physiological range.

Forty days after initial surgeries, rats were anesthetized with ketamine and xylazine and all capsules were removed. Visual inspection confirmed their integrity. Rats that previously underwent sham surgery were ovariectomized (Previous Intact) and rats that were previously ovariectomized underwent sham surgeries (Previous Vehicle, Previous Estradiol). Therefore, no rats had ovarian or exogenously administered estrogens during behavioral testing.

Maze Training

Five days following termination of hormone treatment and at which point circulating hormones should not be detectable (Woolley & McEwen, 1993), rats began training on the spatial memory radial-maze task (Olton & Werz, 1978). Rats were placed on diets and weighed daily to maintain body weights at 85–90% of presurgery weights and trained to obtain food rewards (Froot Loops; Kellogg Co., Battle Creek, MI) from the arms of an eight-arm radial maze purchased from Lafayette Instruments (Lafayette, IN). The maze consisted of black metal floors and clear acrylic walls with arms (10 cm wide $\times$ 70 cm long $\times$ 20 cm high) extending out from an octagonal center (33 cm across). The maze was located in the center of a 3 $\times$ 5 m room and raised approximately 1 m from the floor. Extramaze cues, including overhead fluorescent lights, desk, chairs, sink, and door, were visible from the maze.

To begin a trial, a rat was placed in the center compartment in a pseudorandom orientation with access to all eight arms. Arm choices were recorded by an observer seated in a fixed location approximately 1 m away from the maze. An arm choice was scored if the rat traversed halfway down an arm. Rats could choose arms in any order until all arms were visited or 5 min elapsed. Arm-choice accuracy was measured by mean errors of the first eight choices. Each animal received one trial per day across 24 days of acquisition.

Behavioral Testing

Following acquisition, testing on the first of two sets of delay trials began. During delay trials, various delays (1, 2.5, or 4 hr) were imposed between the fourth and fifth arm choices. Delays required that rats remember over an extended period which arms had already been visited. After four correct arm choices, the animal was removed from the maze and put in a holding cage in a separate room. Following the delay, the animal was returned to the maze until the four remaining, still baited arms had been visited or until 5 min had elapsed. Errors were reentries into previously visited arms. Arm-choice accuracy was measured by mean errors of eight, which included errors before the delay and errors during the first four arm choices after delay. Rats received 1 day of habituation to a 1-min delay trial. Subsequently, two daily trials were conducted for each longer delay. A second set of delay trials took place 2 months later (and 3 months after hormone exposure was terminated in the Previous Intact and Previous Estradiol groups). Between the first and second sets of delays, rats received weekly 1-min delay trials to maintain performance.

Hormone Treatment and Ovariectomy Efficacy

Daily vaginal smears were collected by lavage during the final week of hormone exposure confirmed treatment efficacy. Smears of ovariectomized, vehicle-treated rats were characterized by a predominance of leukocytes, and smears of ovariectomized, estradiol-treated rats were characterized by a predominance of cornified and nucleated epithelial cells. Smears of naturally cycling rats were characterized by slightly extended but normal cycling. When rats were killed, right uterine horns were extracted and weighed to verify ovariectomy. Two rats (one Previous Vehicle and one Previous Estradiol) were dropped because of the presence of large uterine horns. The final number of rats included in the experiment was 24 (Previous Vehicle = 7, Previous Estradiol = 7, Previous Intact = 10).

Statistical Analyses

Acquisition data were averaged across 4 consecutive days (blocks) and analyzed by two-way ANOVA (Treatment $\times$ Block) with repeated measures on block. Delay trial data were averaged across the 2 consecutive days of testing at each set of delays and analyzed by three-way ANOVA (Treatment $\times$ Delay $\times$ Time Point) with repeated measures on delay and time point. Post hoc tests (Fisher's lease significant differences test, $p < .05$) were used to probe group differences following significant main effects.

Results

Acquisition

As illustrated in Figure 1b, rats that had previously experienced extended exposure to either exogenous estradiol or ovarian hormones displayed increased arm choice accuracy during acquisition of the radial-maze task as compared with vehicle-treated rats. There was a significant main effect of training block, $F(1, 21) = 22.743$, $p < .001$, indicating improvement across blocks. There was a significant main effect of treatment, $F(2, 21) = 7.818$, $p = .003$. Post hoc analysis revealed that the Previous Estradiol and Previous Intact groups had significantly fewer errors than the Previous Vehicle group across acquisition. No interaction between block and treatment was revealed.

