Gonadal Hormones Modulate the Dendritic Spine Densities of Primary Cortical Pyramidal Neurons in Adult Female Rat

Adult dendritic arbors and spines can be modulated by environment and gonadal hormones that have been reported to affect also those of hippocampal and prefrontal cortical neurons. Here we investigated whether female gonadal hormones and estrous cycle alter the dendrites of primary cortical neurons. We employed intracellular dye injection in semisixed brain slices and 3-dimensional reconstruction to study the dendritic arbors and spines of the major cortical output cells, layer III and V pyramidal neurons, during different stages of the estrous cycle. Dendritic spines of both pyramidal neurons were more numerous during proestrus than estrus and diestrus, whereas dendritic arbors remained unaffected. Ovariectomy (OHE) reduced dendritic spines by 24-30% in 2 weeks, whereas subcutaneous estrogen or progesterone supplement restored it to normal estrous/diestrous level in 14 days; neither treatment affected the dendritic arbors. Reduction of dendritic spines following OHE was associated with decrease of PSD-95 suggesting decrease of excitatory synapses. Thus, fluctuation of gonadal hormones during the female sex cycle is likely to modulate primary cortical functions and loss of gonadal hormones for instance following menopause might compromise cortical function, and the effect could be reversed by exogenous female sex hormones.

Keywords: estrogen, menopause, ovariectomy, PSD-95, pyramidal neuron

Introduction

Dendrites, the main receiving structures of neurons, are dynamic structures and their arbors and spines change in response to environment changes (Horner 1993), injuries (e.g., Tseng and Hu 1996; Chen et al. 2003), diseases (e.g., Paul and Scheibel 1986; Ferrer et al. 1990), and following aging (Scheibel et al. 1975; Wang et al. 2009). Gonadal hormone is known to influence the dendritic morphology of hippocampal neurons for almost 2 decades (see review by Cooke and Woolley 2005). Recent investigations show that it affects prefrontal cortical neurons as well (Radley et al. 2006; Petanjek et al. 2008). Studies in the rodents on how female gonadal hormone could alter neuronal dendrites showed that brain level of apolipoprotein E (ApoE), a substance that facilitates process outgrowth, changes with estrous cycle and may be modulated by exogenous 17β-estradiol (Struble et al. 2003; Nathan et al. 2004). These suggest that dendritic length could be cyclically regulated during the estrous cycle. Estrous cycle and gonadal hormone also affect the densities of dendritic spines on hippocampal pyramidal neurons (Gould et al. 1990; Woolley and McEwen 1992; Rudick and Woolley 2001). In mice, estrogen enhanced spatial memory and altered the structure of hippocampal neurons (Li et al. 2004) suggesting that estrogen-induced hippocampal structural plasticity can potentially affect cognition. In mammals, females are uniquely endowed with sex cycle that displayed cyclic physiological changes in association with profound sensory and motor influences (e.g., Bonifazi et al. 2004). Behavioral study of female rats shows that grooming, rearing, and motor activity change cyclically during estrous cycle suggesting that brain areas such as the primary cortices may be affected during female sex cycle as well (Scimonelli et al. 1999). To investigate this, we studied the dendritic arbors of layer III and layer V pyramidal neurons of the primary sensory and motor cortices (primary sensorimotor cortex) of adult female rats during different stages of the estrous cycle by using intracellular dye injection and 3-dimensional neuronal reconstruction. The study also sought to ascertain dendritic changes, if any, are female sex hormone-dependent. Additionally, we studied the effect of ovariectomy (OHE) and explored whether subsequent female sex hormone administration, delivered via subcutaneous pellet implantation, would reverse the changes.

