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Effects of long-term treatment with estrogen and medroxyprogesterone acetate on synapse number in the medial prefrontal cortex of aged female rats

Nioka C. Chisholm, MA^{a,2} and Janice M. Juraska, PhD^{a,b,1}

^aDepartment of Psychology, University of Illinois at Urbana – Champaign, Champaign, Illinois 61820

^bNeuroscience Program, University of Illinois at Urbana – Champaign, Champaign, Illinois 61820

Abstract

Objective—The present study investigated the effects of long-term hormone treatment, including the most commonly prescribed progestin, medroxyprogesterone acetate, during aging on synaptophysin labeled boutons, a marker of synapses, in the medial prefrontal cortex (mPFC) of rats.

Methods—Female Long Evans hooded rats were ovariectomized at middle age (12–13 months) and were placed in one of 4 groups: no replacement (n=5), 17 β -estradiol alone (n=6), estradiol and progesterone (n=7) or estradiol and medroxyprogesterone acetate (n=4). Estradiol was administered in the drinking water and progestagens were administered via subcutaneous pellets that were replaced every 90 days. Following seven months of hormone replacement, animals were sacrificed and brains were stained for synaptophysin, a membrane component of synaptic vesicles. The density of synaptophysin labeled boutons was quantified in the mPFC using unbiased stereology and multiplied by the volume of the mPFC to obtain total number.

Results—Animals receiving estradiol and medroxyprogesterone acetate had significantly more synaptophysin labeled boutons in the medial prefrontal cortex than animals not receiving replacement ($p < .03$) and those receiving estradiol and progesterone ($p < .02$). In addition, there was a non significant trend for animals receiving estradiol alone to have more synapses than those receiving estradiol and progesterone.

Conclusions—This study is the first to examine the effects of estradiol and medroxyprogesterone acetate during rat aging on cortical synaptic number. Estradiol with medroxyprogesterone acetate, but not progesterone, resulted in a greater number of synapses in the mPFC during aging than no replacement.

Keywords

estrogen; MPA; synaptophysin; hormone therapy; progesterone

¹To whom correspondence should be addressed: Department of Psychology, University of Illinois, 603 E. Daniel Street, Champaign, IL 61820. Fax: (217) 244-5876. jjuraska@illinois.edu.

²Author previously published as Nioka C. Lowry

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Introduction

Menopause in humans is associated with a loss of ovarian hormones and this decline in estrogen and progesterone has been linked to several of the symptoms related to menopause. Hormone therapies including Premarin (conjugated equine estrogens; CEE) and Prempro (CEE in combination with medroxyprogesterone acetate; MPA), have been approved to alleviate these symptoms. In women with a uterus, MPA, a synthetic analogue of progesterone, is administered in combination with estrogen therapy to prevent endometrial hyperplasia¹. Along with alleviating some of the symptoms of menopause, hormone treatment has beneficial effects on cognition²⁻⁴. However, results from the Women's Health Initiative indicated that CEE alone or CEE administered with MPA results in an increased risk of stroke and dementia⁵⁻⁷. There is evidence that the timing of hormone replacement onset is an important factor and may explain the negative findings of the Women's Health Initiative studies⁸⁻¹⁰.

Although data on the cognitive effects of hormone treatment in post-menopausal women seem inconsistent, several neurobiological studies have found that ovarian hormones increase synapses in the hippocampus of both young adult rats and non human primates. Ovariectomy decreased synapses in the CA1 region of the hippocampus¹¹ and estradiol administration increased the density of spines and synapses in the CA1 of young rats and non human primates¹²⁻¹⁵. Chronic treatment with CEE increased synaptic density in the CA1 of young adult rats^{16,17}. The synaptic response to estrogen is thought to involve estrogen receptor (ER) α and ER β , but the percentage of ER α labeled synapses¹⁸ and the amount of ER β ¹⁹ decreases in the hippocampus during aging indicating that the aged brain might respond differently to estradiol. Indeed, estradiol increased synaptophysin, a membrane component of synaptic vesicles, in the CA1 of young animals but not in middle-aged rats²⁰. Although estradiol increased NR1, a subunit of the NMDA receptor, in the hippocampus of aged animals, there was no increase in synapse number¹³.

