

Original Article

Estrogen and progesterone improve scopolamine-induced impairment of spatial memory

Fumiko Tanabe, Naoyuki Miyasaka, Toshiro Kubota and Takeshi Aso

Comprehensive Reproductive Medicine, Graduate School, Tokyo Medical and Dental University

This study was conducted to investigate the effects of estrogen and progesterone on spatial memory in ovariectomized female rats, specifically, on memory impaired by the cholinergic antagonist scopolamine. Forty-one female rats were divided into five groups: ovariectomized (OVX), estrogen-treated after ovariectomy (OE), progesterone-treated after ovariectomy (OP), estrogen-progesterone-treated after ovariectomy (OEP), and the sham control group (Control). The animals were trained on an eight-arm radial maze with four arms baited to assess both working and reference memory performances. The OE and OEP groups showed significant improvement in the ability to perform a spatial memory task over the OVX group ($P < 0.05$). Spatial memory in the OP group did not differ from that in the OVX group. After thirty-two trials were conducted and all rats learned the eight-radial maze task, scopolamine hydrobromide (0.2 mg/kg i.p.) was administered prior to retesting. After scopolamine injection, the OVX group showed an increased number of working memory errors, reference memory errors than the other groups ($P < 0.05$). The OE, OEP and OP groups showed significant improvement in spatial impairment induced by scopolamine. These findings suggest progesterone alone or in combination with estrogen, improved scopolamine-induced

impairment of working memory and reference memory as effectively as estrogen supplementation.

Key words: estrogen, progesterone, scopolamine, working memory, radial maze

Introduction

Estrogen replacement therapy (ERT) has been prescribed for postmenopausal women not only to treat climacteric disorders but also for osteoporosis¹. Recently, there is growing interest in the effects of estrogen on the central nervous system (CNS), especially, on cholinergic function in cognition^{2,3,4,5}. In Alzheimer's disease patients, acetylcholine (ACh) and choline acetyltransferase (ChAT) activity are reduced in cerebral cortex, hippocampus and nucleus basalis of Meynert, and those reductions correlate with impairment of memory for recent events (working memory)⁶. Recent studies have shown that ERT significantly increases verbal memory^{7,8,9}, and may potentially prevent cognitive dysfunction and Alzheimer's disease in postmenopausal women^{10,11}. And estrogen appears to have many cognitive effects not only on cholinergic system but also on glutamatergic system and other regions of the brain, such as increasing cerebral blood flow, and neuroprotection against glutamate neurotoxicity as well as oxygen radicals^{2,12,13,14,15,16,17}.

Progesterone is an ovarian hormone that is used clinically in combination with estrogen as hormone replacement therapy (HRT). It is well known that progesterone supplementation can reduce the occurrence of endometrial cancer in postmenopausal women

Corresponding Author: Fumiko Tanabe
Comprehensive Reproductive Medicine, Graduate School, Tokyo Medical and Dental University
Yushima 1-5-45, Bunkyo-ku, Tokyo 113-0089, Japan
Tel: +81-3-5803-5322
Fax: +81-3-5803-0148
E-mail address: f.tanabe.gyne@tmd.ac.jp
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receiving ERT¹. However, little is known about the effects of progesterone on cognitive functions in HRT. Progesterone has sedative-like effects^{18,19}. And its inhibitory effects on learning^{20,21,22}; antagonistic effects on σ_1 receptors play a potent neuromodulatory role in cholinergic and N-methyl-D-aspartate (NMDA) receptor-mediated glutamatergic neurotransmissions, have been reported in many behavioral tests in rodents^{23,24,25}. On the contrary, Progesterone injection given after injury was effective to reduce impairments on a Morris water maze spatial navigation task²⁶. And it was reported that medroxyprogesterone in combination with estrogen moderately improved cognitive scores in women^{27,28}. These effects of progesterone on cognition are, therefore, controversial. Recently, Women's Health Initiative (WHI) reported that the risks of invasive breast cancer and coronary heart disease are associated with HRT^{1,29}. To assess the health benefits of the progesterone in combination with estrogen treatment, it is important to clarify the effects of progesterone on cognitive functions.

