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Ovarian Steroids Increase Spinogenetic Proteins in the Macaque Dorsal Raphe

Heidi M. Rivera^a and Cynthia L. Bethea^{a,b,c,d}

^aDivision of Reproductive Sciences Oregon National Primate Research Center Beaverton, OR 97006

^bDivision of Neuroscience Oregon National Primate Research Center Beaverton, OR 97006

^cDepartment of Behavioral Neuroscience Oregon Health and Science University Portland, OR 97201

^dDepartment of Obstetrics and Gynecology Oregon Health and Science University Portland, OR 97201

Abstract

Dendritic spines are the basic structural units of neuronal plasticity. Intracellular signaling cascades that promote spinogenesis have centered on RhoGTPases. We found that ovarian steroids increase gene expression of RhoGTPases (RhoA, Cdc42 and Rac) in laser-captured serotonin neurons. We sought to confirm that the increases observed in gene expression translate to the protein level. In addition, a preliminary study was conducted to determine whether an increase in spines occurs via detection of the spine marker protein, PSD-95. Adult ovariectomized (Ovx) monkeys were treated with estradiol (E), progesterone (P) or E+P for 1 month. Sections through the dorsal raphe nucleus were immunostained for RhoA and Cdc42 ($n = 3-4/\text{group}$). The number and positive pixel area of RhoA-positive cells, and the positive pixel area of Cdc42-positive fibers were determined. Upon combining E and E+P treated groups, there was a significant increase in the average and total cell number and positive pixel area of RhoA-positive cells. E, P and E+P treatments individually or combined, also increased the average and total positive pixel area of Cdc42-positive fibers. With remaining sections from 2 animals in each group, we conducted a preliminary examination of the regulation of PSD-95 protein expression. PSD-95, a postsynaptic scaffold protein, was examined with immunogold silver staining ($n = 2/\text{group}$) and the total number of PSD-95-positive puncta was determined with stereology across 4 levels of the dorsal raphe. E, P and E+P treatment significantly increased the total number of PSD-95-positive puncta. Together, these findings indicate that ovarian steroids act to increase gene and protein expression of two pivotal RhoGTPases involved in spinogenesis and preliminarily indicate that an increased number of spines and/or synapses result from this action. Increased spinogenesis on serotonin dendrites would facilitate excitatory glutamatergic input and in turn, increase serotonin neuronal activity throughout the brain.

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Corresponding Author Heidi M. Rivera, Ph.D. Tel. 503-614-3714 Fax: 503-690-5384 rivera@ohsu.edu.

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Keywords

estradiol; immunocytochemistry; PSD-95; progesterone; RhoGTPase; serotonin

Introduction

Ovarian steroid loss in postmenopausal women is associated with increased depression and anxiety (Conde et al., 2006; Heikkinen et al., 2006; Tangen and Mykletun, 2008; Maki et al., 2010). The serotonin system has been strongly implicated in the treatment of these affective disorders and it has widespread projections throughout the brain that support numerous higher order neural functions (Ressler and Nemeroff, 2000). We have reported that ovarian steroids increase serotonin neural function at multiple levels including gene and protein expression, as well as cellular resilience (Bethea et al., 2002; Bethea et al., 2009). In addition, we have shown that ovariectomized macaques in a semi-free ranging troop exhibit an increase in anxiety-related behaviors compared to tubal-ligated females, which correlated with a significant decrease in serotonin-related gene expression (Bethea et al., 2011; Coleman et al., 2011). In humans, a number of studies indicate that the loss of ovarian steroids, either induced with complete hysterectomy or gradually through menopause, will negatively impact mood in a significant subset of women (Sherwin, 1991; Joffe and Cohen, 1998; Epperson et al., 1999; Kugaya et al., 2003; Soares et al., 2003; Schmidt et al., 2004; Heikkinen et al., 2006).

The basic structural unit of neuronal plasticity in the adult nervous system is the dendritic spine (Ethell and Pasquale, 2005) and dendritic spines represent the morphological basis for excitatory neurotransmission (Butler et al., 1998; McKinney et al., 1999). It has been suggested that antidepressants act by promoting neuronal plasticity (Manji et al., 2003). However, steroid-induced spinogenesis has been explored to a greater extent. In the hippocampus, steroids increase spinogenesis (the number of dendritic spines) and excitatory glutamatergic input (the number of axospinous synapses) (Gould et al., 1990; Woolley and McEwen, 1992; Murphy et al., 1998). Also in the hippocampus, steroids increase expression of spinogenetic proteins, such as spinophilin, Ras homolog gene family member A (RhoA) and postsynaptic density-95 (PSD-95) (Brake et al., 2001; Akama and McEwen, 2003; Choi et al., 2003; Hao et al., 2003; Liu et al., 2008; Spencer et al., 2008; Kramar et al., 2009; Waters et al., 2009). Steroid-induced spinogenesis is also evident, though less studied, in the hypothalamus, amygdala, cerebellum and cortex (Frankfurt et al., 1990; Hao et al., 2006; Sasahara et al., 2007; de Castilhos et al., 2008). We question whether steroid-induced spinogenesis occurs in the dorsal raphe nucleus, where ~60% of forebrain serotonergic neurons originate (Jacobs and Azmitia, 1992).

