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Progesterone in Conjunction With Estradiol Has Neuroprotective Effects in an Animal Model of Neurodegeneration

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VONGHER, J. M. AND C. A. FRYE. *Progesterone in conjunction with estradiol has neuroprotective effects in an animal model of neurodegeneration.* PHARMACOL BIOCHEM BEHAV **64**(4) 777–785, 1999.—Restorative and neuroprotective effects of the steroid hormones estradiol benzoate (EB) and progesterone (P) were investigated in an animal model of neurodegeneration. Rats received EB, P, EB + P, or vehicle/cholesterol control immediately after (Experiment 1) or prior to (Experiment 2) intrahippocampal colchicine infusions, which destroy hippocampal neurons and deplete neurotransmitters, such as acetylcholine. Intrahippocampal colchicine produced long latencies, distances, and increased wandering in the water maze, in addition to hippocampal damage. Chronic administration of hormones immediately after colchicine, which produced circulating concentrations within physiological range, did not affect water-maze performance compared to cholesterol control (Experiment 1). When acute EB (10 μg SC) + P (500 μg SC) was given prior to intrahippocampal colchicine, latencies, distances, and wandering in the water maze were reduced (Experiment 2). The acutely administered EB + P also were in physiological range. Both experiments demonstrate some influence of hormones on neuronal integrity and ChAT that warrants further investigation. Together, these findings suggest that physiological concentrations of EB + P, when administered before hippocampal damage, may have neuroprotective actions on learning and memory impairment and hippocampal damage. © 1999 Elsevier Science Inc.

Neurodegeneration Neuroprotection Estradiol Progesterone ChAT Nongenomic Neurosteroid

HORMONES may play a role in protecting against neural insults because sex differences are often found in the occurrence of, and recovery from, neural insults. For instance, premenopausal women have a lower risk of ischemia (1,3), but the incidence of cerebrovascular events rapidly increases after menopause (68). Also, the incidence of Alzheimer's disease (AD) in postmenopausal women is greater than in men (13,57,70). Women have a 2.7-fold higher risk of developing AD than men (57), and this risk increases with age. Even when examining the incidence rates of AD at different age strata, women are always at higher risk than men, especially among the very old (16). Alzheimer's disease is a dementia characterized by inexorable neurodegeneration; in particular, a loss of acetylcholine in the entorhinal cortex, hippocampus, ventral striatum, basal forebrain, and cerebral cortex (4,29,38), and behavioral pathologies, such as wandering behavior (62).

These sex differences imply an involvement of hormones. Indeed, an important factor associated with female susceptibility to AD is the postmenopausal state (29), which is characterized by decreased ovarian hormone production (43). Postmenopausal women who receive hormone replacement therapy are less likely to develop AD or have a later onset (33,56), and tend to have improved mental status scores (15,34,35,54,55). Epidemiological studies also demonstrate that women who receive estrogen (E_2) replacement therapy are less likely to develop AD or develop AD with a later onset (8,33,56,67). However, some studies have reported no effect of hormones on the development of AD, but do report an improved quality of life (11). The few trials of E_2 replacement therapy for the treatment of AD have revealed that E_2-responsive AD patients tend to have lower baseline levels of E_2 and a higher incidence of osteoporosis (15). Furthermore, E_2 enhances the therapeutic response of women with AD taking Tacrine (63,64). Medroxyprogesterone, in conjunction with E_2, results in moderate improvement in baseline cognitive

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scores (54,55); however, medroxyprogesterone, which cannot be readily metabolized to natural progesterone (P), is associated with negative physical symptoms.

Ovarian hormones have been implicated in AD because of their effects on cognition (5) and acetylcholine, which is markedly decreased in brains of AD patients (42). Although ovariectomy (ovx) in experimental animals leads to a decrease in choline acetyltransferase (ChAT), E_2 replacement increases ChAT in the basal forebrain, hippocampus, and cerebral cortex (44,47). Estrogen replacement, which increases high affinity choline uptake, increases the acquisition of avoidance behavior compared to that seen in ovx rats (65). Furthermore, aged rats, impaired in the radial arm maze, have decreased ChAT levels in forebrain and hippocampal areas (45). Estradiol administered for 30 days prior to training, during testing, or in a combination of pretraining and testing, enhances ovx female rats' performance in the radial arm maze (10). Furthermore, for ovx rats trained in the T-maze, E_2, or E_2 and P-treated rats are protected against the performance decrements of scopolamine, while rats receiving no hormone treatment are not protected (12,14).

Very little research on hormonal modulation of learning and memory has studied the effects of P only or P in conjunction with E_2. Studying effects of P in the presence or absence of E_2 is important because in the female hormonal cycle, E_2 is followed by increases in P. Furthermore, P is usually given in conjunction with E_2 to postmenopausal women, because unopposed E_2 is contraindicated, due to possible increases in endometrial (9) and breast cancer (71).

The present study uses colchicine as an effective agent of neurodegeneration. Colchicine destroys dentate gyrus cells (30,52,53), degenerates hippocampal cholinergic neurons in a dose- and time-dependent manner (53), decreases ChAT in the hippocampus (53,69), blocks axoplasmic transport (58), induces neurofibrillary tangles (52), and is the only animal model known to cause progressive neurodegeneration (53). Colchicine infusions to the hippocampus produce learning deficits for mice in a passive-avoidance task (69), and for rats in the water maze (6,66) and T-maze (53) tasks.

The present experiments investigate the recovery of function (Experiment 1) and neuroprotective (Experiment 2) effects of E_2 and P in a model of neurodegeneration. Estradiol benzoate (EB) and/or P are administered, in a time-dependent manner, immediately after (Experiment 1) or before (Experiment 2) hippocampal colchicine infusion. There are two hypotheses in the present study. First, EB and P administered immediately after colchicine infusion will have recovery of function effects on water maze performance compared to a cholesterol condition (Experiment 1). Second, EB and P administered prior to colchicine infusion will have neuroprotective effects as demonstrated by improved performances in the water maze compared to a vehicle condition (Experiment 2). The effects of colchicine will be validated by examination of neuronal integrity and choline acetyltransferase (ChAT) immunocytochemistry in the hippocampus.

GENERAL METHODS

All methods and procedures described below were preapproved by the Institutional Animal Care and Use Committee.

Subjects and Housing

Adult female Long–Evans rats ($n = 72$; approximately 200 g) were obtained from Charles River Laboratory (Kingston, NY). Rats were housed individually in hanging stainless steel cages in an environment that was temperature (72 ± 2° F) and humidity (30–40%) controlled. The light cycle was reversed (12 L:12 D) with lights on at 20:00, and food and water were provided ad libitum.

Hormonal Manipulations

Estradiol benzoate (1,3,5[10]-Estratriene-3,17β-diol-3 benzoate) and P (4-pregnen-3,20-dione) were purchased from Sigma (St. Louis, MO). For Experiment 1, rats ($n = 32$, eight per group) had a Silastic tubing capsule containing hormone treatments implanted under the skin on the back. The EB tubing (0.062 i.d., 0.125 o.d.) was 10 mm/100 g body weight, the P tubing (0.132 i.d., 0.184 o.d.) was 10 mm long, and the cholesterol tubing (0.132 i.d., 0.184 o.d.) was 10 mm long (7,18,31). The EB + P group received an EB and a P silastic tubing capsule, and a cholesterol-filled capsule was used as a control drug.

