Estrogen Replacement Increases Spinophilin-immunoreactive Spine Number in the Prefrontal Cortex of Female Rhesus Monkeys

While studies have shown that estrogen affects hippocampal spine density and function, behavioral studies in humans and nonhuman primates have also implicated the prefrontal cortex in the effects of estrogen on cognition. However, the potential for similar estrogen-induced increases in spines and synapses in the prefrontal cortex has not been investigated in primates. Moreover, it is not known if such an estrogen effect would be manifested throughout the neocortex or primarily in the regions involved in cognition. Therefore, we investigated the effects of estrogen on dendritic spines in the prefrontal and primary visual cortices of young rhesus monkeys. Young female monkeys were ovariectomized and administered either estradiol cypionate or vehicle by intramuscular injection. Using an antibody against the spine-associated protein, spinophilin, spine numbers were estimated in layer I of area 46 and in layer I of the opercular portion of area V1 (V1o). Spine numbers in layer I of area 46 were significantly increased (55%) in the ovariectomy + estrogen group compared to the ovariectomy + vehicle group, yet spine numbers in layer I of area V1o were equivalent across the two groups. The present results suggest that estrogen’s effects on synaptic organization influence select neocortical layers and regions in a primate model, and provide a morphological basis for enhanced prefrontal cortical functions following estrogen replacement.

Keywords: cognition, dendritic spines, hormone replacement, macaque monkey, plasticity, steroids, visual cortex

Introduction

The effect of estrogen on brain regions that are not directly associated with reproduction has been the subject of numerous studies over the past decade, with hormonal effects on hippocampus as the primary focus of these studies. Spine density on pyramidal neurons in rat hippocampal CA1 varies with the estrous cycle and is sensitive to experimentally induced estrogen depletion and replacement (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992). Estrogen replacement has also been shown to be protective against neocortical ischemic damage in a rodent model of stroke (Wise et al., 2003). Ovariectomy (OVX) and ovariectomy plus estradiol replacement have been shown to affect the density of catecholaminergic and cholinergic fibers in the dorsolateral prefrontal cortex of young adult monkeys (Kritzer and Kohama, 1998, 1999). However, spine and synapse analyses comparable to those existing for the hippocampus are lacking in this region. To reveal the neurobiological underpinnings of estrogen-induced enhancement of memory function mediated by the prefrontal cortex, we investigated the effect of estrogen on dendritic spine numbers in area 46. In order to find out whether estrogen-induced dendritic spine plasticity displays a regional preference for cortical areas that are involved in memory and cognition, we investigated the effect of estrogen on spine numbers in the opercular portion of area V1 (therein, V1o), as a regional control. We hypothesized that dendritic spine plasticity in a classic association region such as the prefrontal cortex would be detectable in young female macaque monkeys, whereas no equivalent changes in spine numbers would occur in the primary visual cortex in response to a manipulation of ovarian hormone levels.

Dendritic spines can be visualized as individual puncta with high-resolution light microscopy using immunoreactivity to the dendritic spine-associated protein, spinophilin, which is an actin- and protein phosphatase-1-binding protein present in dendritic spines. Spinophilin-immunoreactivity (Sp-ir) has been shown to be intense in the majority of dendritic spines of rat hippocampus (Allen et al., 1997). Using electron microscopic localization, we found that spinophilin labeled 93% of the dendritic spines in rhesus monkey hippocampus (Hao et al., 2005), and sparsely labeled any other portion of the dendrite, making it an excellent marker for quantitative assessment of spine numbers. We labeled dendritic spines with a well-characterized anti-spinophilin antibody (Allen et al., 1997) in monkey area 46 and V1o. We used a similar electron microscopic approach to validate Sp-ir as a label for spines in area 46, then used a stereological technique, the optical fractionator, to estimate the total numbers of Sp-ir spines in layer I of area 46, layers II–VI of area 46, and layer I of V1o from ovariectomized young rhesus monkeys which receive vehicle (OVX+Veh) as compared to ovariectomized young rhesus monkeys which received estradiol replacement (OVX+E).

Materials and Methods

Animal Materials

Ten young female rhesus monkeys (Macaca mulatta) ranging in age from 6 to 8 years were used in the present study. The monkeys were...
housed at the California National Primate Research Center at UC-Davis. Animals were housed on a 12 h light/dark cycle with unlimited access to food and water. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals using protocols approved by the Institutional Animal Care and Use Committee at the UC-Davis.