The issue of steroid-induced spinogenesis on serotonin neurons may be important for menopausal women grappling with issues surrounding hormone therapy (HT). Women experience ovarian failure and loss of ovarian steroid production around 50 years of age. Thus, with extended life spans, a woman may live 35-40 years without ovarian steroids. We speculate that dendritic spines on serotonin neurons shrink or atrophy due to lack of steroid supported gene expression, which may decrease serotonergic support of higher neural functions. Furthermore, we hypothesize that ovarian steroids enhance serotonin neuronal plasticity by increasing spinogenesis on serotonin dendrites, which would facilitate excitatory glutamatergic input to serotonin neurons and in turn, increase serotonin neuronal activity throughout the brain. Maintenance of serotonergic function would support mood, cognition and circadian rhythms.

Dendritic spine protrusion, maturation and stabilization involve a complex repertoire of cytoskeletal reorganization, expression of glutamate receptors and synapse assembly. We recently reported that ovarian steroids increase gene expression of the effector guanosine triphosphatases (GTPases), RhoA, cell division control protein 42 homolog (Cdc42) and ras-related C3 botulinum toxin substrate (Rac), in laser-captured serotonin neurons (Bethea and Reddy, 2010). These 3 molecules activate cascades that lead to filopodia extension, spine head enlargement and spine shortening, all of which are necessary for the production of a mature dendritic spine. However, changes in gene expression do not always reflect changes in protein expression; and changes produced at the protein level are more functionally relevant. This study examines whether steroid-induced increases in gene expression of the GTPases, RhoA and Cdc42, are translated to the protein level in the dorsal raphe. In addition, we conducted a preliminary study to determine whether an increase in spines occurs via detection of the spine marker protein, PSD-95. PSD-95 is a major scaffolding protein located at the postsynaptic density of excitatory glutamatergic synapses (Kim and Sheng, 2004).

We utilize a cost effective model of surgical menopause and HT in which adult rhesus macaques are ovariectomized (Ovx) for a relatively short period of time, i.e. 4-9 months, followed by subcutaneous delivery of bioidentical estradiol (E), progesterone (P) or estradiol plus progesterone (E+P) for 1 month. Therefore, we only examine one time point. However, it probably reflects a significant level of spine maturity and stabilization, which were shown to occur by 24 hours after spine protrusion in the hippocampus (De Roo et al., 2008). We examined the effect of E, P, and E+P on 3 proteins related to spinogenesis with single-label immunocytochemistry for RhoA and Cdc42, and with immunogold silver staining for PSD-95.

Experimental Procedures

The Institutional Animal Care and Use Committee of the Oregon National Primate Research Center approved this study, which followed the guidelines of the Animal Welfare Act.

Animals and steroid treatment

Adult female rhesus monkeys (*Macaca mulatta*) were ovariectomized (Ovx) 5.2 ± 0.85 months before assignment to this project. Ovariectomy was performed by the surgical personnel of ONPRC according to accepted veterinary surgical protocol. All animals were born in China, aged between 4.6 and 13 years, weighed between 4 and 9 kg and were in good health. ONPRC operates with a lease for fee arrangement. Thus, animals had been previously used in select reproductive protocols that had culminated in ovariectomy before their return to the available pool. The animals were intact, cycling females, either in stimulation protocols involving egg and granulosa cell retrieval by laparoscopy; or that were ovariectomized for follicle retrieval and 3D culture. They were rested between ovariectomy and assignment to this study.