For Experiment 2 ($n = 32$, eight per group, and eight additional animals for hormone measurement) the hormones were administered via an SC injection. All hormones were dissolved in a sesame oil vehicle. The EB concentration was 10 μg/0.2 ml, P concentration was 500 μg/0.2 ml, and the control group received 0.2 cc of the sesame oil vehicle.

Surgical Procedures

Under sodium pentobarbital (50 mg/kg, IP) anesthesia, experimental rats were ovx by bilateral flank incisions. Two weeks later, rats were stereotaxically infused with colchicine (Research Products International, Natick, MA) under sodium pentobarbital (50 mg/kg, IP) anesthesia following the methods of Nakagawa, Nakamura, Kase, Noguchi, and Ishiharo (53) and Rudy and Sutherland (66). Briefly, two holes were drilled in the skull over the hippocampal region (flat skull from bregma: AP −4.2, LAT ± 2.5, DV −2.1). The colchicine was dissolved in sterile physiological saline to a concentration of 15 μg/μl. Using a Hamilton syringe, a volume of 1 μl was administered to each side of the posterior hippocampus at the rate of 0.1 μl per 10 s. Following the infusion, the needle remained in place an additional minute to allow the colchicine to diffuse away from the tip of the needle. The rats were tested 6 to 7 days after colchicine infusion to maximize the neurodegenerative effects of colchicine.

Behavioral Testing

The Morris water maze tank had a circumference of 555 cm and was 71 cm deep. It was filled with 25–30°C water made opaque with powdered milk so that the platform (5.3 × 5.3 × 33.5 cm) was hidden. The platform was approximately 2.5 cm below the surface of the water and 60 cm from the tank's side. There were many visual cues in the room that rats could use to learn the position of the platform even when starting from different locations. A San Diego Instrument (San Diego, CA) tracking system was used to record total distance swam in centimeters and total time in seconds (maximum of 120 s) to reach the platform. The swimming pattern also was recorded during testing to calculate the number of circumference laps.

Before any experimental manipulation, rats were pretrained for the water maze task in two exposure trials. For each trial, rats were allowed a maximum of 120 s to find the

hidden platform. Either after the platform was found or after being guided to it at the end of 120 s, rats remained on the hidden platform for 45 s.

One week after pretraining and surgery, animals were tested in the Morris Water Maze task for six trials a day for 5 days to assess reference spatial memory. Distances, latencies, and wandering were recorded for each trial. Wandering was defined as swimming around the entire circumference of the pool, and was measured by the number of complete circumference laps. Number of trials until criterion was met also was measured; the criterion for learning the maze was defined as reaching the hidden platform within 25 s in three consecutive trials.

Steroid Hormone Measurement

Competitive Enzyme Linked Immunosorbant Assay (ELISA) kits for E_2, P, and testosterone (T) were purchased from Oxford Biomedical Research (Oxford, MI). The E_2 kit (#EA70) had crossreactivities ranging from 100% for E_2 and less than 2% for the following hormones: T, estriol, estrone, dehydroepiandrosterone, aldosterone, androstenedione, corticosterone, cortisol, cortisone, deoxycorticosterone, 17-hydroxyprogesterone, pregnenolone, and P. The crossreactivities for the P kit (#EA74) were 100% for P and less than 2% for the following hormones: androstenedione, corticosterone, deoxycorticosterone, pregnenolone, cortisol, cortisone, estriol, dehydroisoandrosterone, estrone, E_2, 17-hydroxyprogesterone, and T. The T kit (#EA78) had a 100% crossreactivity for T and dihydrotestosterone, but it had less than 2% crossreactivity for androstendione, estriol, E_2, dehydroepiandrosterone, deoxycorticosterone, aldosterone, corticosterone, cortisol, estrone, P, 17-hydroxyprogesterone, and pregnenolone. The ELISA kits were read with a microplate reader from Bio-Tek Instruments, Inc. (Winooski, VT).

The tritiated P ([1,2-^{3}H] P: specific activity = 48.8 ci/mmol) and tritiated 5α-pregnan-3α-ol-20-one ([^{3}-H] 3α,5α-THP: specific activity = 8000 dpm/μl) used in the radioimmunoassay (RIA) were purchased from New England Nuclear (Boston, MA). The P antibody used in the P RIA was obtained from Dr. G. D. Niswender (Colorado State University), and the 3α,5α-THP antibody was purchased from Dr. Robert Purdy (Veteran's Medical Center, LaJolla, CA).

Following behavioral testing and hormone treatment, rats were exposed to CO_2 for 60 s, and blood samples were obtained by cardiac puncture prior to perfusion. In addition, more samples were obtained for Experiment 2 by injecting additional animals with a hormone condition and taking blood prior to perfusion as in Experiment 1. Samples were centrifuged at 3000 *g* and stored at −20°C until assayed. Plasma was extracted with diethyl ether. The organic layer was dried down and reconstituted in buffer. After extraction, an ELISA was run for E_2, P, and T measurements, and a RIA was run for 3α,5α-THP and P to confirm the ELISA.

For the ELISAs, standard curves were prepared in duplicate (E_2 range = 0–2 ng/ml; P range = 0–2 ng/ml; T range = 0.00–0.04 ng/ml). Fifty microliters of the standards or extracted hormone followed by enzyme conjugate were added to the ELISA plate and allowed to incubate for 1 h at room temperature. The plate was then washed three times with 300 μl of wash buffer, and 150 μl of tetramethylbenzidine was added for a 30-min incubation. The reaction was stopped by adding 50 μl of 1 M HCl. The plate was then read at 450 nm in the microplate reader. The standard curve was plotted with $\%B/B_0$ as the ordinate and the concentration as the abscissa, and the sample concentrations were determined using this standard curve.

The P RIA was performed according to the modified methods of Frye, McCormick, Coopersmith, and Erskine (20). 3α,5α-THP was measured according to previously published methods (19,22,59). Briefly, tritiated P or 3α,5α-THP was added to 0.5 ml plasma and then extracted as outlined above. The P antibody was used in a concentration of 1:30,000, while the 3α,5α-THP antibody concentration was 1:5,000. Both the P and 3α,5α-THP standard curves were prepared in duplicate (P range = 5–800 pg; 3α,5α-THP range = 100–4000 pg). The total assay volume was 800 μl for the P RIA and 950 μl for 3α,5α-THP. Tubes were incubated overnight at 4°C, which was subsequently terminated by the addition of dextran-coated charcoal. Sample tube concentrations were determined using the Logit-Log method of Rodbard and Hutt (60). The intraassay variances were 10.7% (P) and 12.1% (3α,5α-THP), and interassay variances were 9.2% (P) and 15.6% (3α,5α-THP).

Histology

Upon the completion of behavioral testing, four animals in each condition of each experiment were sacrificed with an overdose of pentobarbital solution (given IP) to process the brain tissue for immunocytochemistry (ICC) of ChAT. Rats were perfused intracardially with 0.9% saline followed by 4% paraformaldehyde for the ICC procedure. The remaining rats were exposed to CO_2, and had blood withdrawn by cardiac puncture for assays. Rats exposed to CO_2 were perfused with 0.9% saline followed by 10% formalin. The frozen brains were sliced in 40-μm sections, and alternate slices were stained with cresyl violet.