In learning process, there are two components; short term memory for recent events (working memory) and long term acquired memory for past events (reference memory). Previous behavioral studies in rodents have indicated that estrogen improves working memory^{30,31} and prevents amnesic effects of scopolamine a muscarinic acetylcholine receptor antagonist^{32,33,34}. Therefore, a question of progesterone's effect, alone or in conjunction with estrogen, on spatial memory after cholinergic neural damage induced by scopolamine needs to be answered.

We tested the hypothesis that progesterone alone supplementation could not affect the impairment of spatial memory induced by estrogen depletion or by scopolamine in ovariectomized female rats. The purpose of this study is to determine the effect of progesterone treatment on the impaired working and reference memories induced by scopolamine in ovariectomized female rats, and to compare these effects to those seen with estrogen and/or progesterone treatments in rats subjected to the same spatial memory task.

Materials and Methods

Subjects

Forty-one female Sprague-Dawley rats were used in this experiment. During this study, all the animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals at Tokyo Medical and

Dental University. The rats were housed in a temperature-controlled vivarium, under a 12-hour light 12-hour dark cycle. Food and water were freely available until the maze training began.

Hormone treatment

The rats were divided into five groups: ovariectomized (OVX, $n = 11$), estrogen-treated after ovariectomy (OE, $n = 7$), progesterone-treated after ovariectomy (OP, $n = 8$), estrogen-progesterone-treated after ovariectomy (OEP, $n = 7$), and the sham operation group (Sham control, $n = 8$).

At 10 weeks old, the experimental rats under sodium pentobarbital (50 mg/kg i.p.) anesthesia were subjected to bilateral ovariectomy. A pellet containing 17 β -estradiol (1.5 mg, 90-day-release pellet; Innovative Research of America, Florida, USA) was implanted subcutaneously in each rat of the OE group. A pellet containing progesterone (200 mg, 90-day-release pellet; Innovative Research of America, Florida, USA) was implanted subcutaneously in each rat of the OP group. An estrogen pellet and a progesterone pellet were both implanted subcutaneously in each rat of the OEP group. The rats of the sham control group were subjected to sham operation. Serum estrogen and progesterone levels were measured by radioimmunoassay 8 weeks after operation and pellet administration. (Radioimmunoassay kits: Diagnostic Production Corporation, Los Angeles, USA.). Dose-ranges and administrations routes were selected according to the previous related studies^{30,32,33,35}.

Eight-radial maze

Apparatus

An eight-arm radial maze (Video Image Motion Analyzer AXIS90, TARGET 1, Neuroscience Co., Japan) was utilized for spatial memory testing. Eight arms (10-cm wide, 50-cm length) extended from the octagonal central chamber (30 cm in diameter) and were divided by experimenter-controlled guillotine doors from the central chamber. The walls were made of transparent acrylic resin. At the end of each arm, a recessed food cup was located on the floor. Various extra-maze cues, including a table, chairs, shelves, pictures, and an experimenter were distributed throughout the maze. These cues remained in the same position with respect to the maze until all experiments were complete.

Initial training

Two weeks after the operation, the rats were handled

for 5 minutes a day for 7 days, for weight measurement and initial behavioral training. They were fed approximately 1 hour per day until their body weights decreased to 85% of the free-feeding level to reinforce and stimulate appetite during the behavioral test in each hormone condition. Water was freely available. During the 7-day initial training, all rats were allowed approximately 15 min a day to explore the apparatus containing food pellets at the end of each arm. The initial behavioral training and behavioral test for each rat were administered during the daylight period at each same time of the day.