Ovx monkeys received either a placebo implant for 28 days (Ovx), an E-filled implant for 28 days (E), a placebo implant for 14 days and a P-filled implant for the final 14 of the 28 days (P) or an E-filled implant for 28 days supplemented with P-filled implant for the final 14 of the 28 days (E+P). The placebo-treated monkeys received empty Silastic implants (s.c.) on day 0 and day 14. The E-treated monkeys received one 4.5-cm Silastic implant (i.d. 0.132 in.; o.d. 0.183 in.; Dow Corning, Midland, MI) filled with crystalline E (1,3,5(10)-estratrien-3, 17 β -diol; Steraloids, Wilton, NH) on day 0 and an empty implant on day 14. The P-treated monkeys received an empty implant on day 0 and one 6-cm Silastic implant filled with crystalline P (4-pregnen-3, 20-dione; Steraloids) on day 14. The E+P-treated monkeys received one E-filled implant on day 0 and one P-filled capsule on day 14. All

implants were placed in the periscapular area under ketamine anesthesia (ketamine HCl, 10 mg/kg, s.c.; Fort Dodge Laboratories, Fort Dodge, IA). The E+P treatment regimen has been shown to cause differentiation of the uterine endometrium in a manner similar to the normal 28-day menstrual cycle (Brenner and Slayden, 1994).

All tissue sections for RhoA, Cdc42 and PSD-95 were from a pool of 20 monkeys treated with placebo, E, P or E+P ($n = 5$) for 1 month. Floating sections from 12 monkeys were examined for RhoA immunocytochemistry ($n = 3$ / group); sections from 16 monkeys were examined for Cdc42 immunocytochemistry ($n = 4$ / group) and sections from 8 monkeys were used for PSD-95 immunogold silver staining ($n = 2$ / group), which exhausted all of the floating sections from this pool of animals.

Euthanasia

Monkeys were euthanized at the end of the treatment periods according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association. Each animal was sedated with ketamine in the home cage, transported to the necropsy suite, given an overdose of pentobarbital (25 mg/kg, i.v., Hospira, Lake Forest, IL) and exsanguinated by severance of the descending aorta.

Ovarian steroid assays

Assays for E and P were performed using 2010 Elecsys assay instrument from Roche Diagnostics (Boulder, CO). Prior to these analyses, measurements of E and P on this platform were compared to traditional radioimmunoassay as previously reported (Bethea et al., 2005).

Tissue preparation

The left ventricle of the heart was cannulated and the head of each animal was perfused with 1L of saline followed by 7 L of 4% paraformaldehyde in 3.8% borate, pH 9.5 (both solutions made with 0.1% diethyl pyrocarbonate-treated water to minimize RNase contamination). The brain was removed and dissected. Tissue blocks were post-fixed in 4% paraformaldehyde for 3 h, then transferred to 0.02 M potassium phosphate-buffered saline (KPBS) containing 10%, followed by 20% glycerol and 2% dimethyl sulfoxide at 4°C for 3 days to cryoprotect the tissue. After infiltration, the block was frozen in isopentane, cooled to -55°C and stored at -80°C until sectioning. Coronal tissue sections (25 μ m) were cut on a sliding microtome, collected in a cryoprotectant buffer (30% ethylene glycol and 20% glycerol in 0.05 M sodium phosphate-buffered saline (PBS)) and frozen at -20°C. When tissue sections were ready to be processed they were removed from -20°C storage and washed in 0.02 M KPBS (pH 7.4) for RhoA and Cdc42 or 0.01 M PBS (pH 7.4) for PSD-95 for 1 h.

RhoA and Cdc42 single-label immunocytochemistry

For RhoA, tissue sections were incubated in 0.6 % hydrogen peroxide (H_2O_2) for 30 min, washed in 0.02 M KPBS for 1 h, blocked with 2% normal goat serum (NGS) for 1 h and with avidin and biotin for 20 min each (Vector labs, Burlingame, CA). Tissue sections were then incubated with primary antibody to RhoA for 48 h at 4°C. The RhoA monoclonal antibody was raised in rabbit against the C-terminal end of the human peptide (Cell Signaling, Danvers, MA; Catalog No. 2117). Increasing dilutions of RhoA antibody produced decreased signal up to a titer of 1:8,000. For experimental tissue, the RhoA antibody was diluted to 1:1,000 in a mixture of 2% NGS/0.4% Triton/ KPBS. For control tissue, the highest titer of RhoA antibody that detected a reliable signal (1:6,000) was preabsorbed with an antigen peptide (200 μ g; Cell Signaling; Catalog No. 1878B). After 48

h, tissue sections were washed in KPBS for 1 h, biotinylated goat anti-rabbit antibody (Vector) diluted to 1:100 was placed on tissue and incubated for 1 h at RT. After another 1 h KPBS wash, tissue sections were incubated in 2% VECTASTAIN ABC solution (Vector) for 1 h and washed again in KPBS for 1 h. Diaminobenzidine (DAB; Dojindo Laboratories, Kumamoto, Japan) was used as a chromagen. Tissue sections were incubated in a mixture of 0.05% DAB/0.003% H₂O₂/KPBS for 5 min and washed in KPBS for 30 min. Tissue sections were mounted onto microscope slides and vacuum-dried overnight. The following day, tissue sections were dehydrated with a graded series of ethanols, cleared with Histoclear and coverslipped with dibutyl phthalate and xylene (DPX) mountant.