Fewer studies have looked at the effects of hormone treatment on the prefrontal cortex (PFC), which exhibits greater neuroanatomical loss in both humans and other animals during aging than the hippocampus²¹. Several studies have identified the human PFC as a region that has greater decline in gray matter volume during aging than other brain areas^{22,23}. This change in volume is accompanied by age-related losses of dendrites and spines^{24,25}. There are also age-related losses of dendrites and spines in the PFC of non-human primates^{26,27} and rats²⁸⁻³⁰. Similar to the hippocampus, estrogen can alter the structure of the PFC. Ovariectomy decreased spine density in the young adult rat PFC³¹ and estradiol benzoate increased spine synapse density in the PFC of young non-human primates³². Furthermore, long-term cyclic estradiol treatment increased dendritic spine density in the PFC of both young and aged rhesus monkeys^{33,34}, indicating that estradiol may affect the number of synapses in the aged PFC. In addition, intact females lose fewer spines during aging in the mPFC than males which may be due to the continued secretion of estrogen and progesterone during estropause in rats²⁹. To our knowledge, no study has examined the effects of progestogens on the PFC.

Progestogens can alter the effects of estrogen on behavior in aging females^{35,36} and may also change the effect of estrogen on synaptic numbers. The few studies that have investigated the effects of progestogens on synapse number have examined the hippocampus. Although progesterone administered alone increased the density of synaptophysin in young adult rhesus monkeys, progesterone administered with estrogen resulted in densities similar to ovariectomized controls¹⁵. A more recent study found that estradiol in combination with progesterone increased synaptophysin in hippocampal CA1 of young rats but not middle-aged or aged animals²⁰. MPA is the progestin most commonly

prescribed to women and very little is known about the neural effects of long-term use. Both MPA and progesterone bind with high affinity to the progesterone receptor as well to the androgen and glucocorticoid receptors^{37–39}. However, progesterone is readily metabolized to allopregnanolone⁴⁰ whereas MPA inhibits the enzymes needed for this conversion^{41–43}. Differences in mechanisms of action between these progestogens may result in divergent neural responses. Surprisingly only one study has evaluated the effects of MPA on synapse number. Chronic treatment with MPA alone or in combination with CEE resulted in an increase in the number of synapses in the hippocampus of young rats¹⁷. It is currently unknown if long-term treatment with MPA alters synapse number in the aged brain.

The potential effects of estrogen and the addition of progestogens on the number of synapses in the PFC are especially pertinent, given the dramatic changes in the PFC during aging. Therefore, the present study examined the effects of long-term chronic hormone treatment on the number of synaptophysin labeled boutons in the mPFC of aging females.

Methods

Subjects

Subjects were 22 female Long Evans hooded rats purchased from Charles River Laboratories as retired breeders at the age of 11–12 months. Due to limited availability from the supplier, animals were run in two experimental cohorts. Animals from the same group were pair- or triple- housed, in clear Plexiglass cages in a temperature-controlled environment on a 12:12-hr light–dark cycle. Animals from these cohorts were used in two behavioral experiments prior to sacrifice^{36,44}. Standard rodent chow (Harlan 8604 Tekland) and water were available *ad libitum* to all animals, except during behavioral procedures during which the animals were maintained at 85–90% of their normal body weight. All rats were handled, checked for health problems (tumors), and weighed weekly. At sacrifice, both body and uterine weight were measured. Animal care and experimental procedures were in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee.

Hormone Treatment

All subjects were ovariectomized (OVX) at 12–13 months, because intact female rats continue to secrete low levels of ovarian hormones during aging^{29,35,45}. Subjects were anesthetized with 4% isoflurane and ovaries were removed via bilateral incisions. Animals were administered the analgesic, carprofen (0.05 mg/kg delivered intraperitoneally) prior to surgery and again 12 hours later, in accordance with animal care policy. Subjects were housed individually for five days following surgery to allow for recovery and then returned to pair- or triple-housed conditions. Hormone administration was initiated the day of surgery and continued until sacrifice. Animals were randomly assigned into the following four groups: no replacement (n = 5), 17 β -estradiol (E₂) (n = 6), E₂ and MPA (E₂ + MPA) (n = 4), E₂ and progesterone (E₂ + P) (n = 7).