Behavioral test

After the 7-day initial training, therefore three weeks after operation, the behavioral test was initiated. During the radial arm maze tests four cups were baited and the other four cups unbaited. The locations of the four baited arms and extra-maze cues did not change throughout all the behavioral tests. Before each trial, one food pellet was placed in each of the four cups of the baited arms. At the start of each trial, a rat was placed at the central chamber of the maze, then all eight doors were opened silently. The rat was allowed to freely explore the maze. When it entered an arm, the doors to other arms were closed. The rat was allowed to proceed to the end of the arm, eat the reward, and exit from the arm. When the rat returned to the central chamber, all the doors were closed. After 5 seconds, all the doors were opened again. This sequence was repeated until four rewards had been consumed or until 10 min had elapsed. The rat was considered to have entered an arm when the central point of its body was more than 10 cm in the arm. Performance measures that were recorded during these training trials included *number of errors* defined as either failure to proceed to the end of the arm or failure to eat a reward. Measures also consisted of the *number of correct choices* defined as successful entry into a baited arm to eat the food pellet, the *number of working memory errors* defined as entry to an arm that previously contained food but already consumed, the *number of reference memory errors* defined as entry to an unbaited arm, and the *number of total errors* defined as all errors committed in all arms. The *time* taken to consume all four rewards was recorded, and the *distance* of activity was also measured by the TARGET system, which follows the trail of the rat during the trial.

All the rats continued to perform one trial per day until they reached the criterion, that is the mean of the number of correct choices within 4 days was more than 3, or

until they had undergone 32 days of training.

Scopolamine injection

When the thirty-second maze trial had been performed, each rat was subjected to intraperitoneal-scopolamine hydrobromide injection (0.2 mg / kg). Thirty minutes after the injection, each rat performed the final trial. Dose-range and administration route was selected according to the previous related studies^{33,34,35,36,37,38}.

Data analysis

Hormone condition in rats of sham control group was cyclic (almost 4-day cycle), as those in rats of other groups were constant. To eliminate the effect of estrus, data were grouped into eight blocks, one block includes 4-day data; (first block: 1-4 trials, second block: 5-8 trials, . . . , eighth block: 28-32 trials). The effects of hormone conditions on the required blocks to reach the learning criterion were analyzed using the one-way ANOVA and the Bonferroni/Dunn post-hoc tests. Statistical significance was defined as $P < 0.05$. In order to evaluate the learning ability of spatial task, the effects of hormone conditions in the learning process on the six parameters (correct choice, total error, working memory error, reference memory error, time, distance) were analyzed using the two factor (hormone condition by block) ANOVA with repeated measures on 'block'. Individual group comparisons were made using the Bonferroni/Dunn test.

In order to evaluate the influence of scopolamine, we analyzed differences in the data between pre and post scopolamine injection using the two factor (hormone condition by time) ANOVA with repeated measures on 'time' (pre and post scopolamine injection). Individual group comparisons were made using the Bonferroni/Dunn test.

For hormone measurement analyses, the one-way ANOVA and the Bonferroni/Dunn post-hoc tests were used. Data are shown as means \pm SD. $P < 0.05$ was considered statistically significant.

Results

Eight-radial maze performance

At the start of maze training (1st block), there was no difference in locomotor activity and the mean number of correct choices or other parameters among the rats of all hormone condition groups. All the rats initially showed poor acquisition ability. With advancing the 4-