Based upon Hoffman et al. (2008), the immunosignal was enhanced with Nickel sulfate (NiSO₄), the concentration of H₂O₂ was increased and the concentration of secondary antibody, ABC solution and DAB were decreased in the assays for Cdc42. Tissue sections were incubated in 1 % H₂O₂ for 30 min, washed in KPBS for 1 h, blocked with 2% NGS for 1 h and with avidin and biotin for 20 min each. Tissue sections were then incubated with primary antibody to Cdc42 for 48 h at 4°C. The Cdc42 polyclonal antibody was raised in rabbit against the C-terminal end of the human peptide (Abcam, Cambridge, MA; Catalog No. ab64533). Increasing dilutions of Cdc42 antibody produced decreased signal up to a titer of 1:4,480. For experimental tissue, the Cdc42 antibody was diluted to 1:2,240 in a mixture of 2% NGS/0.4% Triton/KPBS. For control tissue, the highest titer of Cdc42 antibody that detected a reliable signal (1:4,480) was preabsorbed with an antigen peptide (130 µg; Abcam; Catalog No. ab66571). After 48 h, tissue sections were washed in KPBS for 1 h, biotinylated goat anti-rabbit antibody (Vector) diluted to 1:500 was placed on tissue and incubated for 1 h at RT. After another 1 h KPBS wash, tissue sections were incubated in 0.5% VECTASTAIN ABC solution (Vector) for 1 h, washed again in KPBS for 1 h and washed in 175 mM sodium acetate for 5 min. Nickel sulfate (NiSO₄)-enhanced DAB (Wako Pure Chemical Industries, Richmond, VA) was used as a chromagen. Tissue sections were incubated in a mixture of 2.5% NiSO₄/0.02% DAB/0.003% H₂O₂/KPBS for 3 min and 30 sec, washed in 175 mM sodium acetate for 15 min and in KPBS for 45 min. Tissue sections were mounted onto microscope slides and vacuum-dried overnight. The following day, tissue sections were dehydrated with a graded series of ethanols, cleared with Histoclear and coverslipped with DPX mountant.

PSD-95 immunogold silver staining

We used the remaining sections from this series of monkeys to conduct a preliminary study for PSD-95. Tissue sections first underwent protease section pretreatment adapted from a previous study (Fukaya and Watanabe, 2000). Briefly, a tube containing 0.2 N hydrochloric acid (HCl) was warmed to 37°C in a water bath. Frozen aliquots containing 4 mg/ml of pepsin solution (DAKO, Carpinteria, CA; Catalog No. S3002) were thawed and diluted to 1 mg/ml by adding warm HCl. Tissue sections that had been pre-warmed to 37°C in H₂O were transferred to the 1 mg/ml pepsin solution. After 3 min in pepsin solution, tissue sections were washed in 0.01 M PBS (pH 7.4) for 1 h at RT before being processed with immunogold silver staining protocol. The immunogold silver staining protocol used here was adapted from a previous study (Hao et al., 2003). Tissue sections were rinsed in PBS for 10 min, 0.3% Triton for 1 h and blocked with a blocking solution (0.3% Triton, 0.1% cold water fish skin gelatin (Electron Microscopy Sciences, Hatfield, PA; Catalog No. 25560), 0.5% bovine serum albumin (Sigma, St. Louis, MO) and 5% NGS (Vector)) for 1 h. Tissue sections were then incubated with primary antibody to PSD-95 for 72 h at 4°C. The PSD-95 polyclonal antibody was raised in rabbit against the N-terminal of the mouse peptide (Abcam; Catalog No. ab18258). Increasing dilutions of PSD-95 antibody produced decreased signal up to a titer of 1:4,000. For experimental tissue, the PSD-95 antibody was diluted to 1:1,000 in blocking solution. For control tissue, the highest titer of PSD-95

antibody that detected a reliable signal (1:2,000) was preabsorbed with an antigen peptide (100 µg; Abcam; Catalog No. ab18661). After 72 h, tissue sections were washed in PBS for 1 h, an ultra small gold-conjugated goat anti-rabbit antibody (Electron Microscopy Sciences; Catalog No. 25100) diluted to 1:100 in blocking buffer was placed on tissue and incubated for 3 h at RT. After another 1 h PBS wash, tissue sections were post-fixed in 2% glutaraldehyde (Sigma) dissolved in PBS for 10 min, washed in PBS for 2 min, briefly rinsed in H₂O and washed in H₂O for 1 hr. Silver enhancement from Aurion R-Gent-LM kit was used to amplify immunosignal (Electron Microscopy Sciences; Catalog No. 25520). Tissue sections were incubated in silver enhancement solution (18-22°C) for 11 min, washed in H₂O for 15 min and in PBS for 2 min. Tissue sections were mounted onto microscope slides and vacuum-dried overnight. The following day, tissue sections were dehydrated with a graded series of ethanols, cleared with HistoClear and coverslipped in Biomount mountant (Electron Microscopy Sciences; Catalog No. 17894).