**Surgical Procedure**

Bilateral OVX was performed in all 10 young monkeys. Animals were given ketamine HCl (10 mg/kg i.m.) and atropine (0.04 mg/kg s.c.). Then, they were intubated and placed on isoflurane anesthesia. The abdominal wall was opened with a ventral midline incision, and the ovarian vessels and Fallopian tubes were isolated, ligated and severed. The ovaries were subsequently removed. The abdominal wall was then closed, and animals were observed until responsive. Oxytropium (1.5 mg/kg i.m., 3 times/day for 2 days) was provided for post-operative analgesia. Five randomly assigned monkeys were then administered estradiol cypionate (100 μg/1 ml peanut oil i.m.; Pharmacia, Peapack, NJ) 3 months after OVX and again 3 weeks later (group OVX+E). The others were injected with sterile peanut oil/vehicle injection according to the same schedule (group OVX+Veh).

**Urinary Levels of Estradiol and Estrone Conjugates**

Urinary levels of estradiol and estrone conjugates were monitored using protocols approved by the Institutional Animal Care and Use Committee at UC-Davis. Animals were housed in a 12-h light/dark cycle with unlimited access to food and water. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals using protocols approved by the Institutional Animal Care and Use Committee at the UC-Davis.

**Estrogen Assays**

The collective urinary metabolites of estradiol and estrone (estrone conjugates, E1C) and progesterone (pregnanediol-3-glucuronide, PdG) were measured by the immunoenzyme assays described by Shideler et al. (2001), whereas total urinary estradiol was measured following hydrolysis, by radioimmunoassay. Briefly, urine was diluted in distilled water assayed directly for E1C and PdG. In contrast, for total estradiol measurements, urine samples were first spiked with tritiated estradiol in order to monitor for recovery. The samples were then diluted with 2 vol of hydrolysis buffer (pH 5.0) and incubated overnight at 37°C following the addition of 0.02 ml glucosylase (Sigma, St Louis, MO). Samples were subsequently extracted with 10 vol of diethyl ether, reconstituted and separated by discontinuous celite column chromatography. Appropriate eluates were then reconstituted in assay buffer and assayed with a specific estradiol radioimmunoassay. The recovery coefficient of variations for high and low internal controls for these assays were 8% and 12% and 9% and 12% for the high and low internal controls for the direct E1C and PdG assays, respectively, and were 23% and 15% for the high and low internal controls for the combined hydrolysis, extraction, column separation and estradiol assay. OVX- and treatment-induced effects on total urinary estradiol levels were evaluated by comparing the highest estradiol observed prior to OVX to the concentration of estradiol found in the last urine collected immediately prior to treatment (e.g. 3 months after OVX), and to the urinary levels within 24 h after treatment. Given that the pre-OVX measurements reflect the peak levels during the estrous cycle, reasonable physiological levels following treatment are from 40 to 60% of peak pre-OVX levels. A repeated-measure ANOVA was used to compare concentrations between the groups and treatments.

**Perfusion and Tissue Processing**

One day after the second estrogen injection, animals were anesthetized and perfused transcardially with cold 4% paraformaldehyde in phosphate-buffered saline (PBS). One monkey in the OVX+Veh group was excluded from this study due to poor perfusion of the brain resulting from compromised vasculature. Following perfusion, the brains were removed from the skull and hemisected. One hemisphere was cut into 30–50 mm thick blocks, postfixed in 4% paraformaldehyde and cryoprotected. Following postfixation and cryoprotection, the blocks were frozen on dry ice, and series of frozen sections with a section thickness of 40 μm were cut on a cryostat. The sections were kept in anatomical series. To assure that the analysis was conducted blind, the sections from each animal were coded prior to quantitative analysis, and the code was not broken until the analysis was completed. From the sections containing area 46, every 12th section was sampled in a systematic-random manner. The remaining sections were held in reserve and stored in serial order at −20°C in a cryoprotection solution. From the sections sampled, every second section was sampled again in a systematic random fashion to result in two series of sections with 16 sections per series on average. One series of sections was labeled with an antibody against spinophilin and used for stereological analysis. The adjacent series of sections was labeled with an antibody SMI-32 against nonphosphorylated neurofilament protein and counterstained with the Giemsa stain to help delineate the boundaries of area 46 on the spinophilin-labeled sections. From the sections containing area V1o, every 20th section was sampled in a systematic-random manner. The sampled sections were immunoreacted with anti-spinophilin and then used for the stereologic analysis of the total numbers of dendritic spines in layer I of area V1o. The boundaries of area V1o were evident due to the abrupt change in the appearance of layer IV at the border with area V2.