Mean number of correct choices

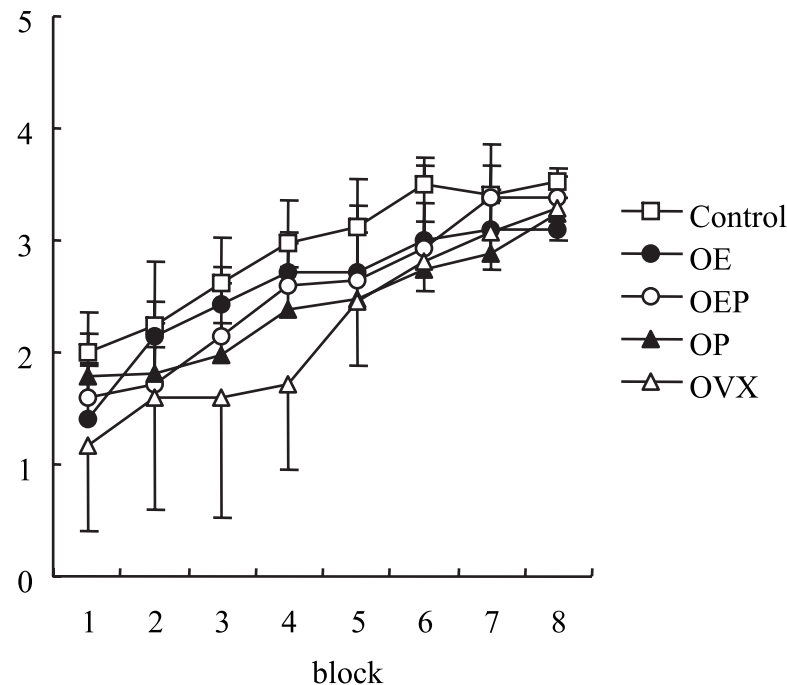


Fig. 1. Effects of each hormone condition on mean number of correct choices in learning curve. The rats in the OVX and OP groups also required more blocks to reach the learning criterion than that of the Control group (Bonferroni/Dunn test, $P < 0.05$). Values are Means \pm SD. Control: sham control group, OVX: ovariectomized group, OE: estrogen-treated group after ovariectomy, OEP: estrogen-progesterone-treated group after ovariectomy, OP: progesterone-treated group after ovariectomy.

day blocks, a learning curve was evident and a significant effect of treatment on the number of correct choices was found in learning process. The number of correct choices was significantly lower in the OVX and OP groups than in the control group [$F(28, 252) = 1.64$, $P = 0.025$, Bonferroni/Dunn test, $P < 0.05$] (Fig. 1). The rats in the OVX and OP groups also required more blocks to reach the learning criterion than that of the Control group [$F(4, 36) = 8.630$, $P < 0.001$, *Bonferroni/Dunn test, $P < 0.05$] (Table 1).

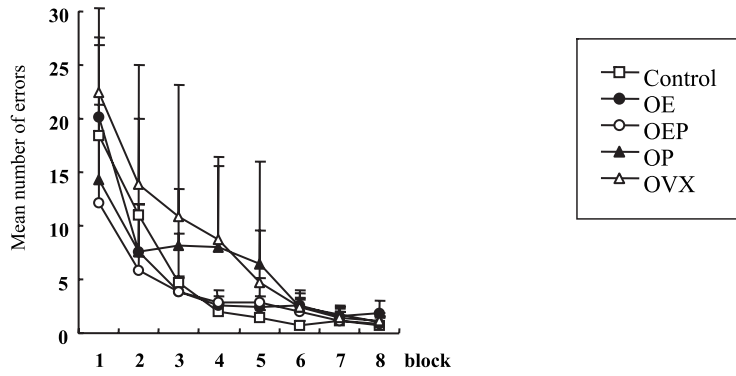
In the learning process, the OVX and OP groups showed not only a decreased number of correct choices [$F(28, 252) = 1.644$, $P = 0.025$] (Fig. 1), but also an increased number of total error [$F(28, 252) = 1.905$, $P = 0.005$], working memory error [$F(28, 231) = 2.572$, $P < 0.0001$], reference memory error [$F(28, 252) = 1.655$, $P = 0.0238$], trial time [$F(28, 252) = 2.115$, $P = 0.0013$] and distance [$F(28, 252) = 1.873$, $P = 0.0064$] than the control group (Bonferroni/Dunn test, $P < 0.05$) (Fig. 2 A-E). On the contrary the OE and

Table 1. Effects of each hormone condition on the number of 4-day time blocks to reach the learning criterion of correct choice

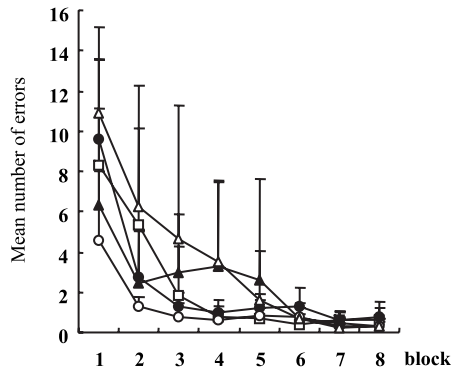
Group	N	block number to reach learning criterion
Control	8	4.13 \pm 0.83
OVX	11	6.64 \pm 1.02 *
OE	7	5.14 \pm 0.90
OEP	7	5.16 \pm 1.72
OP	8	6.50 \pm 1.51 *

Values are Means \pm SD. *statistically significant difference relative to control ($P < 0.05$). Control: sham control group, OVX: ovariectomized group, OE: estrogen-treated group after ovariectomy, OEP: estrogen-progesterone-treated group after ovariectomy, OP: progesterone-treated group after ovariectomy. The rats of OVX and OP groups required more blocks to reach the learning criterion than that of Control group.