After 24 h, the brains for ICC were transferred to a 25% sucrose-buffer solution for an additional 24 h at 4°C. One to 2 days later, the brains were sliced at 40 μm in a freezing microtome. Free-floating sections were washed in phosphate-buffered saline (PBS), hydrogen peroxide, and then incubated in normal goat serum for 1 h to decrease nonspecific binding. A polyclonal antibody specific for ChAT (#AB143); Chemicon, Temecula, CA) was diluted in PBS/0.1% bovine serum albumin with 2% normal goat serum (antibody concentration = 1:500). Sections were then incubated for 48 h at 4°C in ChAT primary antibody. After 48 h, the sections were rinsed in normal goat serum, incubated in a biotinylated secondary antibody for 1 h, washed in PBS, and then incubated in an avidin–biotin solution for 30 min to utilize a peroxidase staining kit for diaminobenzidine (Vector Laboratories Burlingame, CA). Finally, the sections were rinsed in distilled water for 10 min and then in PBS until they were mounted on gelatin-coated slides. Darkly stained, ChAT-immunoreacted presynaptic terminals counted were within a reticle superimposed over a representative section of the CA1 region of the hippocampus similar to the methods of Ishimaru, Takahashi, Ikarashi, and Maruyama (36). All terminals counted were of similar shape and contrast. One representative hippocampal section was counted per animal. Representative sections were sections closest to the infusion site, in the anterior direction, without tissue damage. The CA1 region of the hippocampus was defined as the area of smaller sized pyramidal cells but greater pyramidal cell density compared to the CA2 and CA3 regions. The CA1 was targeted in this study because of susceptibility to neural insults and importance in learning and memory functioning (36). Cresyl violet-stained cell bodies were counted in the same manner.

Statistics

Mixed-design analyses of variance (ANOVAs) were used to reveal any differences between the three steroid (EB, P, EB + P) and control conditions across trials and days with respect to latencies, total distances, and wandering for the postsurgery behavioral testing (1—between, 2—within). For pretraining, one-within ANOVAs were used for the dependent variables, latency, and distance to a hidden platform. When significant effects arose from the factorial ANOVAs, separate one-way ANOVAs and Tukey post hoc analyses were used to determine group differences for that variable.

For the trials to achieve criterion, ICC, and hormone measurement analyses, one-way ANOVAs were used in each experiment to distinguish differences among hormone conditions. Tukey post hoc tests were used to decipher group differences.

Any data from rats that died during testing ($n = 1$ for Experiment 1; $n = 2$ for Experiment 2) were omitted from analyses.

PROCEDURE

Experiment 1: Do Chronically Administered Ovarian Steroid Hormones Have Restorative Effects in a Model of Neurodegeneration?

After depletion of endogenous hormones, 32 rats were allowed to learn the Morris Water Maze through exposure in two trials. Within the next 2 days, rats were stereotaxically infused with colchicine intrahippocampally and implanted with a hormone containing silastic capsule ($n = 8$ per condition: EB, P, EB + P, or cholesterol) immediately after the infusion. One week after surgery, behavioral testing began. After behavioral testing, half of the animals were perfused for ChAT ICC, and the other half had blood withdrawn prior to perfusion so that plasma steroid hormone levels could be measured. As part of the histology, cresyl violet was used as a cell body stain and ICC for ChAT was used to stain presynaptic terminals with ChAT present to observe neuronal integrity and presence of ChAT, respectively. An ELISA and RIA were used to measure plasma E_2, P, 3α,5α-THP, and T, as described earlier, for each condition (EB, P, EB + P, and cholesterol).

Experiment 2: Do Ovarian Steroid Hormones Have Neuroprotective Effects in a Model of Neurodegeneration?

Thirty-two rats were ovx and then allowed to learn the maze through exposure in two pretraining trials the following week. To test for neuroprotection of memory, the EB, P, and sesame oil vehicle groups ($n = 8$ per group) received injections immediately following the pre-training trials. The EB + P group received EB immediately following the pretraining trials and received P 44 h after EB. The EB + P group was designed to represent both ovarian hormones as they normally occur in the female rat estrous cycle. Within the 2 days following pretraining and hormone injection, rats were intrahippocampally infused with colchicine. The hormones were administered at a single time point prior to intrahippocampal colchicine infusion. Blood was collected from the tail vein at the time of surgery for later RIA. The next week, postsurgery behavioral testing began in the Morris Water Maze. As in Experiment 1, latency to the hidden platform, total distance swam, wandering, and trials to criterion were measured. To describe neuronal integrity, cresyl violet cell-body straining was performed. Rats also were perfused for ChAT ICC at the conclusion of behavioral testing to characterize the role of ChAT in this model of neurodegeneration.

RESULTS

Experiment 1: Chronically Administered Ovarian Hormones Have Limited Restorative Effects in a Model of Neurodegeneration

Water-maze performance. On the pretraining trials, the latencies, [$F(1, 30) = 10.87$, $p \leq 0.05$, and distances, $F(1, 30) = 5.36$, $p \leq 0.05$, of the second trial (66.8 ± 8.0 s; 1681.8 ± 190.5 cm) were less than those of the first trial (99.8 ± 6.1 s; 2242.2 ± 150.3 cm).]

However, on the postcolchicine behavioral testing trials, latency performance improved only slightly over days, $F(4, 108) = 5.85$, $p \leq 0.05$, and distances did not change for all groups (see Fig. 1). Furthermore, wandering behavior, which was not evident in the pretraining trials, increased over days, $F(4, 108) = 6.97$, $p \leq 0.05$ (see Fig. 1). Because rats in this experiment performed at or near maximal values, there were no differences among the hormone conditions for latency, dis-

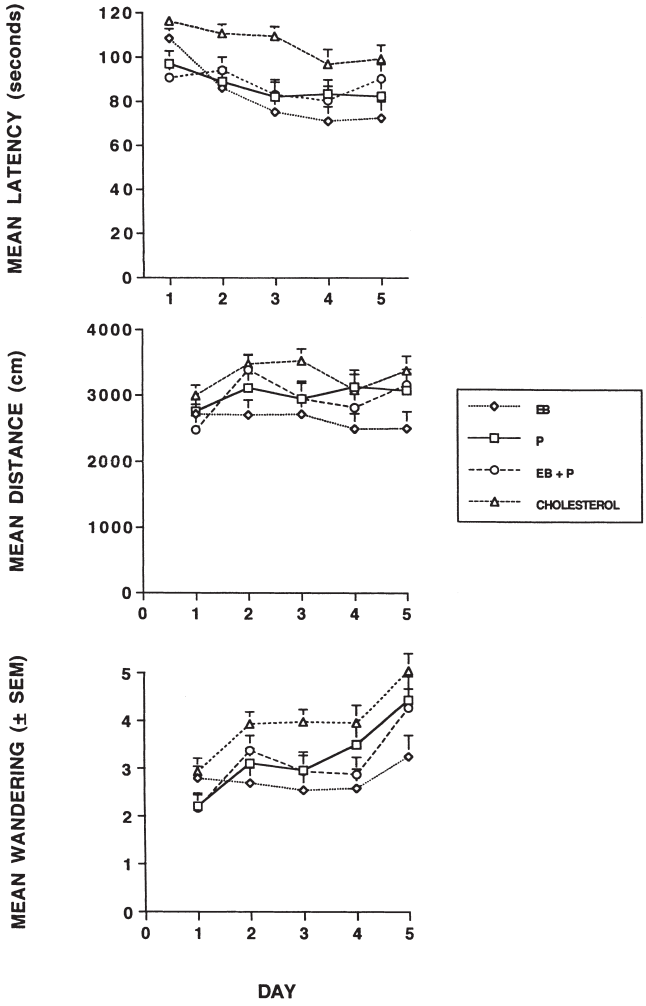

FIG. 1. Mean ± SEM latencies (top), distances (middle), and wandering (bottom) for the chronic hormonal conditions of Experiment 1 as a function of testing day.