RhoA and Cdc42 quantitative image analysis

All images of tissue sections for RhoA and Cdc42 were captured with the Marianas Stereology Workstation and Slidebook 4.2 software. A blue filter was used to capture RhoA and no filter was used to capture Cdc42. A 10X montage of the dorsal raphe was created by Slidebook, which was subjected to further analysis with Image J1.43u software (National Institutes of Health). For each anatomical level, the largest representation of the dorsal raphe was chosen from amongst all monkeys. A rectangle outline was placed over the chosen dorsal raphe and the exact dimensions were recorded. The same size rectangle was then used for all of the monkeys at that anatomical level. On each tissue section, the operator outlined the dorsal raphe with a rectangle and the image was segmented into positive (stained) and negative (unstained) pixels. RhoA and Cdc42 were processed differently since RhoA only labeled cells and Cdc42 labeled both cells and fibers. For RhoA, two measurements were generated from the segmented image containing cells. The area covered by the positive pixels was obtained and called the positive pixel area. Then, the number of positive cells was obtained using size cut-off values.

For Cdc42, the initial image contained both cells and fibers. Thresholding and subtraction features were used to separate cells and fibers into two distinct images. Positive pixel area and number of positive cells were generated from the image containing cells. Positive pixel area was generated from the image containing fibers.

PSD-95 stereological analysis

A series of sections at 500 µm intervals through the entire rostro-caudal extent of the dorsal raphe of each monkey was used. The analyses were conducted with a Zeiss Axioplan microscope and StereoInvestigator software (MicroBrightField, Williston, VT). The probe used to estimate total number of PSD-95-positive puncta in the dorsal raphe was the optical fractionator as previously described and used in West et al. (1991) and Hao et al. (2003). It is an unbiased estimate of the total number of PSD-95 puncta in the dorsal raphe obtained by sampling (via systematic random sampling) a fraction of the dorsal raphe. On each tissue section, the operator outlined the dorsal raphe with a 2.5x objective.

StereoInvestigator placed counting frames uniformly across a grid containing the dorsal raphe. Counting frame dimensions were 3µm width and 3µm height. Grid size dimensions were 250µm width and 150µm height. Approximately 74 imaging sites were visited per dorsal raphe outline. For counting of PSD-95-positive puncta, the operator switched the objective to an oil-immersion 100x objective with a high numerical aperture of 1.3. Counting was conducted through an optical disector height of 3µm. Guard zone was set at 1µm. A puncta was counted if it was partly or fully located within inclusion box and as long

as it did not touch exclusion lines. We used the estimated population with number weighted section thickness obtained from StereoInvestigator for further analysis. The average tissue thickness was 6 μ m and Schmitz Hoff coefficient of error was 0.048.

Photomicrographs and Statistical analysis

Photomicrographs were obtained with a Leica DML microscope mounted with a Leica DFC290 digital camera. Also shown are montages from the Marianas Stereomicroscope that were saved and converted to binary images.

The positive pixel area and the number of cells immunostained for RhoA and Cdc42 were averaged or totaled across the 4 anatomical levels of the dorsal raphe from each monkey, generating one overall average or total per monkey. The positive pixel area of fibers immunostained for Cdc42 were also averaged or totaled for each monkey. The mean $\pm$ SEM of the monkey averages or totals in each steroid-treated group was then obtained for statistical analysis, thus the variance around the mean was due to differences between animals.

The morphology of the dorsal raphe varied from rostral to caudal levels, which resulted in significant variation of PSD-95 puncta present based upon the morphology of the anatomical level. Therefore, the number of puncta for PSD-95 was totaled for the 4 anatomical levels, generating one overall total for each monkey. The mean $\pm$ SEM of the total for the monkeys in each steroid-treated group was then obtained for statistical analysis, thus the variance around the mean was due to differences between animals.

The animals were treated and euthanized at the same time in sets consisting of one OvX, one E, one P and one E+P treated animal (matched). Therefore, all data were analyzed with repeated-measures ANOVAs. Any significant group differences ($p < 0.05$) were followed by Newman-Keul's post hoc tests. There was no posthoc difference between E and E+P groups so they were further examined as a combined group, which was compared to the OvX group, using an unpaired t-test (two-tailed, confidence interval of 95%). All statistical analyses were conducted using Prism v5.0 software (Graph-Pad, San Diego, CA).