**Immunocytocchemistry**

Pre-embedding Electron Microscopy (EM)

Silver-enhanced nanogold localization of spinophilin similar to that used for the light microscopy was prepared for cryosubstitution EM in order to assess the suitability of spinophilin as a spine marker in prefrontal cortex. After immunocytocochemy, freeze substitution and low-temperature embedding of the specimens was performed as described previously (van Lookeren Campagne et al., 1991; Hjelle et al., 1994; Chaudhry et al., 1995). Slices were cryoprotected by immersion in increasing concentrations of glycerol in phosphate buffer (10, 20, 30%) and were plunged rapidly into liquid propane cooled by liquid nitrogen (−190°C) in a Universal Cryofixation KF80 (Reichert-Jung, Vienna). The samples were immersed in 1.5% uranyl acetate (for en bloc fixation) in anhydrous methanol (−90°C, 24 h) in a cryosubstitution Automated Freeze Substitution Unit (Leica, Vienna). The temperature increased in steps of 4°C/h from −90°C to −45°C. The samples were washed with anhydrous methanol and infiltrated with Lowicryl HM20 resin (Electron Microscopy Sciences, Washington, PA) at −45°C with a progressive increase in the ratio of resin to methanol for 2 h each, followed with pure Lowicryl (overnight). Polymerization was performed with ultraviolet light (360 nm) at −45°C for 48 h, followed by 24 h at room temperature. Layer I of area 46 in prefrontal cortex was identified and sectioned. Serial ultrathin sections were cut with a diamond knife on a Reichert-Jung ultramicrotome and mounted on formvar-coated slot grids for analysis. A total of 100 axospinous synapses were imaged through a series of five sections to determine the total percentage of Sp-ir synapses in a three-dimensional serial analysis. A synapse was considered immunoreactive if it contained more than one silver-intensified product in any individual section within the series of sections through an identified axospinous synapse. The sections were analyzed on a JEOL 1200EX electron microscope (JEOL, Tokyo, Japan). Images were captured using a high-resolution Advantage CCD camera (Advanced Microscopy Techniques Corporation, Danvers, MA).

**Spinophilin Light Microscopy**

The sections sampled were rinsed in 0.1 M PBS containing 0.3% Triton X-100. They were then incubated in a blocking solution containing 0.3% Triton X-100, 0.1% cold water fish gelatin, 0.5% bovine serum albumin, and 5% normal goat serum for 1 h at room temperature. The free-floating sections were incubated for 60 h at 4°C with a fully characterized polyclonal antibody against spinophilin (Allen et al., 1997). The primary antibody was used at a dilution of 1:240 000 in PBS containing 0.3% Triton X-100. The antibody solution was removed by rinsing repeatedly with PBS containing 0.3% Triton X-100. The sections were then incubated in secondary antibody (goat-anti-rabbit IgG, ultra-small, EM grade; Auron) for 3 h at room temperature. The secondary antibody was used at a dilution of 1:100 in PBS containing 0.3% Triton X-100. After rinsing with PBS containing 0.3% Triton X-100 and with PBS alone, the sections were postfixed in 2% glutaraldehyde diluted in PBS for 10 min. The sections were then rinsed with PBS and with distilled water. The silver enhancement reagent was prepared by mixing equal amounts of Auran R-Gent Developer and Enhancer (Auron) before use. The sections were then processed with
the prepared silver enhancement reagent at room temperature for 15 min. After being rinsed with distilled water and PBS, the sections were mounted on slides, and dried overnight. The sections were finally dehydrated in a graded series of ethanol, cleared with xylene, and covered with Biomount mounting medium (Electron Microscopy Sciences, Washington, PA).

**Neurofilament Protein Localization with SMI-32**

The second series of sections sampled from area 46 was rinsed in PBS containing 0.3% Triton X-100. After incubation in a blocking solution as described above for 1 h, the free-floating sections were incubated for 48 h at 4°C with the monoclonal antibody SMI-32 (Sternberger Monoclonals, Lutherville, MD) at a dilution of 1:5000 in PBS containing 0.3% Triton X-100 and 0.5 mg/ml bovine serum albumin. The monoclonal antibody SMI-32 recognizes nonphosphorated epitopes on the medium (168 kD) and heavy (200 kD) molecular weight subunits of the neurofilament protein (Sternberger and Sternberger, 1983; Lee et al., 1988). The sections were rinsed with PBS, then incubated with a biotinylated secondary antibody diluted to 1:200 for 1.5 h at room temperature in PBS containing 0.3% Triton X-100. Antibody/antigen complexes were visualized with the avidin-biotin method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The reaction was visualized with chromogen 3,3'-diaminobenzidine. The sections were rinsed with PBS, mounted on slides, and dried overnight. The immunoreactivity was subsequently intensified with 0.005% osmium tetroxide and the sections were lightly counterstained with the Giemsa stain to enhance visualization of neurons.