Materials and Methods

Animal and Tissue Preparation

A total of 35 virgin female Sprague-Dawley rats (200-300 g) were studied. Animals were housed and cared for according to guidelines of animal researches of the National Chung-Hsing University. All efforts were taken to minimize animal suffering during and following surgery. For studying the dendritic characteristics of cortical pyramidal neurons during different phases of the estrous cycle, the estrous cycle of 13 rats, 7 proestrus, 3 estrus, and 3 diestrus were confirmed with vaginal smears. To study the effect of OHE, 6 rats were such operated and 2 additional rats were sham-operated and allowed to survive for 2 weeks. Sham-operated rats were, in addition, confirmed to be at the proestrus with vaginal smear before sacrifice. Three each of the normal proestrus and OHE rats were processed for western blotting of the glutamatergic postsynaptic marker PSD-95 (Furuyashiki et al. 1999). To apply gonadal hormones, we followed previous studies in rats and used the same delivery system and dosage, namely 0.5 mg estrogen and 10 mg progesterone pellets (21-day release, Innovative Research of America, Sarasota, FL), for testing the effects of these 2 hormones (Sudoh et al. 2001; Hoffman et al. 2003). Twelve OHE rats 2 weeks following surgery were divided into 4 groups to receive subcutaneous implantation, in the posterior neck, of estrogen pellet (n = 5), progesterone pellet (n = 3), both estrogen and progesterone pellets (n = 5), and placebo pellet (n = 3, 21-day release, Innovative Research of America), respectively. These animals were allowed to survive for 14 days following implantation and then processed for fixed tissue intracellular dye injection. Briefly, they were deeply anesthetized with chloral hydrate (0.45 mg/100 g body weight) and perfused with a fixative containing 2% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3, at room temperature for 30 min. Immediately following the perfusion, the whole brain was carefully removed and sectioned with vibratome into 350-μm-thick coronal slices.
**Intracellular Dye Injection and Subsequent Immunoconversion of the Injected Dye**

We used Lucifer yellow (LY; Sigma, St Louis, MO) as the intracellular dye to reveal the dendritic arbors. For dye injection, brain slices prepared above were first treated with 0.1 M PB containing 10^{-7} M 4′, 6-diamidino-2-phenylindole (Sigma) for 30 min to make cell nuclei fluoresced blue under the same filter set that visualized the yellow fluorescence of LY. This enabled us to select individual cells of specific cortical layers for dye injection. Slice was then placed on a dish on the stage of a fixed-stage, epifluorescence microscope (Olympus BX51) and covered with thin layer of 0.1 M PB. An intracellular micropipette filled with 4% LY in water mounted on a 3-axial hydraulic micromanipulator (Narishige, Tokyo, Japan), and a long working distance objective lens (×20) was used to facilitate the selection of layer III and layer V pyramidal neurons of the studied cortex for dye injection. An intracellular amplifier (Axoclamp-IIB) was used to generate the constant negative current for injecting LY till all terminal dendrites fluoresced brightly. Several well-separated neurons could be injected in each slice. The slice was then removed, rinsed in 0.1 M PB, and postfixed in 4% paraformaldehyde in 0.1 M PB for 3 days. They were then rinsed thoroughly in 0.1 M PB, cryoprotected, and carefully sectioned into 60-μm-thick serial sections with a sliding microtome.

To convert the intracellular dye LY into nonfading material, sections were preincubated with 1% H2O2 in PB for 30–60 min to remove endogenous peroxidase activity. They were then rinsed 3 times in phosphate-buffered saline (PBS) and incubated for an hour in PBS containing 2% bovine serum albumin and 1% Triton X-100. Sections were then treated in solution containing bovineylated rabbit anti-LY (1:200; Molecular Probes, Eugene, OR) in PBS for 18 h at 4°C. Following subsequent rinses in PBS, sections were incubated with standard avidin-biotin horseradish peroxidase reagent (Vector, Burlingame, CA) for 3 h at room temperature. They were then reacted at room temperature with a solution containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.01% H2O2 in 0.05 M Tris buffer. Sections were then treated in solution containing biotinylated rabbit anti-LY (1:200; Molecular Probes, Eugene, OR) in PBS, followed by 0.3% H2O2 in PB for 30 min that yielded band intensity linearly reflecting the amount of protein. A low-powered picture of the neuron from one of the 60-μm-thick brain slice. The neuron was first injected with LY intracellularly in a 350-μm-thick brain slice. The slice was then resectioned and processed to polyvinylidene difluoride membranes, and subjected to western analysis for PSD-95. Mouse anti-PSD-95 (Chemicon, Temecula, CA) diluted (1:1000) in 10 mM Tris, pH 7.4, with 150 mM NaCl, and 5% skim milk was used for immunoblotting. Monoclonal antibody to GAPDH (Chemicon) was used as the internal standard. We followed the supplier’s recommendation on the dilution of the primary antibodies that we used. Similar dilution of the same antibody was used to detect PSD-95 in rat brains (Ansari et al. 2008). Antibody thus diluted was further tested by varying the amount of protein loaded to different lanes of the same gel to ensure that it linearly detected the protein. Exposure time was selected by developing the gel in steps from 10 s to 1 min or longer if necessary, and a proper exposure time of around 1 min that yielded band intensity linearly reflecting the amount of protein tested was adopted for this study. Relative optical densities (ROD) of the bands corresponding to the proteins of interest were quantified using a densitometer (Image ProPlus; Media Cybernetics, Silver Spring, MD) and presented relative to the ROD of GAPDH for that lane.