17 β -estradiol (E₂) Administration—As in Lowry et al.³⁶, all groups receiving estradiol were given E₂ in their drinking water. A pilot study indicated that an E₂ dose of 47ug/kg/day produced estrogen levels in the physiological range for this age group (25–30 pg/ml)^{29,35}. E₂ was first dissolved in 95% ethanol (2mg/ml) and then dissolved in water as described in Gordon et al.⁴⁶. Water bottles were filled with new estrogen water every third day and stock estrogen water was stored in a dark refrigerator. Water consumption was measured for each cage and remained between 60–80 ml/kg/day throughout the experiment for all groups. This range in water consumption resulted in E₂ doses between 40–55 ug/kg/day. The dose of E₂ was calculated by taking the amount of water consumed by a cage and dividing by the sum

of the weights in that cage. This value was then multiplied by the E₂ concentration in the water.

Progestogen Treatment—On the day of OVX, one hormone pellet of either progesterone or MPA was inserted through a small incision in the nape of the neck in the appropriate groups. Progesterone pellets were made from silastic tubing (Dow Corning) packed with crystalline hormone. Studies have shown that 40 mm implants produce hormone levels between those found in aging female rats in persistent estrus and persistent diestrus⁴⁷. The MPA pellets (1.5mg) were purchased from Innovative Research of America. The 1.5mg 90-day release pellets result in a dose similar to that in women taking 2.5 mg per day when expected daily release and average body weight are factored in. Progesterone and MPA pellets were replaced every 90 days. All other groups received sham surgeries at the time of pellet replacement.

Histology

At 19–20 months, after approximately 7 months of hormone treatment, rats were deeply anesthetized with sodium pentobarbital (2 mg/kg of a 50 mg/ml solution) and perfused intracardially with phosphate buffered saline followed with a solution of 4% paraformaldehyde, 4% sucrose and 1.4% sodium cacodylate in dH₂O. The brains were removed and stored in the same solution for 24 hours. Brains were then transferred to a sodium cacodylate buffer solution and shipped at room temperature to Neuroscience Associates (Knoxville, TN) for sectioning. Briefly, brains were cryoprotected in a glycerol and DMSO-based formulation prior to sectioning. Fixed brains from each cohort were embedded together in a gelatin block that was frozen-sectioned at 30μm. Every tenth section was stained for synaptophysin, a membrane component of synaptic vesicles, and other sections were saved. Adjacent sections were stained with methylene blue/azure II, a cell body stain, in our laboratory for volume calculations.

Volume estimation

Using cytoarchitectonic criteria^{48,49} the ventral mPFC (prelimbic (PL) and infralimbic (IL)) regions were parcellated at 31.25× using a camera lucida on coded slides stained with methylene blue/azure II. The ventral mPFC was parcellated starting with the first section containing white matter continuing through the first section in which the genu of the corpus callosum appeared. This resulted in parcellation of both hemispheres in four to five sections per subject. Parcellation criteria used for the ventral mPFC have been described in Markham et al.⁵⁰. For the present study, the PL and IL were not separated. The border between PL and dorsal anterior cingulate (ACd) is marked by a broadening of layer V and an increase in the density of layer 3 cells in the ACd as compared to the PL (Figure 1). Layers 2/3 and 5/6 were measured separately (rat mPFC lacks layer 4). Camera lucida tracings were scanned into a computer and Image J (version 1.44, 2010) was used to measure the area of each parcellation. The volume was then calculated by multiplying this area by the mounted tissue thickness between sections. Mounted tissue thickness was measured by determining the difference between the focal depth of the top and bottom of the tissue using the StereoInvestigator software program (MicroBrightField). Ten measurements of thickness were taken per animal. An average section thickness was calculated per animal and used in the calculations for that animal. Mounted section thickness was equivalent among all groups. Randomly, animals were selected for re-parcellation and the area of the ventral mPFC was recalculated. This was done to insure consistency in parcellation criterion. Area measurements remained within 5% between parcellation drawings for a given animal.