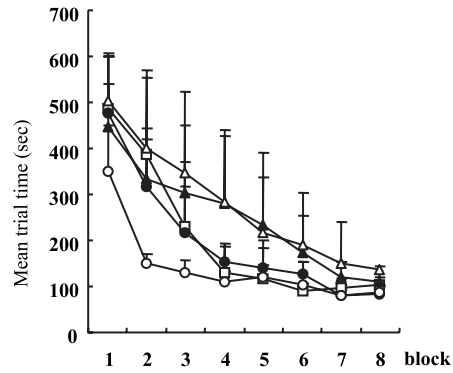
A : Total errors



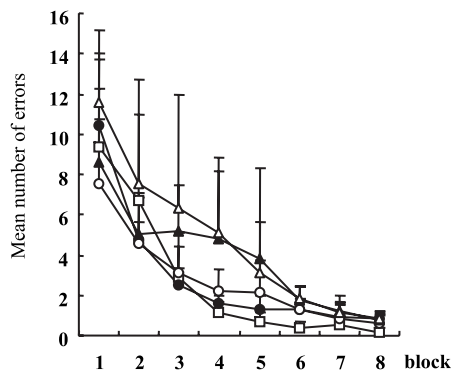
B : Working memory errors



D : Mean trial time



C : Reference memory errors



E : Mean distance

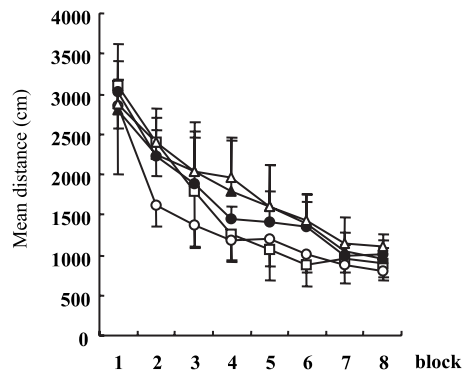


Fig. 2. Effects of each hormone condition on number of total errors (A), working memory errors (B), reference memory errors (C), time (D), and distance (E). In the learning process, the OVX and OP groups showed an increased number of total error, working memory error, reference memory error, trial time and distance than the control group (Bonferroni/Dunn test, $P < 0.05$). Values are Means \pm SD. Control: sham control group, OVX: ovariectomized group, OE: estrogen-treated group after ovariectomy, OEP: estrogen-progesterone-treated group after ovariectomy, OP: progesterone-treated group after ovariectomy.

OEP groups showed significantly improved acquisition ability of a spatial memory task based on six parameters. There were no differences in acquisition ability among members of the OE and OEP and sham control groups based on each of these six parameters. Throughout thirty-second trials all the rats learned the eight-radial maze task and no difference in performances was observed among all groups.

Scopolamine injection

Analysis of the differences before and after scopolamine injection revealed that there was no significant difference in the mean number of correct choices among all groups (Fig. 3 A). However, the OVX group had a significant deficiency in performing the tasks after scopolamine treatment when compared to the other groups for the other parameters, namely, total error [F(4, 36) = 5.713, P = 0.0013], working memory error [F(4, 36) = 5.072, P = 0.0026], reference memory error [F(4, 36) = 2.910, P=0.0363], trial time [F(4, 36) = 6.080, P = 0.0008] and trial distance [F(4, 36) = 7.984, P<0.0001] (*Bonferroni/Dunn test, P<0.05) (Fig. 3 B-F). On the other hand, the OE, OEP and OP groups showed similarly improved acquisition based on the above five parameters when compared to the control group. There was no difference in acquisition ability after scopolamine treatment based on these five parameters among these groups (Fig. 3 B-F).