**Stereological Analysis**

The optical fractionator (West et al., 1991) was used to estimate total numbers of spines in areas 46 and V1o. The entire prefrontal cortex and occipital cortex were serially sectioned at 40 µm as described above. The tissue was sampled in a systematic-random manner from the entire cytoarchitectonically defined areas of 46 and V1o. The number of spines inside the sampled materials was counted with the optical disector method. One series of sections was sampled from area 46 in a systematic-random manner with a section-sampling fraction of 1/24, resulting in 16 sections per series on average. One series of sections was sampled in area V1o with a section-sampling fraction of 1/20, resulting in 12 sections per series on average. The sampled sections were immunoreacted with the anti-synaptopin antibody. A contour was traced at 5× magnification around layer I of area 46, layers II–VI of area 46, and layer I of V1o, respectively. It was not possible to subdivide layers II–VI into individual layers with the precision required for stereologic analysis. Thus, this approach has limited application for single layer analyses of deeper layers. The counting fields were sampled from the delineated region of the sections with constant intervals in the x and y axes. The x and y distances between sampling frames were set at 300 µm in layer I and at 1200 µm in layers II–VI of area 46. The x and y distances between sampling frames were set at 250 µm in layer I of area V1o. The height and width of the counting frame were both set at 1 µm. A 100× oil immersion lens with a numerical aperture of 1.4 was used to count the spines in each of the counting fields sampled. A 2 µm ‘guard zone’ depth was set at the top surface of the sections. Counting was performed with the optical disector through a depth of 3 µm. The optical disector height divided by the overall average section thickness yields the section thickness sampling fraction, 3/8.48 on average, in the present study. On average, 700 spines were sampled per region and per animal. Once the analysis was completed, the total numbers of spines in layer I and layers II–VI of area 46 and layer I of area V1o of each animal were calculated by multiplying the total numbers of spines counted in the sampled volume of tissue by the reciprocal of the sampling fraction.

**Statistics**

Group means were compared using unpaired, two-tailed Student’s t-tests. Significance was set at 0.05. Variability within groups is expressed as the dimensional coefficient of variation (CV = standard deviation/mean). Coefficients of error were calculated according to the method described by Schmitz and Hof (2000).

**Results**

**Estrogen Measurements**

The endocrine manipulations and measurements employed in this study allowed for monitoring of urinary estrogen levels at three critical time points: (i) baseline levels at a high estrogen phase of the cycle prior to O VX; (ii) efficacy of OVX as reflected in urinary estrogen levels at least 3 months following surgery; and (iii) efficacy of replacement immediately after estrogen injection, (i.e. 24 h). This protocol was designed to mimic the normal cycle through administration of estrogen only once every 5 weeks, in an oil medium that allowed for relatively slow absorption into the blood stream. Serum assays in a different cohort of animals with identical treatment suggests that estrogen levels return to untreated post-OVX levels within 3 days (Rapp et al., 2005). The results show that the model succeeded in both depleting estrogen and replacing it to reasonable physiological levels.

All animals were judged to be exhibiting normal ovarian function prior to OVX as indicated by urinary E1C and PdG profiles and menstrual calendars. E1C peaks preceded sustained elevations of PdG that declined at the time vaginal bleeding was detected. Mean total urinary estradiol exhibited an 8–10-fold drop following OVX for both treatment groups (P < 0.001). The urinary estradiol levels in the treated animals increased ∼4-fold compared to post-OVX levels (P < 0.001) whereas the vehicle group exhibited no change (Table 1). Urinary concentrations of estradiol were not significantly different between the groups before and following OVX while the urinary estradiol concentrations were higher in the treated animals following estradiol treatment compared to the vehicle group (P < 0.001). Post injection levels of total estradiol were significantly increased (P < 0.001) over the pre-injection levels found post-

**Table 1**

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Pre-OVX: the concentrations of estradiol in hydrolyzed urine samples taken prior to OVX; Post-OVX: estradiol following OVX; Injection 1: estradiol after the first injection; Injection 2: estradiol after the second injection. The Pre-OVX samples were collected near the time of the pre-ovulatory peak and therefore represent concentrations of estradiol that are both higher and more variable than most days of the menstrual cycle. In both groups, urinary estradiol exhibited a significant drop following OVX (P < 0.001). The urinary estradiol levels after the first and second injections in the treated group significantly increased compared to Post-OVX levels (P < 0.001) whereas the vehicle group exhibited no change. Urinary concentrations of estradiol were not significantly different between the treated group and control group before and following OVX, whereas they were significantly higher in the treated group following estradiol treatments compared to the central group (P < 0.001).
OVX. We did not expect estradiol replacement in ovariectomized animals to result in pre-ovulatory peak concentrations in this cohort of young adult females, rather to intermediate levels (40–50% of peak values) as a reasonable physiological reflection of average levels throughout the menstrual cycle.