Results

Specificity of RhoA antibody

RhoA immunostaining in the dorsal raphe of a P-treated monkey is depicted in Figure 1. Panel A depicts immunostaining at the antibody concentration used for experimental sections (1:1,000). Immunostaining was restricted to cells. Panel B depicts immunostaining at a lower concentration of antibody (1:6,000) that reached the limit of detection. Fewer RhoA-positive cells were observed demonstrating the signal was concentration-dependent. As a further control, sections were incubated with RhoA antibody alone (1:6,000) or in combination with antigen peptide (200 μ g). Panel C depicts immunostaining after preabsorption of RhoA antibody with antigen peptide. The RhoA signal was significantly reduced by the antigen peptide.

Effect of steroid treatment on expression of RhoA immunostaining

Representative images of RhoA-positive cells in steroid-treated monkeys are depicted in Figure 2. There was robust expression of RhoA-positive cells in E, P and E+P groups, but it was reduced in the OvX group. Quantitative analyses of the average number of RhoA-positive cells and the average positive pixel area of the cells are depicted in Figure 3. There was a significant difference between groups in average number of detectable cells (ANOVA, $p = 0.02$). E, P and E+P increased the average number of cells relative to the OvX group

(Newman-Keul's, $p < 0.05$ all). In addition, there was a significant increase in average number of cells in the combined E & E+P group relative to OvX group (t-test, $p = 0.005$). No group differences were observed in average cell pixel area (ANOVA, $p = 0.13$), but there was a significant increase in average cell pixel area in the combined E & E+P group relative to the OvX group (t-test, $p = 0.008$).

Quantitative analyses of the total number of RhoA-positive cells and the total positive pixel area of the cells are depicted in Figure 4. No group differences were observed in total number of cells or total cell pixel area (ANOVAs, $p = 0.08$ and 0.16 , respectively), but there was a significant increase in total number of cells and total cell pixel area in the combined E & E+P group relative to the OvX group (t-tests, $p = 0.004$ and 0.0012 , respectively).

Specificity of Cdc42 antibody

Cdc42 immunostaining in the dorsal raphe of a P-treated monkey is depicted in Figure 5. Panel A depicts immunostaining at the concentration used for experimental sections (1:2,240). Immunostaining was expressed in cells and in fibers. Panel B depicts immunostaining at a lower concentration of antibody (1:4,480) that reached the limit of detection. Fewer Cdc42-positive cells and fibers were observed indicating the signal was concentration-dependent. For controls, sections were incubated with Cdc42 antibody alone (1:4,480) or in combination with antigen peptide (130 μ g). Panel C depicts immunostaining after preabsorption of Cdc42 antibody with antigen peptide. The Cdc42 signal was significantly reduced by the antigen peptide.

Effect of steroid treatment on expression of Cdc42 immunostaining

Representative images of Cdc42-positive cells and fibers as separate images and reassembled images from steroid-treated monkeys are depicted in Figure 6. There was robust expression of Cdc42-positive cells and fibers in steroid-treated monkeys, which were subjected to image analysis. In addition, the images containing cells and fibers were pseudocolored and reassembled for visual reconstruction of the dorsal raphe; cells were red and the fibers were green.

Cells—Quantitative analyses of the average and total number of Cdc42-positive cells and the average and total positive pixel area of the cells were conducted. A significant difference between groups was observed in the average positive pixel area of the cells (ANOVA, $p = 0.04$). E+P increased average cell pixel area relative to the OvX group (Newman-Keul's, $p < 0.05$). However, there was no group difference in the average number of cells, total number of cells or total cell pixel area (ANOVAs, $p = 0.06 - 0.69$). In addition, there was no difference between the combined E & E+P group relative to the OvX group in any of the t-tests ($p = 0.06 - 0.31$). Overall, little change was observed in the cellular immunostaining for Cdc42 [Data not shown].

Fibers—Quantitative analyses of the average and total positive pixel area of Cdc42-positive fibers are depicted in Figure 7. A significant difference between groups was observed in average and total fiber pixel area (ANOVAs, $p = 0.02$ and 0.02 , respectively). E, P and E+P increased average and total fiber pixel area relative to OvX group (Newman-Keul's, $p < 0.05$ all). In addition, there was significant increase in average and total fiber pixel area in the combined E & E+P group relative to OvX group (t-tests, $p = 0.006$ and 0.008 , respectively). Overall, significant steroid regulation of Cdc42 was observed in fibers and not cells.