Hormone levels

In the control rats, mean diestrus serum levels of 17 β -estradiol and progesterone were 11.2 \pm 4.2 pg/ml and 16.6 \pm 7.5 ng/ml after sham operation (MEAN \pm SD). In the ovariectomized rats, mean serum levels of 17 β -estradiol and progesterone were significantly reduced after ovariectomy than that of sham control rats (*Bonferroni/Dunn test, P<0.05). Estrogen and progesterone replacements revealed that chronically administered hormones were within the nearly same ranges of sham control group rats, when measured 8 weeks after administration. The levels of 17 β -estradiol, F(4, 26) = 3.897, P = 0.0131, and progesterone, F(4, 25) = 4.018, P = 0.011, were significantly influenced by hormone replacement (Table 2).

Discussion

The purpose of this study is to determine the effect of progesterone treatment on the impaired working and reference memories induced by scopolamine in

Table 2. Mean estradiol and progesterone serum levels of rats in each hormone condition

Group	17 β Estradiol (pg/ml)	Progesterone (ng/ml)
Control	11.2 \pm 4.2	16.6 \pm 7.5
OVX	5.3 \pm 2.3 *	6.7 \pm 3.1 *
OE	9.8 \pm 3.6	9.7 \pm 6.1 *
OEP	10.2 \pm 3.8	16.5 \pm 3.4
OP	6.5 \pm 1.5 *	15.0 \pm 1.9

Values are Means \pm SD. *statistically significant difference relative to control (P<0.05). Control: untreated group, OVX: ovariectomized group, OE: estrogen-treated group after ovariectomy, OEP: estrogen-progesterone-treated group after ovariectomy, OP: progesterone-treated group after ovariectomy.

ovariectomized female rats, and to compare these effects to those seen with estrogen and/or progesterone treatments in rats subjected to the same spatial memory task. Our results indicate that progesterone alone supplementation could not affect the impairment of spatial memory induced by estrogen depletion in ovariectomized female rats, but reduce the impairment by scopolamine against the hypothesis.

It is well known that cholinergic systems in the brain play important roles in learning and memory. Previous studies have demonstrated that estrogen treatment can enhance working memory performance during acquisition of the radial maze task^{30,32,33,34}. This effect has been explained to be due to the induction of ChAT activity by estrogen. Indeed recent report indicated that estrogen but not progesterone restored the up-regulated muscarinic Ach receptor subtype due to ovariectomy-induced lack of Ach to that of normal rats^{39,40}. Furthermore estrogen improves memory processing by enhancing not only the cholinergic but also the glutamatergic system through σ_1 receptor^{14,15}. Thus, these reports support our results that estrogen treatment affected the impairment of spatial memory induced by hormone depletion or scopolamine in ovariectomized female rats.

On the other hand, progesterone exerts sedative-like effect²⁰ and is used in generating an amnesia model^{21,22,23,24}. Allopregnanolone a metabolite of progesterone acts like a sedative by enhancing the function of an inhibitory transmitter GABA (γ -aminobutyric acid) though GABA_A receptor and inhibits learning the