TABLE 1

MEAN PLASMA HORMONE LEVELS OF E_2, P, 3α,5α-THP, AND T FOR EACH OF THE EXPERIMENAL HORMONE CONDITIONS

	E_2 pg/ml		P ng/ml		3α,5α-THP ng/ml		T ng/ml	
Condition	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Control	6.4 ± 2.0	6.5 ± 1.3	0.1 ± 0.1	1.4 ± 0.4	1.2 ± 0.6	0.9 ± 0.1	0.005 ± 0.001	0.005 ± 0.001
EB	48.9 ± 8.0*	45.4 ± 8.0*	0.6 ± 0.4	0.7 ± 0.1	0.3 ± 0.2	0.9 ± 0.8	0.005 ± 0.002	0.005 ± 0.002
P	9.5 ± 1.2	8.4 ± 1.3	46.3 ± 0.7*	62.7 ± 11.3*	4.8 ± 1.6*	5.6 ± 0.7*	0.005 ± 0.004	0.005 ± 0.001
EB + P	59.7 ± 25.4*	54.8 ± 9.3*	48.7 ± 1.4*	63.4 ± 27.3*	6.7 ± 2.8*	8.0 ± 0.6*	0.005 ± 0.001	0.005 ± 0.001

*Represents a significant difference from control group ($p \leq 0.05$).

tance, or wandering. The maximum number of trials to learn the maze was 30, and hormone groups did not significantly differ from this maximum when examining trials to reach criterion ($p = 0.54$).

Steroid concentrations. Plasma hormone measurements of E_2, P, 3α,5α-THP, and T revealed that the chronically administered hormones were within the proestrous-physiological range for female rats (see Table 1). Levels of E_2, $F(3, 12) = 5.38$, $p \leq 0.05$, P, $F(3, 12) = 1010.83$, $p \leq 0.05$, 3α,5α-THP, $F(3, 12) = 5.55$, $p \leq 0.05$, but not T ($p = 0.59$) were significantly influenced by hormone administration.

Hippocampal morphology. When representative sections of the CA1 region of the hippocampus were counted, EB + P-treated rats tended to have more neurons than rats administered cholesterol, $F(3, 12) = 3.40$, $p = 0.07$ (see Fig. 2, top). The number of ChAT immunoreactive presynaptic terminals of the CA1 region of the hippocampus, $F(3, 12) = 52.40$, $p \leq 0.05$, differed significantly by hormone condition (see Fig. 2, bottom). There were ChAT increases of 17.8, 23.5, and 53.0% for EB, P, and EB + P, respectively, compared to cholesterol.

Experiment 2: Ovarian Hormones Have Neuroprotective Effects in a Model of Neurodegeneration

Water-maze performance. As in the first experiment, the latencies, $F(1, 29) = 15.22$, $p \leq 0.05$, and distances, $F(1, 29) = 9.74$, $p \leq 0.05$, for the pretraining trials were shorter for the second trial (61.8 ± 7.7; 1192.9 ± 127.1 cm) compared to the first trial (93.5 ± 7.4; 1654.7 ± 166.5 cm).

On the postcolchicine behavioral testing trials, performance was dampened. Latencies, $F(4, 104) = 20.71$, $p \leq 0.05$, and distances, $F(4, 104) = 13.28$, $p \leq 0.05$, improved slowly over days before reaching the levels of the pretraining trials for all groups (see Fig. 3). Similar to the first experiment, wandering behavior was present in the postcolchicine trials, but not in the pretraining trials with all groups. However, in this experiment, wandering decreased over days $F(4, 104) = 3.87$, $p \leq 0.05$ (Fig. 3). Furthermore, on day 1, there were significant effects of hormone condition on latencies, $F(3, 26) = 6.28$, $p \leq 0.05$, and distances, $F(3, 26) = 3.37$, $p \leq 0.05$. As shown in Fig. 3, the EB + P group had significantly shorter latencies compared to all other groups, and significantly shorter distances than the EB group on day 1. Although there was a trend for EB + P to significantly decrease wandering on day 1 compared to the EB group ($p = 0.06$), by day 5 all hormone groups were wandering less than the vehicle group, $F(3, 26) = 3.14$, $p \leq 0.05$ (see Fig. 3). Similar to the first experiment, all hormone groups did not differ significantly from the maximum of 30 trials when analyzing trials to reach criterion ($p = 0.31$).

Steroid concentrations. Plasma hormone measurements of E_2, P, 3α,5α-THP, and T revealed that the acutely administered hormones were all within proestrous-physiological range for the female rat (see Table 1). Levels of E_2, $F(3, 12) = 51.38$, $p \leq 0.05$, P, $F(3, 12) = 12.65$, $p \leq 0.05$, 3α,5α-THP, $F(3, 12) = 21.99$, $p \leq 0.05$, but not T ($p = 0.53$), were significantly influenced by the hormone administration.

Hippocampal morphology. When representative sections of the CA1 region of the hippocampus were counted, the EB + P group had more neurons than the vehicle and the P groups,