Specificity of PSD-95 antibody

PSD-95 immunostaining in the dorsal raphe of a P-treated monkey is shown in Figure 8. Panel A depicts immunostaining at the concentration used for experimental sections (1:1,000). Dark punctate staining was observed. Panel B depicts staining at a lower concentration of antibody (1:2,000) that reached the limit of detection. Fewer PSD-95-positive puncta were observed indicating the signal was concentration-dependent. For controls, sections were incubated with PSD-95 antibody alone (1:2,000) or in combination with antigen peptide (100 μ g). Panel C depicts immunostaining after preabsorption of PSD-95 antibody with antigen peptide. The PSD-95 signal was significantly reduced by the antigen peptide.

Effect of steroid treatment on expression of PSD-95 immunogold staining

Representative segmented images of PSD-95-positive puncta in steroid-treated monkeys are shown in Figure 9. There was robust expression of PSD-95-positive puncta in E, P and E+P groups, but it was reduced in OvX group. Quantitative analysis of the total number of PSD-95 puncta is shown in Figure 10. There was a significant difference between groups in the total number of puncta (ANOVA, $p = 0.01$). E, P and E+P increased the total number of puncta relative to OvX group (Newman-Keul's, $p < 0.05$ all). However, with an $n = 2$ /group there is insufficient statistical power. In addition, there was a significant increase in total number of puncta in the combined E & E+P group relative to the OvX group (t-test, $p = 0.009$).

Verification of steroid treatment on serum E and P concentrations

Serum was collected at the end of the study to verify the efficacy of Silastic implants. The concentration of E in the serum of E and E+P-treated groups was 78.6 ± 7 pg/ml and 89.0 ± 13 pg/ml, respectively. The concentration of P in the serum of P and E+P-treated groups was 6.17 ± 0.65 ng/ml and 3.89 ± 0.73 ng/ml, respectively. The concentrations of E and P are similar to that observed in the mid-luteal phase (Hotchkiss et al., 1982). The concentrations of E and P in the serum of the OvX group were 8.0 ± 2 pg/ml and 0.09 ± 0.03 ng/ml, respectively (significantly different from steroid-treated monkeys by ANOVAs, $p = 0.0001$).

Discussion

Dendritic spine protrusion and remodeling is a multi-step process that requires actin reorganization. Filopodia precede spine formation and glutamate promotes filopodia morphogenesis. Filopodia may be transformed into spines, which involves decreased motility, shortening of the neck and growth of the head (Butler et al., 1998; McKinney et al., 1999; Ethell and Pasquale, 2005). Spinogenesis requires synthesis of new proteins (Papa and Segal, 1996).

The dynamic organization of the actin cytoskeleton provides the force for filopodia protrusion and spine morphogenesis. It is regulated by small GTPases of the Rho family, in particular RhoA, Cdc42 and Rac. When activated, these RhoGTPases alter spine morphology through distinct, but synergistic mechanisms (Hall, 1998). RhoA induces bundling of actin and myosin filaments to produce stress fibers and clustering of integrins to produce focal adhesion contacts. Both Cdc42 and Rac cause actin filament assembly at the cell periphery to produce either filopodium or microspikes (Cdc42) or lamellipodium and membrane ruffles (Rac). We previously found that E treatment increased gene expression of all 3 RhoGTPases in laser-captured serotonin neurons (Bethea and Reddy, 2010).

Our present findings indicate that ovarian steroids increase pivotal GTPases involved in dendritic spine proliferation. Moreover, preliminary data suggests that indeed, there are significantly more spines or synapses in the dorsal raphe of steroid-treated animals; further indicating that ovarian steroid treatment supports serotonin neuroplasticity. E or P treatment increase RhoA, Cdc42 or Rac signaling in various cell types, such as endothelial cells, breast cancer cells, and primary cultures of cortical cells (Simoncini et al., 2006; Fu et al., 2008a; Fu et al., 2008b; Giretti et al., 2008; Sanchez et al., 2009). E also increased RhoA (GTP-bound) protein levels in the hippocampus of Ovx rats (Kramar et al., 2009). Our data are consistent with these studies and extend the action of ovarian steroids to GTPases in dorsal raphe neurons of macaques. Of note, we were unable to find a specific commercial antibody against Rac for immunostaining in macaque brain.