Delay Trials

As illustrated in Figure 2, rats that had previously experienced extended exposure to exogenous estradiol displayed enhanced memory as indicated by increased arm choice accuracy during delay trials as compared with vehicle-treated rats as well as compared with rats that had previously experienced extended exposure to endogenous ovarian hormones. There was a significant effect of treatment, $F(2, 21) = 3.671$, $p = .045$. Post hoc analyses revealed that the Previous Estradiol group made significantly fewer errors than both the Previous Vehicle and Previous Intact groups across trials (Figure 2c). There was a near significant effect of delay ($F[2], 42 = 3.155$, $p = .053$), indicating that as the time delay increased, the number of errors increased (Figure 2a and b). There were no significant effects of time point nor an interaction of time point and treatment, indicating that the effect of treatment was

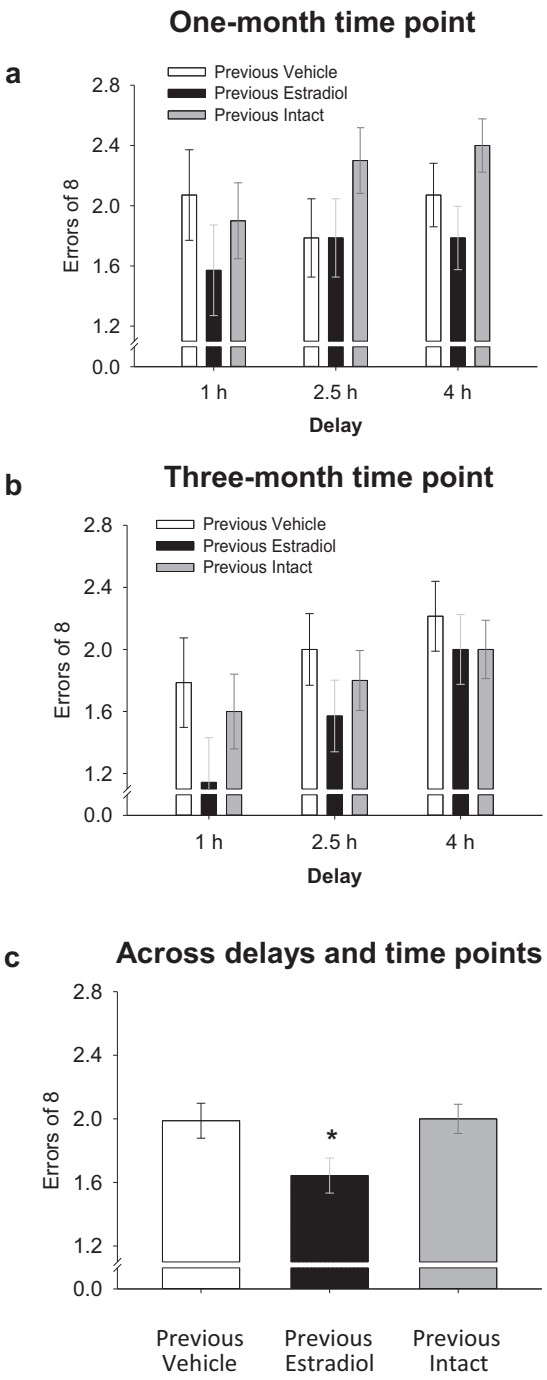

Figure 2. Effects on delay trials on a radial-arm maze task in middle-aged ovariectomized rats of previous exposure to 40 days of exogenous estradiol treatment (Previous Estradiol), previous exposure to the same period of endogenous ovarian hormones (Previous Intact), or previous exposure to vehicle treatment (Previous Vehicle). Delays were imposed between the fourth and fifth arm choices. Rats were tested at (a) 1 and (b) 3 months following termination of hormone exposure in Previous Estradiol and Previous Intact groups (a and b). Mean ($\pm SEM$) in first eight arm choices presented across delays. (c) Mean number of errors ($\pm SEM$) of first eight arm choices averaged across delays and time points. * $p < .05$ versus Previous Vehicle and Previous Intact.

consistent across the 1-month and 3-month time points. There were no other significant interactions.