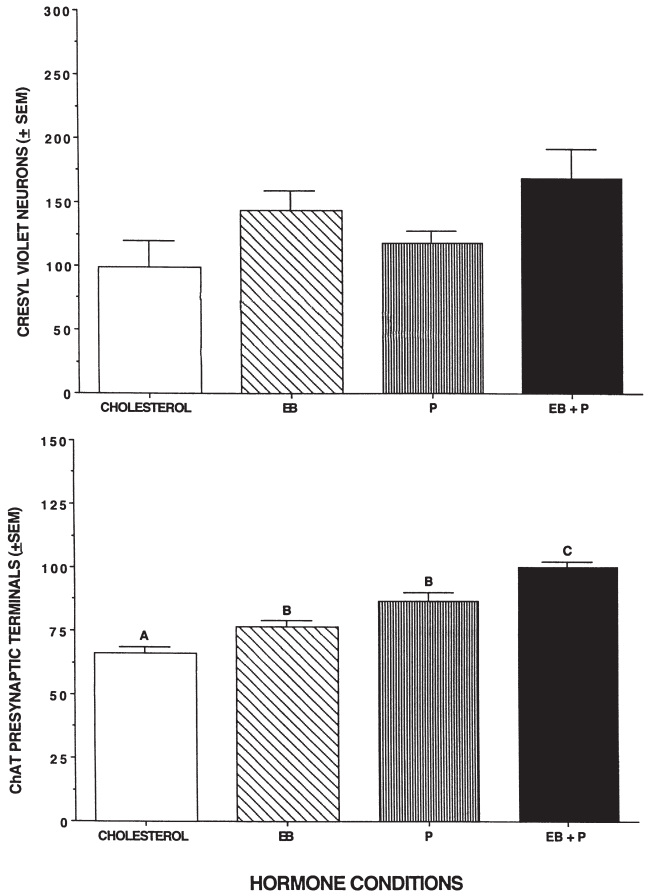

FIG. 2. (Top) Mean ± SEM cresyl violet neurons in the CA1 region of the hippocampus as a function of chronic hormonal condition for Experiment 1. (Bottom) Mean ± SEM of ChAT immunoreacted presynaptic terminals in the CA1 region of the hippocampus as a function of chronic hormonal condition for Experiment 1. Letters that are different represent a significant difference at $p < 0.05$.

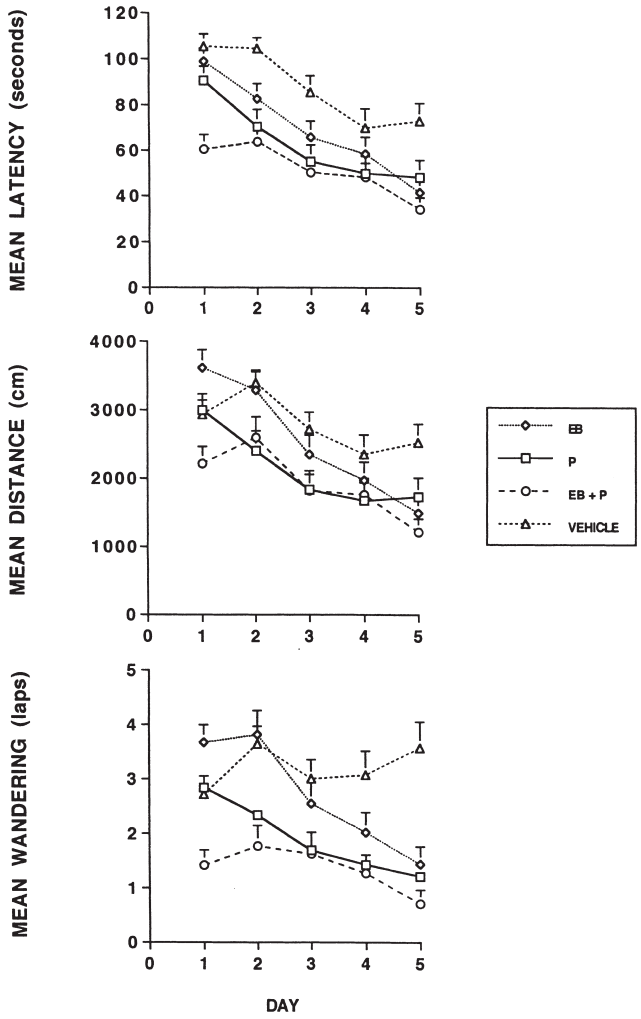

FIG. 3. Mean ± SEM latencies (top), distances (middle), and wandering (bottom) as a function of testing day for the acute hormone conditions of Experiment 2.

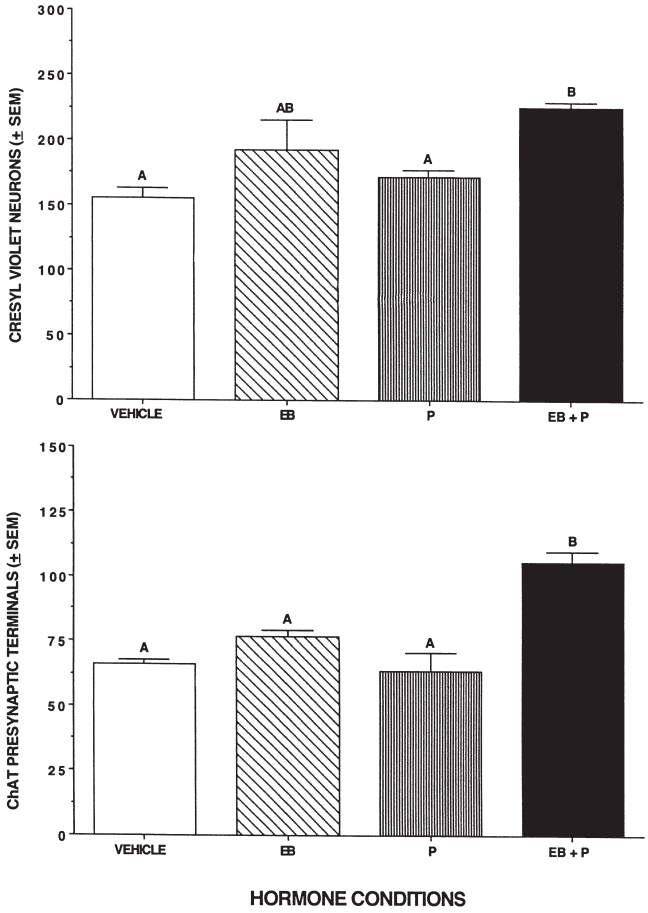

FIG. 4. (Top) Mean ± SEM cresyl violet neurons in the CA1 region of the hippocampus as a function of acute hormonal condition for Experiment 2. Letters that are different represent a significant difference at $p < 0.05$. (Bottom) Mean ± SEM of ChAT immunoreacted presynaptic terminals in the CA1 region of the hippocampus as a function of acute hormonal condition for Experiment 2. Letters that are different represent a significant difference at $p < 0.05$.

$F(5, 12) = 5.14$, $p < 0.05$ (see Fig. 4, top). The number of ChAT immunoreactive presynaptic terminals in the CA1 region of the hippocampus, $F(3, 12) = 23.77$, $p \leq 0.05$, also differed between hormonal conditions (see Fig. 4, bottom). The EB + P group had more ChAT immunoreacted neurons than the vehicle, EB, or P groups. The percent increases in ChAT compared to the vehicle only condition were 15 and 61% for EB and EB + P, respectively, while there was an 8.1% decrease in ChAT for P alone.

DISCUSSION

The results of the present study are mixed. Ovarian hormones offer more amelioration when administered prior to a neural insult than when administered after a neural insult. Hence, the first hypothesis that ovarian hormones would have a restorative effect after colchicine infusion was not supported. However, the second hypothesis that EB and P treatment would have neuroprotective effects when administered prior to colchicine was supported.

Intrahippocampal colchicine produced neurodegeneration as evidenced by long latencies and distances to the hidden platform, as well as wandering the water maze. When hormones were administered after intrahippocampal colchicine, the latencies, distances, and wandering were not improved. In comparison, when EB + P were administered after pretraining and before intrahippocampal colchicine, postinfusion water maze performances were improved compared to vehicle controls. Estradiol + P administered before colchicine decreased latencies, distances, and wandering in the water maze compared to the vehicle condition. Although the significant hormonal effects of Experiment 2 were not found for each testing day (days 1 and 5 had the most differences between groups), it is possible that with a larger number of subjects per group statistical power would be increased, and hormonal effects over all testing days would be evident.