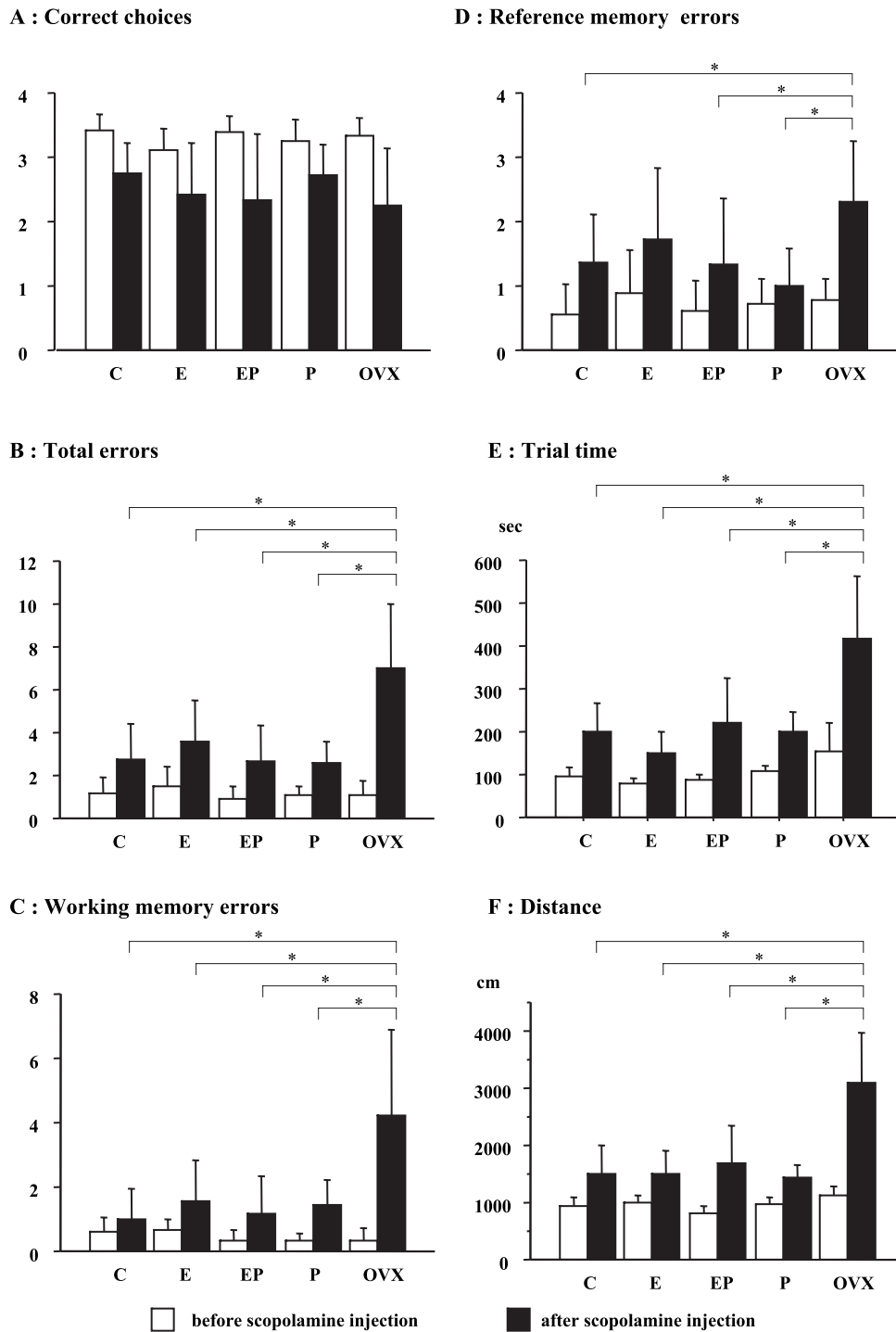


Fig. 3. Effects of each hormone condition on scopolamine-induced impairment. Behavior scores before (white bars) and after (black bars) injection. After scopolamine injection, decrease in Mean \pm SD number of correct choices (A), and increase in Mean \pm SD number of total errors (B), working memory errors (C), reference memory errors (D), time (E), and distance (F). Only the OVX group showed a significant increased number of total error, working memory error, reference memory error, trial time and distance than the control group. *statistically significant difference relative to OVX (Bonferroni/Dunn test, $P < 0.05$). Control: sham control group, OVX: ovariectomized group, OE: estrogen-treated group after ovariectomy, OEP: estrogen-progesterone-treated group after ovariectomy, OP: progesterone-treated group after ovariectomy.

Morris water maze^{21,22}. In other previous studies progesterone also antagonized the ameliorating effects of the selective σ_1 receptor agonist SA4503 on the working and reference memory deficits induced by dizocilpine (a non-competitive NMDA receptor antagonist)^{23,24}. Therefore, progesterone acts as a σ_1 receptor antagonist. These previous reports could support our result that progesterone alone could not improve the learning impairment induced by estrogen deprivation. This result could be explained to be due to the inhibitory effects of progesterone on neurotransmission.