**Ultrastructural analysis**

The pre-embedding immunoelectron microscopic analysis demonstrated that spinophilin is localized in dendritic spines of layer I of the prefrontal cortex (Fig. 1). A qualitative analysis of multiple fields suggested that the majority of visible axospinous synapses were labeled on single EM ultrathin sections. This was supported by a pilot quantitative study in which the percentage of labeled axospinous synapses was ~70% in randomly chosen fields within layer I (Fig. 1). Silver-coated ultra-small gold particles were clustered in the spine neck and especially in the spine head, and disappeared abruptly at the junction between the spine neck and dendritic shaft. Within dendritic spines, the immunoreaction product adhered to the cytoplasmic surface of the plasmalemma, the spine apparatus, and the postsynaptic density. There were occasional sparse single gold particles present in dendritic shafts, but these single particles were clearly below the level of detection of a light microscope, and thus would not have been included in the stereologic spine counts. There were also occasional labeled synapses on shafts.

A pilot quantitative serial section analysis of 100 axospinous synapses from randomly selected fields was carried out to determine the percentage that contained spinophilin immunoreactivity in a more accurate fashion than possible in randomly chosen single sections (Fig. 1). While not all individual sections through a given axospinous synapse contained immunoreactivity, 94% of the synapses in this analysis contained robust labeling in at least one section in the series, demonstrating that Sp-ir accounts for the vast majority of axospinous synapses in layer I of area 46, the target layer for the stereological analysis.

**Figure 1.** Pre-embedding silver-intensified EM demonstrating spinophilin label in monkey area 46 (layer 1). (Ai–x) Serially sectioned images demonstrate that the majority of axospinous synapses are spinophilin immunopositive. A two-dimensional (single section) analysis suggested that while the majority of spines (70%) were immunopositive (i.e. sp2 and sp4), a subpopulation were unlabeled (sp5). However, a subpopulation of spines would have been inaccurately scored as spinophilin immunonegative in such an analysis. A quantitative analysis of serially sectioned axospinous synapses demonstrated that the overall percentage of immunoreactive synapses exceeds 90% (see results). Additionally, note the low level of non-specific background label. (Bi–iv) High-magnification serially sectioned images demonstrating spinophilin distribution within and throughout an axospinous synapse. Multiple silver-intensified particles are seen throughout the cytoplasm, as well as in close apposition with the postsynaptic density (PSD). Asterisk (*) denotes spine. Note the high level of immunoreactive puncta seen in Bi and Biii, as compared to the lack of label in Bii and Biv. Sp = spine. Scale bars: Ai–x = 0.5 µm, Bi–iv = 0.25 µm.
described below. A similar analysis in CA1 showed that 93% of axospinous synapses were Sp-ir (Hao et al., 2003).

**Quantitative Light Microscopic Immunohistochemical Observations**

Our analysis of dorsal prefrontal cortex centered on area 46, the region associated with the principal sulcus as depicted in Figure 2. SMI-32 immunohistochemistry was employed to help define the boundaries of area 46 (Fig. 3A). SMI-32-ir neurons were predominantly located within the deep portion of layer III and within layers V and VI of area 46 as previously described (Fig. 3A; Hof et al., 1990; Hof and Morrison, 1995). On inspection of the sections labeled with antibody SMI-32 and counterstained with Giemsa, the boundary between area 46 and area 9 can be defined where layer IV thins out dorsomedially (Fig. 3A). The boundary between area 46 and area 45 can be delineated where SMI-32-ir neurons in layer III become irregularly organized laterally (Fig. 3A). There was an intense spinophilin labeling in all six cortical layers (numerous small puncta, 0.5–1 μm in diameter) whereas the white matter had negligible spinophilin staining (Fig. 3B). The neuronal somata are apparent as 'ghosts' surrounded by the typical punctate spinophilin labeling. Thus, the lack of apparent neuronal somata in layer I allowed us to delineate its boundary with layer II (Fig. 3B). This demarcation allowed us to quantify spine number (see below) in layer I as a single layer. However, we were not able to delineate any other laminar boundaries in the spinophilin-labeled materials with sufficient accuracy for a stereological analysis, so the other layers were grouped as II–VI. Careful z-axis focusing revealed that the immunocytochemical protocol used in the current study yielded high-quality staining with complete penetration of the antibody through the entire section thickness (Fig. 3C,D). On inspection of the sections immunoreacted with the anti-spinophilin antibody, there appeared to be a higher spine density in layer I of monkey area 46 in the OVX+E group as compared to the OVX+Veh group (Fig. 4A, 4B), which was borne out by our quantitative analyses.