Overall, rats in Experiment 1 showed much more post-colchicine behavioral decline than rats in Experiment 2. There were even some slight benefits of sesame oil vehicle in Experiment 2 compared to cholesterol in Experiment 1, sug-

gesting that the presence of cholesterol-rich substances may offer some neuroprotection compared to cholesterol given after insult. Cholesterol-rich sesame oil may have had some effects because cholesterol is the biochemical precursor to hormones or because cholesterol can alter membrane fluidity, thereby changing the availability of binding sites. It is also possible that the observed neuroprotective effects seen in Experiment 2 are not due specifically to hormones but to cholesterol-based substances in general. However, the beneficial effects of EB + P strongly suggest hormones offer some neuroprotection greater than sesame oil vehicle.

The present experiments have demonstrated that intrahippocampal colchicine infusion is an efficacious model of neurodegeneration. Colchicine led to impairments in the water maze that worsened over time. Intrahippocampal lesioning produced learning deficits comparable to those noted for mice in a passive-avoidance task (69) and for rats in the water maze (6,66) and T-maze tasks (53). Furthermore, colchicine produced the behavioral pathology of wandering (62) often seen in AD.

The findings of the current study suggest that the effects of hormones are more robust when present before a neural insult, as opposed to during neurodegeneration. The differences in the hormone's effectiveness in the two experiments suggests the importance of whether or not hormones are present at the time of a neural insult or during neurodegeneration. These findings are important, as they relate the role of hormone replacement therapy in postmenopausal women. Future studies should investigate the importance of hormonal status maintenance on decreasing many of the risks commonly observed in postmenopausal women, such as AD.

The present study suggests there may be a relationship between hormonal status and neuron count and ChAT immunoreactivity that warrants further investigation. Indeed, previous research indicates that EB and EB + P can regulate cholinergic function. Estradiol (27,28,44,46) and E_2 followed by P (23) increase the expression of ChAT in a dose- and time-dependent manner (24,25), and facilitate potassium-stimulated acetylcholine release in the hippocampus and overlying cortex (26). Hormone depletion decreases ChAT, but ChAT depletion is attenuated when rats receive EB (65). The present results are consistent with findings of increased ChAT in the hippocampus after EB treatment (25). Although E_2 facilitates acetylcholine synthesis by increasing ChAT and the binding sites of hypothalamic nicotinic acetylcholine receptors (51), P is the most potent steroid to act noncompetitively and extracellularly at inhibiting nicotinic acetylcholine receptors when compared with E_2, corticosterone, and dexamethasone (39). Hence, E_2 + P may work synergistically to provide neuroprotective effects via ChAT. More intensive investigation is needed to demonstrate how E_2 and P may work via acetylcholine to influence neuroprotection.

There are many possible mechanisms by which P in conjunction with E_2 may decrease colchicine deficiencies; however, the majority of studies that explore possible mechanisms have only investigated E_2's effects. For example, E_2 has received attention for its neuroprotective actions via neurotrophs, such as nerve growth factor (24), or its antioxidant properties (2,32). Moreover, E_2 can protect cells when they are exposed to the toxic form of the beta-amyloid protein found in AD patients' brains (33). Estrogen can also influence the metabolism or synthesis of 3α,5α-THP by increasing 5α-reductase activity (49).

It is also possible that functional neuroprotective effects observed in this study may be due to P's metabolism to 3α,5α-THP. Circulating P and 3α,5α-THP were similarly increased in the EB + P condition that produced neuroprotective effects. Progesterone and 3α,5α-THP have previously demonstrated neuroprotective effects in animal models of edema (17,41,61) and epilepsy (18,19,21,22,40). Interestingly, P and 3α,5α-THP have different mechanisms of action. Progesterone binds well to P receptors but is a weak agonist at γ-aminobutyric acid/benzodiazepine receptor complexes (GBRs); 3α,5α-THP does not bind well to P receptors, but is a very effective agonist at GBRs (37,48). The only common mechanism of P and 3α,5α-THP could be through P's metabolism to 3α,5α-THP and its subsequent actions at GBRs. This suggests that a possible substrate for some protective effects of EB + P may be GBRs or through the GBR-mediated influence on acetylcholine. Another alternative mechanism of neuroprotection is that EB, P, or EB + P could have effects through actions at glucocorticoid receptors, which are abundant in the hippocampus (50).

Taken together, the present experiments indicate that EB + P can have neuroprotective effects in an animal model of neurodegeneration. Neuroprotective effects were evident by the reduced latencies, distances, and wandering behavior in the water maze of rats that received EB + P before colchicine (Experiment 2). The present findings are exciting, and indicate that P, alone or in conjunction with EB, may have beneficial effects in other models of neurodegeneration. Future studies should investigate the possible neuroprotective mechanisms of hormones in this acetylcholine depletion model of neurogeneration. Although negative effects of synthetic progestins have been reported, in particular, with the treatment of people with AD, natural P is yet to be examined in this population. Natural P may exert more neuroprotective and restorative effects through mechanisms not possible with synthetic progestins such as medroxyprogesterone.

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REFERENCES

1. Alkayed, N. J.; Harukuni, I.; Kimes, A. S.; London, E. D., Traysman, R. J.; Hum, P. E.: Gender-linked brain injury in experimental stroke. Stroke 29:159–166; 1997.
2. Ayres, S.; Tang, M.; Subbiah, M. T. R.: Estradiol-17-β as an antioxidant: Some distinct features when compared with common fat-soluble antioxidants. J. Lab. Clin. Med. 128:367–375; 1996.
3. Barret-Conner, E.; Bush, T. L.: Estrogen and coronary heart disease in women. JAMA 265:1861–1867; 1991.
4. Bartus, R. T.; Dean, R. L.; Beer, B.; Lippa, A. S.: The cholinergic hypothesis of geriatric memory dysfunction. Science 217:408–417; 1982.
5. Berman, K.; Schmidt, P.; Rubinow, D.; Danaceau, M.; Van Horn, J.; Esposito, G.; Ostrem, J.; Weinberger, D.: Modulation of cognition-specific cortical activity by gonadal steroids: A positron-emission tomography study in women. Neurobiology 94:8836–8841; 1997.