Discussion

Results of the present study indicate that the ability of previous exposure to chronic midlife estradiol treatment in ovariectomized rats to exert long-term benefits for memory (Rodgers et al., 2010; Witty et al., 2013) is uniquely due to the impact of the exogenous estradiol and not simply due to postponing the negative impacts of ovarian hormone deprivation. We found that ovariectomized rats that had previous extended hormone exposure, either via estradiol administration or prolonged exposure to endogenous ovarian hormones, learned a spatial memory radial-maze task better than ovariectomized controls that were not exposed to extended estrogens. However, rats that had previously received exogenous estradiol treatment displayed enhanced memory across delay trials, when memory load is increased, as compared with ovariectomized controls as well as those with previous prolonged exposure to endogenous ovarian hormones. The enhancement was long term because it was evident up to 3 months following the termination of estradiol treatment. These data support our hypothesis that previous exposure to midlife estradiol treatment is unique in its ability to enhance cognition over the long term.

Forty days of previous exposure to exogenous estradiol treatment enhanced performance during acquisition of the radial-maze task, which began shortly after the removal of the estradiol treatment, as well as during delay trials conducted at 1 and 3 months following removal of the estradiol treatment. The fact that the same period of previous exposure to endogenous ovarian hormones enhanced performance only during acquisition but not during subsequent delay trials could be due to differences in time since ovariectomy or to differences in memory demands of the tasks. In humans, surgical menopause impairs cognition in an age-dependent manner, such that the earlier in life oophorectomy occurs, the greater the risk for cognitive decline later (Rocca et al., 2007). However, to our knowledge there is no evidence that undergoing delayed natural menopause has any impact, positive or negative, on cognition.

The present study demonstrates that previous chronic estradiol exposure, rather than cyclical, endogenous exposure, is necessary to enhance cognition long after termination of hormone exposure. There is evidence that in nonhuman primates ongoing phasic estradiol treatment can improve cognition (Rapp, Morrison, & Roberts, 2003). There are also multiple reports that cyclical injections of estradiol following ovariectomy can improve spatial cognition in rodents (Aenlle, Kumar, Cui, Jackson, & Foster, 2009; Frick, Fernandez, & Bulinski, 2002; Gresack & Frick, 2006; Walf, Koonce, & Frye, 2008; Xu & Zhang, 2006). Whereas phasic estradiol treatment is meant to mimic the natural cyclical nature of endogenous estrogens, it is unclear whether phasic treatment can enhance hippocampal-dependent cognition long after treatment has ended, as we have demonstrated using chronic, unopposed estradiol treatment (Rodgers et al., 2010; Witty et al., 2013).

Beyond demonstrating that previous, short-term chronic estradiol treatment enhances cognition long term, our laboratory has also found that previous estradiol treatment increases hippocampal protein expression of estrogen receptor (ER)-α at 1 (Black, Witty, & Daniel, 2016; Witty et al., 2013), 2.5 (Rodgers et al., 2010), and

8 (Rodgers et al., 2010) months following termination of previous estradiol treatment. Furthermore, increased levels of hippocampal ERα mediate lasting cognitive benefits of short-term estradiol treatment. We found in aging, ovariectomized rats increasing levels of hippocampal ERα via lentivirus is sufficient to enhance cognition (Witty, Foster, Semple-Rowland, & Daniel, 2012). In a complementary experiment, we found activation of ERs is necessary to enhance cognition following short-term estradiol treatment in aging, ovariectomized rats (Black et al., 2016). Together these experiments demonstrate that ERs, primarily ERα, mediate cognitive enhancement following short-term estradiol treatment. Further work is required to determine protein expression of hippocampal ERα in aged rodents that underwent delayed ovariectomy, but we hypothesize they would have decreased expression as compared with rats that received 40 days of chronic estradiol treatment. In conclusion, the current experiment provides evidence that previous estradiol exposure following ovariectomy is unique in its ability to enhance cognition long term following termination of hormone treatment.

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