Although progesterone alone has an antagonistic effect on σ_1 receptors in working memory as compared to action of estrogen as σ_1 receptor agonist^{23,24}, our results showed that progesterone in combination with estrogen did not attenuate the ameliorating effects of estrogen on working and reference memory deficits induced by ovariectomy or scopolamine. Previous studies demonstrated positive effects of progesterone that following the administration of progesterone, the effects of estrogen in ChAT mRNA expression or the dendritic spine density or the vesicular Ach transporter (VAcHT) as an index of cholinergic synaptic terminal density were significantly enhanced^{41,42}. Not only acute but also chronic progesterone administration in combination with estrogen also increased ChAT presynaptic terminals in the CA1 region of the hippocampus significantly compared to estrogen⁴³. Furthermore, Gibbs demonstrated significant increases in the brain derived neurotrophic factor (BDNF) mRNA levels in regions of the hippocampus and pyriform cortex after acute treatment with estrogen followed by progesterone⁴⁴. Thus, these previous findings suggest that the interplay between progesterone and estrogen affects the effects of estrogen on the regulation of neuron spine growth and cholinergic system. It may be due to the relative change of the interplay between estrogen and progesterone, and due to predominant effect of estrogen, as compared with progesterone, on interaction with the σ_1 receptor. And these results suggest that the chronic administration of progesterone in combination with estrogen could be effective in improving cognitive dysfunction in menopausal women.

In contrast to current results using scopolamine, our study also indicated that progesterone treatment, either alone or in combination with estrogen, exerted significant effects on improvement of deficiency induced by scopolamine. Previous studies reported progesterone had an antagonistic effect on σ_1 receptors and behave as an antagonist of the attenuating

effects of neuroactive steroids on the scopolamine-induced memory deficits in acetylcholine-dependent memory processes⁴⁵. One possible reason is that the effects of low dosage progesterone on low dosage scopolamine-induced memory deficits in ovariectomized female rats could be different from that of high dosages of scopolamine (2 mg/kg) and progesterone (20 mg/kg) used in previous reports in male rodents. Al-Dahan and Thomas reported that progesterone in combination with estrogen reduced [3H] N-methyl scopolamine (NMS) binding capacity and progesterone (1 mg/kg) could acted independently of estradiol to reduce the NMS binding capacity⁴⁶. It was reported that progesterone could indirectly compete with NMS for the cardiac M2 muscarinic binding site^{47,48}. It could be speculated that low dosage progesterone reduced scopolamine binding in spatial memory task.

Another possible reason is that the duration and administration of hormone replacements could change the effectiveness and sensitivity to the hormones as a result of long-term hormone deprivation. It is important to note that the kinetics of repeated vs. continuous hormone replacement is quite different.

Third possible reason is the active effects of progesterone on other neurotransmission. Progesterone, co-administrated with cocaine, increases the levels of serotonin⁴⁹. Cocaine increases Ach release in regions of frontal cortex⁵⁰ and alter monoamine levels according to prevent their reuptake, and produces analeptic EEG arousal effects. These effects were blocked by scopolamine^{51,52}. Therefore, progesterone effects on modulation of cocaine-induced alternations in serotonin system may be an important element in the neural events on behavior. Progesterone also was reported to increase the NMDA-induced dopamine release and enhanced the glutamate-induced rise in $[Ca^{2+}]_i$ ⁵³, and to facilitate the cognitive recovery in neurodegeneration induced by hippocampal colchicine infusion⁵⁴, ischemia and cortical contusion injury^{26,55}. It could be speculated that progesterone might increase NMDA receptor activation.

Thus, although the functions of progesterone on learning memory are still unclear, progesterone may be important factor in the regulation of cognitive neurotransmission and have neuroprotective effects against scopolamine.

This study has several limitations. In our study, the ovariectomized rats received progesterone and estrogen capsules that released hormones at constant levels, not cyclically. The levels of metabolites of progesterone were not examined. And natural progesterone

was used to compare to other maze reports in which natural progesterone treatment was used in rats, though the medroxyprogesterone is used extensively in HRT for menopausal women. Future studies with cyclic hormone replacement treatments using multiple doses of progesterone or medroxyprogesterone should be needed to explore the effects of progesterone and its metabolites in cyclic hormone condition, and the difference between medroxyprogesterone and progesterone.

Although the effects of progesterone on cognitive memory are still unclear, our result suggests that chronic progesterone with or without estrogen may have neuroprotective effects against working or reference memory impairment induced by scopolamine.

Conclusion

Progesterone, alone or in combination with estrogen can improve the impairments of working memory and reference memory induced by scopolamine.

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