6. Brandeis, R.; Brandys, Y.; Yehuda, S.: The use of the Morris Water Maze in the study of memory and learning. Int. J. Neurosci. 48:29–69; 1989.
7. Brandi, A. M.; Joannidis, S.; Peillon, F.; Joubert, M.: Changes of prolactin response to dopamine during the rat estrous cycle. Neuroendocrinology 51:449–454; 1990.
8. Brenner, D. E.; Kukull, W. A.; Stergachis, A.; van Belle, G.; Bowen, J. D.; McCormick, W. C.; Teri, L.; Larson, E. B.: Postmenopausal estrogen replacement therapy and the risk of Alzheimer's disease: A population-based case–control study. Am. J. Epidemiol. 140:262–267; 1994.
9. Cramer, D. W.; Knapp, R. C.: Review of epidemiological studies of endometrial cancer and exogenous estrogen. Obstet. Gynecol. 54:521–526; 1979.
10. Daniel, J. M.; Fader, A. J.; Spencer, A. L.; Dohanich, G. P.: Estrogen enhances performance of female rats during acquisition of a radial arm maze. Horm. Behav. 32:217–225; 1997.
11. Ditkoff, E. C.; Crary, W. G.; Cristo, M.; Lobo, R. A.: Estrogen improves psychological function in asymptomatic postmenopausal women. Obstet. Gynecol. 78:991–995; 1991.
12. Dohanich, G. P.; Fader, A. J.; Javorsky, D. J.: Estrogen and estrogen/progesterone treatments counteract the effect of scopolamine on reinforced T-Maze alteration in female rats. Behav. Neurosci. 108:988–992; 1994.
13. Evans, D. A.: Descriptive epidemiology of Alzheimer's Disease. In: Khachaturian, Z. S.; Radebough, T. S., eds. Alzheimer's disease: Cause(s), diagnosis, treatment, and care, New York: CRC Press; 51–59; 1996.
14. Fader, A. J.; Hendricson, A. W.; Dohanich, G. P.: Estrogen improves performance of reinforced T-maze alternation and prevents the amnestic effects of scopolamine administered systemically or intrahippocampally. Neurobiol. Learn. Mem. 69:225–240; 1998.
15. Fillit, H.; Weinreb, H.; Cholst, I.; Luine, V.; McEwen, B.; Amador, R.; Zabriskie, J.: Observations in a preliminary open trial estradiol therapy for senile dementia-Alzheimer's type. Psychoneuroendocrinology 11:337–345; 1986.
16. Fratiglioni, L.; Viitanen, M.; von Strauss, E.; Tontodonati, V.; Herlitz, A.; Winblad, B.: Very old women at highest risk of dementia and Alzheimer's disease. Neurology 48:132–138; 1997.
17. Fritts, M. E.; Roof, R. L.: Pregnanalone may mediate neuroprotective effects of progesterone after brain injury. Soc. Neurosci. Abstr. 27:268; 1997.
18. Frye, C. A.: Estrus-associated decrements in a water maze task are limited to acquisition. Physiol. Behav. 57:5–14; 1995.
19. Frye, C. A.; Bayon, L. E.: Seizure activity is increased in endocrine states characterized by decline in endogenous levels of the neurosteroid 3α,5α-THP. Neuroendocrinology 68:272–280; 1998.
20. Frye, C. A.; McCormick, C. M.; Coopersmith, C.; Erskine, M. S.: Effects of paced and non-paced mating stimulation on plasma progesterone, 3α-diol and corticosterone. Psychoneuroendocrinology 21:431–439; 1996.
21. Frye, C. A.; Scalise, T. J.: Anti-seizure effects of P and 3α,5α-THP in kainic acid and perforant pathway models of epilepsy. Psychoneuroendocrinology (in revision).
22. Frye, C. A.; Scalise, T. J.; Bayon, L. E.: Finasteride blocks the reduction in ictal activity produced by exogenous estrous cyclicity. J. Neuroendocrinol. 10:291–296; 1998.
23. Gibbs, R. B.: Fluctuations in relative levels of choline acetyltransferase mRNA in different regions of the rat basal forebrain across the estrous cycle: Effects of estrogen and progesterone. J. Neurosci. 16:1049–1055; 1996.
24. Gibbs, R. B.: Effects of estrogen on basal forebrain cholinergic neurons vary as a function of dose and duration of treatment. Brain Res. 757:10–16; 1997.
25. Gibbs, R. B.; Aggarwal, P.: Estrogen and basal forebrain cholinergic neurons: Implications for brain aging and Alzheimer's disease-related cognitive decline. Horm. Behav. 34:98–111; 1998.
26. Gibbs, R. B.; Hashash, A.; Johnson, D. A.: Effects of estrogen on potassium-stimulated acetylcholine release in the hippocampus and overlying cortex of adult rats. Brain Res. 749:143–146; 1997.
27. Gibbs, R. B.; Pfaff, D. W.: Effects of estrogen and fimbria/fornix transection on p75(NGFR) and ChAT expression in the medial septum and diagonal band of Broca. Exp. Neurol. 116:23–39; 1992.
28. Gibbs, R. B.; Wu, D.; Hersh, L. B.; Pfaff, D. W.: Effects of estrogen replacement on the relative levels of choline acetyltransferase, trkA, and nerve growth factor messenger RNAs in the basal forebrain and hippocampal formation of adult rats. Exp. Neurol. 129:70–80; 1994.
29. Gilman, S.: Alzheimer's disease. Perspect. Biol. Med. 40:230–245; 1997.
30. Goldschmidt, R. B.; Steward, O.: Neurotoxic effects of colchicine: Differential susceptibility of CNS neuronal populations. Neuroscience 7:695–714; 1982.
31. Goodman, R. L.: A quantitative analysis of the physiological role of estradiol and progesterone in the control of tonic and surge secretion of LH in the rat. Endocrinology 102:142–150; 1978.
32. Gridley, K. E.; Green, P. S.; Simpkins, J. W.: Low concentrations of estradiol reduce beta-amyloid 925-350-induced toxicity, lipid peroxidation and glucose utilization in human SK-N-SH neuroblastoma cells. Brain Res. 778:158–165; 1997.
33. Henderson, V. W.; Paganini-Hill, A.; Emanuel, C. K.; Dunn, M. E.; Buckwalter, J. G.: Estrogen replacement therapy in older women: Comparisons between Alzheimer's disease cases and nondemented control subjects. Arch. Neurol. 51:896–900; 1994.
34. Honjo, H.; Ogino, Y.; Naitoh, K.; Urabe, M.; Kitawaki, J.; Yasuda, J.; Yamamoto, T.; Ishihara, S.; Okada, H.; Yonezawa, T.: In vivo effects by estrone sulfate on the central nervous system-senile dementia (Alzheimer's type). J. Steroid Biochem. 34:521–525; 1989.
35. Honjo, H.; Tanaka, K., Kashiwagi, T.; Urabe, M.; Okada, H.; Hayashi, M.; Hayashi, K.: Senile dementia-Alzheimer's type and estrogen. Horm. Metab. Res. 27:204–207; 1995.
36. Ishimaru, H.; Takahashi, A.; Ikarashi, Y.; Maruyama, Y.: Immunohistochemical and neurochemical studies on hippocampal cholinergic neurons after ischemia. Neuroreport 6:557–560; 1995.
37. Iswari, S.; Colas, A. E.; Karavolas, H. J.: Binding of 5α-dihydroprogesterone and other progestins to female rat anterior pituitary nuclear extracts. Steroids 47:189–203; 1986.
38. Kasa, P.; Rakonczay, Z.; Gulya, K.: The cholinergic system in Alzheimer's disease. Prog. Neurobiol. 52:511–535; 1997.
39. Ke, L.; Lukas, R. J.: Effects of steroid exposure on ligand binding and functional activities of diverse nicotinic acetylcholine receptor subtypes. J. Neurochem. 67:1100–1112; 1996.
40. Kokate, T. G.; Cohen, A. L.; Karp, E.; Rogawski, M. A.: Neuroactive steroids protect against pilocarpine- and kainic acid-induced limbic seizures and status epilepticus in mice. Neuropharmacology 35:1049–1056; 1996.
41. Konen, J. A.; Seymore, P. M.; Fritts, M. E.; Powell, R. A.; Stein, D. G.; Roof, R. L.: Delayed treatment with progesterone is effective at reducing edema, delayed treatment with methylprednisolone is not. Soc. Neurosci. Abstr. 27:268; 1997.
42. Kuller, L. H.: Hormone replacement therapy and its potential relationship to dementia. J. Am. Geriatr. Soc. 44:878–880; 1996.
43. Longcope, C.: Hormone dynamics at the menopause. Ann. NY Acad. Sci. 592;21–30; 1990.
44. Luine, V. N.: estradiol increases choline acetyltransferase activity in specific basal forebrain nuclei and projection areas of female rats. Exp. Neurol. 89:484–490; 1985.
45. Luine, V.; Hearns, M.: Relationship of gonadal hormone administration sex, reproductive status and age to monoamine oxidase activity within the hypothalamus. J. Neuroendocrinol. 2:423–428; 1990.
46. Luine, V.; McEwen, B.: Sex differences in cholinergic enzymes of diagonal band nuclei in the rat preoptic area. Neuroendocrinology 36:475–482; 1983.
47. Luine, V.; Park, D.; Joh, T.; Reis, D.; McEwen, B.: Immunochemical demonstration of increased choline acetyltransferase concentration in rat preoptic area after estradiol administration. Brain Res. 191:273–277; 1980.
48. Majewska, M. D.; Harrison, N. L.; Schwartz, R. D.; Barker, J. L.; Paul, S. M.: Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. Science 232:1004–1007; 1986.