![Figure 2](https://example.com/figure2.png)

**Discussion**

**Methodological and Quantitative Considerations**

The defining element of the spine in this analysis is the presence of Sp-ir that is detectable as a clear, individual punctum with high-resolution light microscopic visualization of silver-enhanced immunogold. Although we cannot be sure that every spine and only spines are labeled by Sp-ir, it appears to be a reliable spine marker and any failure to label spines due to methodological considerations should be similar across groups. Previous studies demonstrated the very limited distribution of this protein to spines in the rat and rhesus monkey cortex and hippocampus, suggesting that it is a specific marker for spines (Allen et al., 1997; Muly et al., 2001; Hao et al., 2003). Spinophilin regulates glutamatergic synaptic transmission and dendritic morphology (Feng et al., 2000), suggesting that it is essential for mature spine function. Our pre-embedding EM analysis demonstrated that clustered gold particles label few profiles other than spines, with labeling generally ending abruptly at the junction between the spine neck and dendritic shaft. In addition, the serial section analysis that was performed on material equivalent to that used for the quantitative light microscopic analysis, showed that the vast majority (i.e. 94%) of axospinous synapses in layer I of area 46 are Sp-ir. A similar EM analysis of Sp-ir axospinous synapses in rhesus monkey CA1 demonstrated that >90% of the axospinous synapses were labeled (Hao et al., 2003). Thus, we are confident that spinophilin visualized with silver-intensified gold particles represents a specific and reliable spine marker for stereological analyses of spine number. However, we cannot rule out estrogen-related shifts in spinophilin levels within spines as a contributor to our quantitative observations. Related ongoing studies employing electron microscopy and neuronal reconstructions will help clarify this issue, though our present interpretation is that our observations reflect primarily a structural difference in spine number, as has been demonstrated with other approaches in rat and monkey hippocampus (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992; Adams et al., 2001; Leranth et al., 2002).

The estimates for Sp-ir spine numbers in layer I of areas 46 and V1o that emerge from this analysis are remarkable for both their consistency and the dramatic effect of estrogen. In both the OVX+Veh and OVX+E groups, Sp-ir spine numbers in layer

**Quantitative Analysis**

The mean total number of Sp-ir spines in layer I of area 46 in the OVX+E group averaged $4.25 \times 10^9$ and $2.74 \times 10^9$ in the OVX+Veh group (Table 2, Fig. 4C), representing a significant increase of 55% ($P = 0.0215$). The mean total number of Sp-ir spines in layers II–VI of area 46 in the OVX+E group was not significantly different from the OVX+Veh group. Given our result in layer I of area 46, layer I of the primary visual cortex was then analyzed to test whether a primary sensory area would display an estrogen-induced increase in Sp-ir spine numbers comparable to that observed in layer I of area 46. The mean total number of Sp-ir spines in layer I of area V1o in the OVX+E group ($3.2 \times 10^9$) was not significantly different from the OVX+Veh group ($3.25 \times 10^9$, Table 2, Fig. 4D). On average, 700 spines were sampled per region/animal, which provided a low CE (range 0.04 – 0.07) and a low variance (CVs within 25%, Table 2).
I of area V1o are nearly identical at 3.25 billion and 3.2 billion spines, respectively. Sp-ir spine numbers for layer I of area 46 are in the same range, 2.75–4.25 billion spines, but in this layer, the estimate for OVX+E animals is a striking 55% higher than for the OVX+Veh animals, which represents an increase of 1.5 billion layer I Sp-ir spines in area 46 of the estrogen-treated monkeys. While no such change was observed in layers II–VI, implying that layer I is particularly sensitive to estrogen treatment, it should be kept in mind that an analysis with a laminar level of precision in layers II–VI might reveal a localized increase in OVX+E animals, but the SP-ir materials do not lend themselves to a strict delineation of the laminar boundaries of any single layer other than layer I.