**Western Blotting of PSD-95**

To determine whether changes of the densities of dendritic spines reflect alterations of the quantities of excitatory synapses, we studied the amount of PSD-95, glutamatergic postsynaptic marker (Furuyashiki et al. 1999), in the primary somatosensory cortex of the brains of proestrous and OHE rats. Briefly, the cortex of interest was rapidly dissected and homogenized immediately at 4°C. Following centrifugation at 2000 g, 50 mM sodium vanadate hydroxyethyl-1-piperazineethanesulfonic acid (pH 7.4)-containing 21 000 cells and nuclei, the postnuclear supernatant was centrifuged at 176 000 g. Following centrifugation at 6000 g × 30 min, fractions from the pellet were obtained by resuspending and homogenizing with another buffer containing 0.25 M sucrose, protease inhibitor cocktail, 50 mM sodium vanadate, and 5 mM MgCl2 and processed with a Dounce homogenizer. The postnuclear supernatant was then centrifuged at 176 000 g. The pellets were then resuspended in the same buffer containing 0.25 M sucrose, protease inhibitor cocktail, 50 mM sodium vanadate, and 5 mM MgCl2 and rehomogenized. The supernatants were then treated in solution containing biotinylated rabbit anti-LY (1:200; Molecular Probes, Eugene, OR) in PBS, followed by 0.3% H2O2 in PB for 30 min that yielded band intensity linearly reflecting the amount of protein. A low-powered picture of the neuron from one of the 60-μm-thick sections of the injected slice. The dendritic segments (B–E) indicated are illustrated at higher magnification to the right. prox., proximal; dist., distal; Bar = 70 μm for (A) and 10 μm for (B–E).

**Data Analysis**

To look into changes of dendritic arbor and length of layer III and layer V pyramidal neurons, the complete dendritic arbors of 4 neurons each of the proestrus (n = 4), 5 neurons of the estrous (n = 5), and 5 neurons from OHE animals (n = 3) were reconstructed 3-dimensionally with Neurolucida (MicroBrightField, Williston, VT) and analyzed with the same software. Statistical significances were tested by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test to find out any difference between treatment groups where n represents the number of animals. To find out whether gonadal hormones affect the density of dendritic spines, we analyzed representative layer III and layer V pyramidal neurons each from each animal of each treatment group. Dendrites of the studied layer III and layer V pyramidal neurons were distinguished into proximal and distal segments of the apical and basal dendrites following the criteria established by Chen et al. (2004). Briefly, for layer III pyramidal neurons, proximal and distal basal dendrites were defined as the segments 25–75 μm (around the first to second branch) and 100–150 μm (around the last 1 or 2 branches) from the soma, respectively. Because layer V pyramidal neurons were relatively large, their proximal and distal basal dendrites were defined as the segments 50–100 μm (around the first to second branch) and 150–200 μm (around the last 1 or 2 branches) from where they originate from the soma, respectively. On the other hand, proximal apical dendrites were the first...
or second branch of the apical trunk and distal apical dendrites were the terminal dendrites after the last branch point in both layer III and layer V pyramidal neurons. Representative dendritic segments of each neuron were reconstructed with ×100 oil immersion objective lens, and the corresponding spine densities of each cell determined. Mean spine densities on each category of the dendritic segments on layer III and layer V cell of each animal were calculated from all 6 neurons studied. Statistical significance was tested with 1-way ANOVA followed by the Newman-Keuls test to find out any difference between treatment groups where $n$ represents the number of animals.