Synapse Number

Synaptophysin boutons were quantified in the PL and IL of the mPFC using the StereoInvestigator software program (MicroBrightField). The optical disector was used to obtain stereologically unbiased counts of synaptophysin density in each layer of the mPFC (Figure 2). Using this program, contours were drawn of layers 2/3 and layers 5/6 in the ventral mPFC. Both hemispheres from two sections containing the mPFC were used for counts. At least 200 synaptophysin boutons were counted within each layer (2/3, 5/6) for each subject. The area of the counting frame used was $4\ \mu\text{m} \times 4\ \mu\text{m}$ with approximately 20 counting sites per section in both layers 2/3 and layers 5/6. Section thickness was used for disector height excluding the $.5\ \mu\text{m}$ guard zones. Section thickness was measured at every fifth site on counted sections. Boutons fully inside the counting frame or those that contact the 'inclusion' line without contacting the 'exclusion' line were included in counts (Figure 2). Average counts for each layer were divided by the volume of the counting frame to get the density of synaptophysin boutons. This density was then multiplied by the volume of the mPFC to obtain synapse number.

Statistical Analysis

Body weights, uterine weights, volume of the mPFC, and the total number of synaptic boutons, as well as those in layers 2/3 and in layers 5/6, were each analyzed using a one-way ANOVA with cohort as a covariate. Fisher's LSD tests were used for all post hoc comparisons.

Results

Body and Uterine Weights

The ANOVA revealed a significant effect of treatment on body weight ($p < .01$). Post-hoc Fisher's LSD revealed that the no replacement group weighed significantly more than all groups that received hormone treatment (E_2 : $p < .01$; $E_2 + P$: $p < .01$; $E_2 + \text{MPA}$: $p < .01$). No other comparisons reached significance (Table 1.)

The ANOVA resulted in a significant effect of treatment on uterine weight ($p < .02$). Post-hoc Fisher's LSD revealed that uterine weight in the no replacement group was significantly lower than all groups that received hormone treatment, indicating that hormone treatment was physiologically effective (E_2 : $p < .01$; $E_2 + P$: $p < .01$; $E_2 + \text{MPA}$: $p < .01$).

Synapse Number

The volume of the mPFC was not significantly different between any of the groups. There was an overall effect of hormone treatment on the total number of synaptophysin boutons in the mPFC ($p < .05$). Post hoc tests revealed that animals receiving $E_2 + \text{MPA}$ had more synaptophysin boutons than those receiving no replacement ($p < .03$) and $E_2 + P$ ($p < .02$). There was a non significant trend for animals receiving estrogen alone to have more synaptophysin boutons than those receiving $E_2 + P$ ($p < .09$) (Figure 3). For layers 2/3, hormone treatment did not alter synapse number ($p = .17$) (Figure 4a). Analysis of layers 5/6 found a significant effect of hormone treatment on the number of synaptophysin labeled boutons ($p = .04$). Post hoc tests revealed that animals receiving $E_2 + \text{MPA}$ had more synaptophysin boutons than those receiving no replacement ($p < .02$) and $E_2 + P$ ($p < .02$) (Figure 4b).

Discussion

Long-term treatment with estradiol in combination with MPA to middle aged female rats resulted in greater numbers of synapses, as indicated by synaptophysin labeled boutons, in

the mPFC as compared to ovariectomized controls. This is in agreement with the only other study to evaluate the effects of MPA on synapse number which found that MPA alone or administered with CEE increased synapses in the CA1 of young adult rats¹⁷. Also, we have preliminary data showing that animals receiving estradiol in combination with MPA cyclically have greater numbers of synapses than ovariectomized controls²¹. Importantly, synapse number decreases during aging^{51,52} and several measures related to synapse number are altered by aging in the PFC. There are age-related losses of dendrites and spines in the PFC of humans^{24,25}, non human primates^{26,27}, and rats^{28–30}. These changes have been linked to age related cognitive decline. For example, during aging, non human primates experience a decrease in the density of axospinal synapses in the PFC which correlates with acquisition of a delayed non-match to sample task⁵³. Furthermore, age related deficits on an object recognition memory task are associated with decreases in dendritic spine density in the mPFC of rats³⁰. Because aging has been associated with a loss of synapses and this loss has been linked to cognitive deficits, the greater number of synapses in the aged PFC following long-term exposure to estradiol and MPA could result in beneficial effects on behavioral tasks mediated by the mPFC.