Regional and Laminar Implications

The density of dendritic spines on hippocampal CA1 pyramidal cells fluctuates naturally during the 4 day estrous cycle in the adult female rat (Woolley et al., 1990), and is also sensitive to experimentally induced estrogen depletion and replacement (Gould et al., 1990), although the estrogen-induced increase is blunted in aged rats (Adams et al., 2001). Both effects show specificity for CA1, with spine density in CA3 and dentate gyrus unaffected by changes in estrogen status. In addition, recent studies have shown a comparable effect on spine densities (Leranth et al., 2002) and number (Hao et al., 2003) in the African green and rhesus monkey hippocampus, respectively. Thus, extensive data suggest that estrogen affects synaptic circuitry in hippocampus, and given the role of the hippocampus in explicit memory, such an effect could provide a substrate for estrogen enhancing memory performance. However, here we describe a profound effect of estrogen on spine numbers in a cortical area that plays a critically important role in many of the more complex aspects of cognition, such as working memory, attention, planning, temporal organization of behavior, and supramodal integration (Goldman-Rakic, 1987, 1996; Petrides, 1995; Owen, 1997; Miller, 2000). For example, a delayed-response (DR) task, a well-characterized test of working memory, requires the functional integrity of

Figure 3. (A) Photomicrograph showing the boundaries of macaque monkey area 46. The boundary between area 46 and area 9 is delineated where layer IV thins out (white arrow). The boundary between area 46 and area 45 is delineated where SMI-32-labeled neurons in layer III become less regularly arranged (black arrow). The tissue was labeled with antibody SMI-32 and counterstained with Giemsa. AS: lower limb of the arcuate sulcus; PS: principal sulcus; L, M, D, V: lateral, medial, dorsal, ventral. The principal sulcus is shown vertically in this picture. Scale bar = 750 µm. (B) Photomicrograph demonstrating spinophilin labeling. There is an intense spinophilin labeling in all of the cortical layers, but none in the white matter (wm). The lack of neuronal somata in layer I permitted the delineation of its boundary with layer II. Scale bar = 100 µm. (C, D) Higher-magnification photomicrographs of layer I. (C) Photomicrograph showing Sp-ir profiles at intermediate magnification. Spines not visualized at their optimal focal plane appear blurred. (D) Spinophilin labeling at the magnification used for stereologic analysis. An unbiased counting frame is shown. The red border of the frame and its extensions represent the exclusion line and the green border represents its inclusion line. Spines were counted when they came into focus within the height of the optical disector and within the counting frame when moving the focal plane continuously through the section. A spine was considered to be within the counting frame if it was entirely within the counting frame or partially within it without touching or intersecting the exclusion line when in focus. Scale bar = 10 µm in (C) and 5 µm in (D).
the dorsolateral prefrontal cortex (Goldman-Rakic, 1987; Levy and Goldman-Rakic, 2000; Fuster, 2001).

The prefrontal cortex is highly susceptible to age-related functional decline (Rapp and Amaral, 1989) and exhibits structural changes with aging, particularly in layer I (Peters et al., 1998). Some studies of human and nonhuman primates suggest that the prefrontal cortical functions are regulated by estrogen. Peri- and postmenopausal monkeys exhibit significant decline in performance of a DR task compared to either age-matched premenopausal monkeys or young monkeys, suggesting that menopause is coupled to a decline in prefrontal cortical function in monkeys (Roberts et al., 1997). In a study of humans, estrogen users exhibited significantly better performance on a verbal performance and on a spatial task, each with a prominent working memory component, but did not differ from non-users on control tasks involving simple passive recall. These results suggest that estrogen is capable of influencing working memory, a function dependent on prefrontal cortex (Duff and Hampson, 2000). Moreover, the equivalent hormone treatment to that used in this study in aged ovariectomized monkeys substantially reversed age-related cognitive impairment assessed by the DR task (Rapp et al., 2003). These behavioral results taken together indicate that estrogen can enhance performance on cognitive tasks dependent on the prefrontal cortex.

Our data on increased Sp-ir spine numbers in layer I of area 46 provide a compelling anatomic substrate for these observed behavioral effects. Furthermore, the lack of any similar effect in layer I of area V1o suggests that areas directly associated with cognition, such as area 46, may be preferred neocortical targets of estrogen. Future studies will target Sp-ir spine numbers in area 46 of aged monkeys with and without estrogen replacement, and such studies may shed light on the interaction of endocrine status with neural aging.