### Results

The combination of methodologies we used enabled us to routinely reveal the profuse dendritic arbor of individual cortical pyramidal neuron in clear isolation. Figure 1A showed a representative layer III cortical pyramidal neuron in one of the

60-μm-thick sections of the brain slice in which the cell was filled. Higher magnification of segments of this intracellular dye-filled neuron (Fig. 1B–E) showed that dendritic spines were adequately revealed for subsequent analysis. Dendritic spines on both layer III and layer V pyramidal neurons of the primary sensorimotor cortex were dynamically regulated during estrous cycle. Numbers of spines on all segments of layer III and layer V pyramidal neurons were higher during proestrus than estrus and diestrus, whereas spine densities during the latter 2 phases of the estrous cycle were comparable (Figs 2 and 3). Spine densities on layer III and layer V pyramidal neurons during the estrus/diestrus were approximately 7–16% and 7–19%, respectively, fewer than that of the proestrus. The dendritic arbors of layer III and layer V pyramidal neurons were 3-dimensional reconstructed and analyzed, and there was no

![Figure 2](https://academic.oup.com/cercor/article-abstract/19/11/2719/383219)

**Figure 2.** Changes of the dendritic spines of layer III pyramidal neurons during different stages of estrous cycle and 2 weeks following OHE. (A) Composite micrographs showing representative distal segments of the basal and apical dendrites. Spine densities per 10 μm length of the dendritic segment were counted and plotted in (B). Spine densities on proximal basal, distal basal, proximal apical, and distal apical dendritic segments were determined from the mean of 6 representative neurons studied in each rat and 4, 3, 3, 3, and 2 animals were studied in proestrus, estrus, diestrus, OHE, and sham-operated groups, respectively. OHE rats survived for 2 weeks. *$P < 0.05$ between the marked and proestrous rat; **$P < 0.01$ between the marked and proestrous rat; #*$P < 0.05$ between the marked and estrous rat, 1-way ANOVA followed by Newman–Keuls tests. Bar = 10 μm for all micrographs.
difference in the number of trunks, length, or branching profuseness (data not shown) between the different stages of estrous cycle (Figs 4 and 5).

To find out whether these estrous cycle-associated dendritic changes were regulated by the gonad, female rats were subjected to OHE and their dendritic characteristics studied 2

Figure 3. Changes of dendritic spines of layer V pyramidal neurons during different phases of the estrous cycle and the level of glutamatergic postsynaptic marker protein in the primary sensorimotor cortex of proestrus and OHE rats. (A) Composite micrographs to show representative segments of distal basal and apical dendrites during different stages of estrous cycle and 2 weeks following OHE. (B) Plot of the changes of spine densities in the groups of animals studied. Spine densities were determined from the mean of 6 representative neurons studied in each rat, and 4 proestrus, 3 estrus, 3 diestrus, 3 OHE, and 2 sham-operated rats were studied. OHE rats survived for 2 weeks. (C) Western blotting of PSD-95 in normal proestrus and OHE rats (n = 3 each). GAPDH is the internal standard. *P < 0.05 between the marked and proestrous rat; $P < 0.01 between the marked and estrous rat; #P < 0.05 between the marked and proestrous rat, 1-way ANOVA followed by Newman-Keuls tests. Bar = 10 μm for all micrographs in (A).
weeks afterward. Numbers of spines on all dendritic segments of both layer III (Fig. 2A) and layer V pyramidal neurons (Fig. 3A) were significantly reduced. Spine densities of layer III and layer V pyramidal neurons were approximately 22–30% and 28–30%, respectively, less than those of both sham-operated and normal proestrous animals and 10–21% and 11–23% fewer, respectively, than those of the normal estrous and diestrous animals (Figs 2 and 3). There was no difference between the spine densities of the sham-operated animals that we examined during proestrus and normal animals of the same estrous cycle (Figs 2B and 3B). To investigate whether loss of dendritic spines represents functional downregulation of excitatory synapses, we performed western blotting and measured the amount of PSD-95, a protein known to associate with glutamatergic excitatory postsynaptic densities, in the studied cortex. The level of PSD-95 in the cortex was reduced by 27% following OHE as compared with that of the normal proestrous rats (Fig. 3C). The shape, length, and branching profuseness of the dendritic arbors of the layer III and layer V cortical pyramidal neurons were, however, not altered 2 weeks following OHE (Fig. 5).