However, few studies have evaluated the effects of estradiol in combination with MPA on cognition. MPA administered without estradiol impaired performance on the water radial arm maze and water maze^{54,55}. Although progesterone enhanced performance on the water maze and object recognition, MPA alone did not alter performance⁵⁶. A subset of the animals in the present study was tested on the water maze, and estradiol plus MPA resulted in impaired performance as compared to other hormone treated groups³⁶. In contrast, when many of the same subjects were tested on a delayed alternation t-maze task, treatment with estradiol in combination with MPA resulted in animals requiring fewer sessions to reach criterion⁴⁴. The brain region mediating performance on a task may play an important role in determining the behavioral outcome of hormone treatment, and the studies that have found that MPA impairs cognition have used tasks that rely heavily on the hippocampus^{36,54,55}. The present study found that estradiol plus MPA results in more synaptophysin labeled boutons as compared with ovariectomized controls in the mPFC, suggesting that this combination of hormone treatment may be beneficial on tasks that rely more heavily on the mPFC.

Unlike estradiol and MPA, estradiol plus progesterone did not affect the number of synaptophysin labeled boutons. Although MPA is a synthetic analogue of progesterone, studies have found that these two progestogens do not share identical biological properties. MPA has a higher affinity for androgen and glucocorticoid receptors than progesterone⁵⁷, and progesterone is readily metabolized to allopregnanolone⁴⁰ while MPA inhibits the enzymes needed for this conversion^{41–43}. Several studies have found that these two progestogens result in differential neural outcomes. For example, MPA, but not progesterone, suppresses cytokine production after an inflammatory stimulus in vitro³⁸. In addition, in vitro studies have found that progesterone protected against kainic acid-induced neuronal loss while MPA did not⁵⁸. Progesterone alone and in combination with estrogen protected against glutamate toxicity while MPA was not protective and prevented estradiol's influence on neuroprotection^{59,60}. Also, treatment with estradiol and progesterone but not MPA, increased proliferation of neuroprogenitor cells in culture⁶¹. Furthermore, progesterone increased levels of brain-derived neurotrophic factor while MPA decreased this measure⁶². Although MPA and progesterone treatments without estrogen decreased levels of glutamic acid decarboxylase in the hippocampus, this decrease was only significant in those receiving MPA⁵⁵. Interestingly, most of these studies indicate a beneficial effect of progesterone on the measures evaluated, which was not found in the present study; however none of these studies examined synapse number or the consequence of neuroprotection for normal aging. The current study administered hormones for approximately seven months in

order to evaluate the long-term effects of hormone treatment, and many of the previous studies have used acute treatments. It has been found that chronic treatment of ovarian hormones results in different outcomes than more acute treatments^{63–65}. It is possible that long-term hormone treatment results in receptors that are less sensitive and that these two progestagens differ in this long term dynamic. Furthermore, previous studies comparing these progestogens have examined the effects on the hippocampus, and it is known that the anatomical structure of the PFC is particularly vulnerable to aging, while the hippocampus is not (reviewed in ²¹). It is possible that because the hippocampus and mPFC respond differently to aging they are differentially affected by progestogens administered during this time.

Estrogen is known to alter several aspects of synaptic communication in young rats. For example, estrogen administered to ovariectomized animals, returns synaptophysin levels and spine densities in the CA1 to levels observed in intact controls^{11,20,66}. Also, ovariectomy decreases synaptophysin levels in the inner layer of the dentate gyrus and estrogen restores this⁶⁷. However, the aged hippocampus appears to be less responsive to the effects of estrogen. Estrogen treatment did not increase synapse density in the CA1 of aged animals¹³ and there was decreased amounts of synaptic ER α immunoreactivity in this brain region¹⁸. In addition, estradiol increased the amount of synaptophysin and opioid peptides in the CA1 and dentate gyrus of young animals, but did not alter the amount in aged animals²⁰. The present study found that long-term estrogen marginally increased synapses in the aged mPFC suggesting that in contrast to the hippocampus, the mPFC may remain responsive to estrogens during aging. This is in agreement with previous studies in non human primates. Long-term cyclic treatment with estradiol increased apical and basal dendritic spine density in the PFC of aged female rhesus monkeys and reversed age-related impairments on a delayed response task mediated by the PFC^{34,68}. It is important to note that the means in the present study were in the direction of estrogen treated animals having more synapses although it did not reach significance. Studies have found that rodent diets high in phytoestrogens result in a greater density of spines in both the hippocampus and PFC⁶⁹. As in most of the literature, animals in the present study were maintained on a standard rodent diet and it is possible that the low levels of soy in the diet increased the number of synaptophysin boutons in our no replacement animals minimizing differences between groups³². The differences that were found are especially notable in light of this possibility.