**Synaptic Implications**

One of the few other analyses of estrogen’s effects on primate area 46 demonstrated that the dopaminergic innervation of area 46 was compromised in ovariectomized animals, and essentially fully restored by treatment with estrogen plus progesterone (Kritzer and Kohama, 1998). This effect was particularly profound in layer I, which receives a fairly dense dopaminergic innervation in area 46 (Lewis et al., 1987; Williams and Goldman-Rakic, 1993). Dopaminergic modulation of neuronal networks in the dorsolateral prefrontal cortex is believed to play an important role in information processing during working memory tasks in both humans and nonhuman primates (Goldman-Rakic, 1996; Miller, 2000). Depletion of dopamine in the dorsolateral prefrontal cortex of monkeys is

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**Table 2**

Optical fractionator analysis of spine numbers

<table>
<thead>
<tr>
<th>Animals</th>
<th>Area 46 layer I</th>
<th>Area 46 layers II–V1</th>
<th>Opercular part of area V1 layer I</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Numbers (10^3)</td>
<td>CE</td>
<td>Numbers (10^3)</td>
</tr>
<tr>
<td>OVX+Veh</td>
<td>2.78</td>
<td>0.06</td>
<td>52.8</td>
</tr>
<tr>
<td></td>
<td>3.43</td>
<td>0.07</td>
<td>51.0</td>
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<tr>
<td></td>
<td>1.93</td>
<td>0.06</td>
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</tr>
<tr>
<td></td>
<td>2.81</td>
<td>0.07</td>
<td>56.0</td>
</tr>
<tr>
<td>Mean</td>
<td>2.74 (0.23)</td>
<td>0.07</td>
<td>50.7 (0.11)</td>
</tr>
<tr>
<td>OVX+E</td>
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<td>0.05</td>
<td>58.2</td>
</tr>
<tr>
<td></td>
<td>5.10</td>
<td>0.06</td>
<td>64.7</td>
</tr>
<tr>
<td></td>
<td>3.95</td>
<td>0.05</td>
<td>57.9</td>
</tr>
<tr>
<td></td>
<td>3.13</td>
<td>0.06</td>
<td>47.2</td>
</tr>
<tr>
<td></td>
<td>3.93</td>
<td>0.05</td>
<td>49.4</td>
</tr>
<tr>
<td>Mean</td>
<td>4.25 (0.20)</td>
<td>0.05</td>
<td>55.7 (0.13)</td>
</tr>
</tbody>
</table>

*Estimates of total number of spines in each region were obtained with the optical fractionator. OVX+Veh indicates the ovariectomized monkeys that did not receive estrogen replacement. OVX+E indicates the ovariectomized monkeys that received estrogen replacement. The numbers in parentheses are the interindividual coefficients of variation. CE indicates coefficients of error for each individual estimate. Statistically significant differences between group means are shown in bold type (P = 0.0215).
associated with significant deficits on working memory tasks (Arnsten and Goldman-Rakic, 1985; Sawaguchi and Goldman-Rakic, 1991, 1994), and age-associated cognitive decline involves dopaminergic innervation (Arnsten et al., 1995). Dopamine receptor-associated proteins have been localized to the distal dendrites and spines of pyramidal cells (Bergson et al., 1995), and dopaminergic terminals innervate pyramidal cell spines in prefrontal cortex (Lambe et al., 2000). Thus, the estrogen-induced increase in Sp-ir spines reported here would result in a dramatic increase in a key dopamine target, namely the spines on the distal dendrites of pyramidal neurons. In fact, the dopaminergic innervation of spines in the primate prefrontal cortex forms a ‘triad’ involving the postsynaptic spine, the dopaminergic terminal, and importantly, an adjacent excitatory glutamatergic input (Goldman-Rakic et al., 1989), providing a synaptic substrate for direct dopaminergic/glutamatergic interactions influencing NMDA receptors. Given the critical role of NMDA receptors in estrogen-mediated effects on spines in hippocampus (Gazzaley et al., 1996; Woolley et al., 1997; Adams et al., 2001), it appears that estrogen is particularly well-suited to influence positively both NMDA receptor function and dopaminergic inputs at the level of individual spines in layer I of area 46.

**Conclusion**

The present study represents the first stereological investigation of the effects of estrogen on the dendritic spines in monkey prefrontal cortex. Young adult ovariectomized rhesus monkeys with estrogen replacement have ~1.5 billion more Sp-ir spines in layer I of area 46 than those without estrogen replacement. Recent behavioral studies and the present morphological study raise the possibility that estrogen plays an important role in maintaining certain cognitive functions associated with area 46 in female macaque monkeys. These data extend the targets for estrogen’s effects on cognition well beyond the hippocampus, and have important implications for estrogen replacement and enhancement of cognitive function in peri- and postmenopausal women.

**Notes**

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