To find out whether female gonadal hormones were responsible for the observed regulation, we studied the effects of exogenous gonadal hormone in female rats that received OHE 2 weeks in advance. Stable diffusion gonadal hormone pellet was implanted subcutaneously as the source of exogenous gonadal hormone in these animals for 14 days. Estrogen pellet increased the spine densities on all segments of the layer III pyramidal neurons of OHE rats by 16–22% to the level close to those of normal estrous and diestrous rats. Exogenous progesterone also increased the spine densities of these neurons by 15–19%, again approximated the levels of those of the normal estrous and diestrous rats. Similar levels of spine density increases were observed on layer V pyramidal

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**Figure 4.** Representative 3-dimensionally reconstructed layer III (A–C) and layer V (D–F) pyramidal neurons of the primary sensorimotor cortices of normal proestrous (A and D) and estrous (B and E) and OHE (C and F) rats. Different colors depict branches of different dendritic trunks. Bar = 100 μm for (A–C) and Bar = 150 μm for (D–F).
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neurons (Figs 6 and 7). Simultaneous implantation of estrogen and progesterone pellets failed to increase the dendritic spine densities further (Figs 6 and 7); the spine densities on all segments of the dendrites of layer III and layer V pyramidal neurons were comparable to those of the normal estrous and diestrous but less than that of the proestrous rats (compare Fig. 7 with Fig. 2). As expected, placebo did not change the spine densities (data not shown).

Discussion

Effects of Gonadal Hormones on Dendritic Spines

Key finding of the present study is that in female rats dendritic spines on the major output neurons, layer III and layer V pyramidal neurons, of the primary sensorimotor cortex were cyclically regulated during the estrous cycle. These changes occurred consistently on all segments of the basal and apical dendrites. Dendritic spine densities were higher during the proestrus than estrus and diestrus. It is known that blood gonadal hormone level changes during the estrous cycle, the concentration of estrogen during proestrus, approximately 60 pg/mL, is about twice that at the other 3 stages namely estrus,
1999) when spine/synapse numbers are elevated. Estrogen appeared to have a similar function in the cortex; it excites brain activity in animal models of epilepsy (Woolley and Schwartzkroin 1998) and in women with seizures as well (Towanabut et al. 1998). Researchers found seizure frequency to be maximal when estrogen rises (Paul and Scheibel 1986; Weiland 1992; Towanabut et al. 1998; Woolley and Schwartzkroin 1998; Bazan et al. 2005; Hattemer et al. 2006). In the clinic, threshold to evoke peripheral motor responses to transcranial magnetic stimulation of the primary cortical motor area was decreased in the presence of high blood level of estrogen, and this was attributed to result from an estradiol-induced potentiation of glutamatergic excitatory synaptic inputs from corticospinal neurons to motoneurons (Bonifazi et al. 2004). It remains to be ascertained whether estrogen directly modulates the excitatory action of NMDA receptor on cortical pyramidal neurons.