49. Matzkin, H.; Chen, J.; Lewyshon, O.; Ayalon, D.; Braf, Z.: Effects of long term treatment with finasteride (MK-906), a 5-α reductase inhibitor, on circulating LH, FSH, prolactin, and estradiol. Horm. Metab. Res. 24:498–499; 1992.
50. McEwen, B. S.: Possible mechanisms for atrophy of the human hippocampus. Mol. Psychiatry 2:255–262; 1997.
51. Morley, B. J.; Rodriguez-Sierra, J. F.; Clough, R. W.: Increase in hypothalamic nicotinic acetylcholine receptors in prepuberal female rats administered estrogen. Brain Res. 278:262–265; 1983.
52. Mundy, W. R.; Tilson, H. A.: Neurotoxic effects of colchicine. Neurotoxicology 11:539–548; 1990.
53. Nakagawa, Y.; Nakamura, S.; Kase, Y.; Noguchi, T.; Ishihara, T.: Colchicine lesions in the rat hippocampus mimic the alterations of several markers in Alzheimer's disease. Brain Res. 408:57–64; 1987.
54. Ohkura, T.; Isse, K.; Akazawa, K.; Hamamoto, M.; Yaoi, Y.; Hagino, N.: Long-term estrogen replacement therapy in female patients with dementia of the Alzheimer type: 7 case reports. Dementia 6:99–107; 1995.
55. Ohkura, T.; Isse, K.; Azakawa, K.; Hamamoto, M.; Yaoi, Y.; Hagino, N.: Evaluation of estrogen treatment in female patients with dementia of the Alzheimer type. Endocrinol. Jpn. 41:361–371; 1994.
56. Paganini-Hill, A.: Oestrogen replacement therapy and Alzheimer's disease. Br. J. Obstet. Gynaecol. 103:80–86; 1996.
57. Payami, H.; Montee, K.; Grimslid, H.; Shattuc, S.; Kaye, J.: Increased risk of familial late-onset Alzheimer's disease in women. Neurology 46:126–129; 1996.
58. Perisic, M.; Cuenod, M.: Synaptic transmission depressed by colchicine blockade of axophasmic flow. Science. 175:1140–1141; 1972.
59. Purdy, R. H.; Moore, P. H.; Narasimha Roa, P.; Hagino, N.; Yamaguchi, T.; Schmidt, P.; Rubinow, D. R.; Morrow, A. L.; Paul, S. M.: Radioimmunoassay of 3α-hydroxy-5α-pregnan-20-one in rat and human plasma. Steroids. 55:290–296; 1990.
60. Rodbard, D.; Hutt, D. M.: Statistical analysis of radioimmunoassay and immunoradiometric assays: A generalized, weighted iterative, least squares method for logistic curve fitting. In: International Atomic Energy Agency. Symposium on Radioimmunoassay and related procedures in medicine. New York: Uniput; 1974.
61. Roof, R. L.; Duvdevani, R.; Stein, D. G.: Gender influences outcome of brain injury: Progesterone plays a protective role. Brain Res. 607:333–336; 1993.
62. Ryan, J. P.: Wandering in Alzheimer's disease: Clinical and neurobiological perspectives. In: Spear, N. E.; Spear, L. P.; Woodruff, M. L., eds. Neurobehavioral plasticity: Learning, development, and response to brain insults. Hillsdale, NJ: Lawrence Erlbaum Associates; 1995.
63. Schneider, L. S.; Farlow, M. R.; Henderson, V. W.; Pogoda, J. M.: Effects of estrogen replacement therapy on response to tacrine in patients with Alzheimer's disease. Neurology 46:1580–1584; 1996.
64. Schneider, L. S.; Farlow, M. R.; Pogoda, J. M.: Potential role for estrogen replacement in the treatment of Alzheimer's dementia. Am. J. Medicine 1903:46A–50S; 1997.
65. Singh, M.; Meyer, E. M.; Millard, W. J.; Simpkins, J. W.: Ovarian steroid deprivation results in a reversible learning impairment and compromised cholinergic function in female Sprague–Dawley rats. Brain Res. 644:305–312; 1994.
66. Sutherland, R. J.; Rudy, J. W.: Place learning in the Morris place navigation task is impaired by damage to the hippocampal formation even if the temporal demands are reduced. Psychobiology 16:157–163; 1988.
67. Tang, M.-X.; Jacobs, D.; Stern, Y.; Marder, K.; Schofield, P.; Gurland, B.; Andrews, H.; Mayeux, R.: Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. Lancet 348:429–432; 1996.
68. Wenger, N. K.; Speroff, L.; Packard, B.: Cardiovascular health and disease in women. N. Engl. J. Med. 329:247–256; 1993.
69. Yu, Z. F.; Cheng, G. J.; Hu, B. R.: Mechanism of colchicine impairment on learning and memory, and protective effect of CGP36742 in mice. Brain Res. 750:53–58; 1997.
70. Zhang, M.; Katzman, R.; Salmon, D.; Jin, H.; Cai, G.; Wang, Z.; Qu, G.; Grant, I.; Yu, E.; Levy, P.; Klauber, M. R.; Liu, W. T.: The prevalence of dementia and Alzheimer's disease in Shanghai, China: Impact of age, gender, and education. Ann. Neurol. 27:428–437; 1990.
71. Zumoff, B.: Does postmenopausal estrogen administration increase the risk of breast cancer? Contributions of animal, biochemical, and clinical investigative studies to a resolution of the controversy. Proc. Soc. Exp. Biol. Med. 217:30–37; 1998.