The effects of estrogen on synapse number have been shown to be mediated through estrogen receptors^{70,71}. However, because the effects of estrogen alone were subtle in the current study and the addition of MPA led to significantly more synapses as compared to no replacement, it seems likely that this effect was mediated by a different mechanism. Research suggests that synapse number is also regulated by IGF-1. IGF-1 null animals have decreased dendritic length and spines in the frontoparietal cortex⁷² and over expression of IGF-1 increases the number of synapses in the dentate gyrus⁷³. Importantly, IGF-1 protein levels are decreased during aging⁷⁴ and modulated by steroid hormones⁷⁵. Estrogen alone has been found to decrease IGF-1 levels; however, estrogen administered with MPA resulted in an increase in IGF-1 in post-menopausal women⁷⁶ while estradiol administered with natural progesterone does not⁷⁷. Therefore, the effects of estrogen in combination with MPA on synapse number seen in the current study may be mediated in part by increasing IGF-1 levels.

Given the vulnerability of the PFC to the effects of aging, the alteration of the mPFC by hormone treatment during aging may have implications for age-related cognitive deficits. Results from the current study indicate that the aged PFC remains responsive to certain hormone treatments and thus these treatments may protect against age-related cognitive deficits. Indeed, treatment with acute estrogen alone given to postmenopausal women

benefited tasks relying on the PFC to a greater extent than those relying on the hippocampus³. In addition, women not receiving estrogen replacement performed worse on several PFC dependant tasks than those receiving hormone treatment⁷⁸. Future studies in humans should evaluate the effects of long-term hormone treatment, including the addition of a progestogen on PFC dependant tasks.

Conclusions

This study identifies the prefrontal cortex as a brain region that is altered by long-term chronic hormone treatment during aging in the rat. Specifically, treatment with estradiol and MPA resulted in more synaptophysin labeled boutons in the mPFC relative to females with no replacement or replacement with estradiol and progesterone. These findings provide insight into the neural effects of long-term hormone treatment.

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Figure 1.

Coronal section, cut at 60 microns, through the medial prefrontal cortex with cell bodies stained using Methylene blue/Azure II. Borders of the prelimbic and infralimbic regions are shown based on the cytoarchitectonic characteristics revealed by this stain^{48,49}. Reprinted from Markham et al.⁵⁰.

Figure 2. High magnification image of the mPFC stained for synaptophysin, a membrane component of synaptic vesicles. The counting frame used to stereologically quantify the number of boutons was 4 μm by 4 μm .

Figure 3.

Total number (mean + SEM) of synaptophysin labeled synaptic boutons in the mPFC. Animals receiving E₂ + MPA had more synaptophysin boutons than those receiving no replacement and E₂ + P. There was a non significant trend for animals receiving estrogen alone to have more synaptophysin boutons than those receiving E₂ + P (*p < .03, #p < .09).

Figure 4.

Figure 4 A. Total number (mean + SEM) of synaptophysin labeled synaptic boutons in Layers 2/3 of the mPFC. Hormone treatment did not significantly alter the number of synapses. B. Total number (mean + SEM) of synaptophysin labeled boutons in Layers 5/6 of the mPFC. Animals receiving E_2 + MPA had more synaptophysin labeled boutons than those receiving no replacement and E_2 + P (* $p < .02$).

Table 1

Body and Uterine Weight

Hormone Group	Mean Body Weight (g)	Mean Uterine Weight (g)
No replacement	619.6 ± 37.7	.05 ± .01
Estrogen	501.8 ± 35.0 *	.12 ± .02 *
Estrogen & P	472.7 ± 28.1 *	.11 ± .01 *
Estrogen & MPA	451.3 ± 66.2 *	.12 ± .02 *

Body and uterine weights were taken at sacrifice for all groups. The no replacement animals weighed significantly more and had lower uterine weights than all hormone treated groups.

*
p < .01