In the present study, exogenous progesterone alone was as effective as estrogen in restoring the dendritic spine densities of the primary sensorimotor cortical pyramidal neurons of OHE rats to normal diestrous level. Simultaneous administration of estrogen and progesterone to OHE rats failed to further augment this recovery. On the contrary, progesterone was shown to have a spine-promoting effect, which was additive to that of the estrogen on CA1 pyramidal neurons (Gould et al. 1990). The effect of progesterone could be more complex than it appears considering that seizure frequency is maximal in early estrous cycle when estrogen is high but lower in metestrous or pregnant rats when progesterone is high (Woolley and Schwartzkroin 1998). Woolley and McEwen (1993) proposed that progesterone has a biphasic effect on spine density in that progesterone treatment following estradiol initially increases spine density for a period of 2–6 h but then results in a much sharper decrease than is observed following estrogen alone. In our study, the effect of progesterone was examined 2 weeks following pellet implantation. More thorough studies are needed to resolve the effect of progesterone on cortical pyramidal neurons.

In the present study, we performed OHE that removed the ovary, uterus, and uterine tube, instead of ovariectomy alone. Number wise, dendritic spines of the layer III or layer V pyramidal neurons of the OHE rats were reduced by approximately 22–30% than that of the proestrus rats in accompany with a 27% reduction of PSD-95 suggesting a dramatic reduction of glutamatergic excitatory synapses. These support that OHE reduced primary cortical excitatory action. Exogenous estrogen and/or progesterone restored the spine densities of OHE rats to estrous/diestrous level but failed to reach the proestrus level of normal female rats suggesting that structures other than uterus, uterine tube, and ovary, for instance other secondary female sex
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Notes

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References


weeks did not affect these parameters either. Estrous cycle was found not to alter the dendritic length of hippocampal CA1 pyramidal neurons and prefrontal cortical pyramidal neurons as well (Rasia-Filho et al. 2004). Thus, although gonadal hormone promotes neurite outgrowth and increases ApoE expression in culture cells and developing neurons (Struble et al. 2003; Nathan et al. 2004), it appeared not to alter the dendritic arbors of pyramidal neurons of adult brain.

In conclusion, our results show that in female rats, primary sensorimotor cortical pyramidal neurons changed their dendritic spine densities cyclically during estrous cycle and this appeared to be regulated by the accompanying changes of female gonadal hormones. The supporting effect of female gonadal hormones on the dendritic spines of primary sensorimotor cortical neurons brings up the possibility that the female cortical function is likely to be regulated by the level of circulating sex hormone during sex cycle and compromised following menopause.

Effects of Gonadal Steroids on Dendritic Length

In this study, combination of intracellular dye injection in semifixed brain slices with 3-dimensional reconstruction enabled us to measure the dendritic arbors of the studied neurons more accurately (Chen et al. 2003). Neither the dendritic length nor the branching profuseness of the studied neurons was different during different phases of the estrous cycle. OHE for 2

Pyramidal neurons of the primary sensorimotor cortices of OHE and OHE with estradiol or/and progesterone supplemented rats. Please see the legend of Figure 6 for abbreviation. Six representative layer III and layer V neurons were analyzed in each group. * P < 0.05 between the marked and the corresponding OHE rats, 1-way ANOVA followed by Newman-Keuls tests where n = 3 each.

Figure 7. Analysis of the densities of dendritic spines on layer III (A) and layer V (B) pyramidal neurons of the primary sensorimotor cortices of OHE and OHE with estradiol or/and progesterone supplemented rats. Please see the legend of Figure 6 for abbreviation. Six representative layer III and layer V neurons were analyzed in each of the OHE, OHE + E2, OHE + P4, and OHE + E2P4 rats (n = 3 each). * P < 0.05 between the marked and the corresponding OHE rats, 1-way ANOVA followed by Newman-Keuls tests where n = the number of animals.

hormone sources, the pituitary gland or hypothalamus could be involved in modulating the dendritic spines of primary sensorimotor cortical neurons as well. In this study, we implanted the 21-day release estrogen pellet for 14 days and the resulting blood estrogen level would not have reached as high as that of rats 3 weeks following the same implantation (Sudoh et al. 2001; Hoffyan et al. 2003) but should have reached the normal proestrous level (Guerra-Araiza et al. 2000) as our ongoing studies in aging female rats show that implantation of the same estrogen pellet for 14 days resulted in blood estrogen of 99 pg/mL (Chen JR, Wang YJ, Tseng GF, unpublished data). This further argues that factors other than gonadal hormones might also regulate the dendritic spines on cortical neurons.

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