In the hippocampus, both RhoA and Cdc42 are localized in the soma and dendrites of neurons (O'Kane et al., 2003; Santos Da Silva et al., 2004). In the dorsal raphe, ovarian steroid regulation of Cdc42 was more robust in fibers than cells. Because many of the Cdc42-positive fibers were beaded, we have probably immunostained axons and dendrites, both of which utilize Cdc42 for membrane protrusion (Negishi and Katoh, 2005; Hall and Lalli, 2010). Our earlier data clearly indicate an increase in gene expression, which is nuclear (Bethea and Reddy, 2010). However, we can only speculate on whether Cdc42 mRNA or protein is transported into dendrites and axons (Kennedy and Ehlers, 2006; Doyle and Kiebler, 2011). Unlike Cdc42, steroid regulation of RhoA only occurred in cell soma of the dorsal raphe. Restricted RhoA expression is consistent with a previous study conducted in patients following traumatic brain injury (Brabeck et al., 2004). Our present findings suggest that, in the dorsal raphe, ovarian steroids may increase RhoA in the soma through local protein synthesis and increase Cdc42 in dendrites by enhancing transport and/or local translation.

Steroid-induced spinogenesis in other parts of the brain involves increased glutamate signaling. In the hippocampus, it involves activity of the ionotropic glutamate receptor, N-methyl-D-aspartate (NMDA) (Woolley et al., 1997). Moreover, inhibition of synaptic transmission in hippocampal slices causes spine loss and atrophy (Fischer et al., 2000; Matus et al., 2000). We have recently found that there is an increase in gene expression of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and NMDA receptor subunits (Bethea and Reddy, 2011b), and an increase in the expression of genes coding for pivotal proteins in synapse assembly in laser-captured serotonin neurons (Bethea and Reddy, 2011a).

The spinogenetic protein, PSD-95, is a member of the membrane-associated guanylate kinase family. It can bind directly to NMDA receptors or bind indirectly to AMPA receptors (Kornau et al., 1995; Schnell et al., 2002). Moreover, overexpression of PSD-95 promotes spinogenesis (El-Husseini et al., 2000). It can also regulate the surface expression and synaptic strength of NMDA and AMPA receptors (Migaud et al., 1998; El-Husseini et al., 2000; Lin et al., 2006; Schluter et al., 2006). Therefore, PSD-95 is also a positive indicator of synaptic strength as well as a marker protein for dendritic spines. Our preliminary findings indicate that ovarian steroids produce a morphological increase in spines as detected by the increase in the expression of PSD-95 in the dorsal raphe. We are currently preparing more monkeys to increase the power of this observation.

In the hippocampus and cortex of monkeys, ovarian steroids increased spinophilin, another marker of spines, as detected with immunogold silver staining and puncta counts (Hao et al., 2003; Tang et al., 2004). The data is consistent with our study of PSD-95 in the dorsal raphe. However, PSD-95 studies of the hippocampus in monkeys are lacking. Previous research

has shown that PSD-95 protein expression is increased in the hippocampus during proestrus in intact mice and after E treatment in Ovx rats (Spencer et al., 2008; Waters et al., 2009).

Of interest from a steroid receptor perspective is our observation that P alone increased the expression of spinogenetic proteins and preliminarily, the number of spines. Furthermore, E +P treatment was similar to treatment with E alone or P alone on these parameters, showing neither an attenuating nor a potentiating effect. We have previously shown that serotonin neurons contain estrogen receptor subtype β and that administration of E causes an induction of nuclear progesterone receptor (PR), which is maintained with supplemental P (Bethea, 1993,1994; Gundlah et al., 2001). However in the absence of E, there is little or no nuclear PR expression. Hence, we propose that the stimulatory effect of P alone on spinogenesis is independent of nuclear PR. Rather, these data suggest that the action of P alone may be dependent upon the conversion of P to its active metabolite, allopregnanolone, which acts through the gamma-aminobutyric acid receptor subtype A (GABA_A) (Majewska et al., 1986). The convergence of this action upon the spinogenetic signaling cascade requires more investigation. In addition, it is noteworthy that E alone stimulated spinogenetic proteins (and nuclear PR expression as previously published), but the addition of P to the E regimen had no further effect. Thus, P action via nuclear PR was neutral on 3 spinogenetic proteins when added to the E regimen. Moreover, the speculated action of P through the GABA_A receptor was not additive with E. We have observed this pattern of response, as well as both augmentation of E action and blocking of E action by P on different endpoints within serotonin neurons (Bethea et al., 2002; Sanchez et al., 2005; Bethea et al., 2009). These results highlight our lack of understanding of the interactions of E and P in the serotonin system. We hope that future examination of the promoter regions of the numerous steroid-responsive genes in serotonin neurons will advance our comprehension of the mechanisms of action of P in this important system.

The responses of the serotonin neurons to ovarian steroids may be a general phenomenon throughout the brain since steroid-induced spinogenesis occurs in the hypothalamus, amygdala, hippocampus, cerebellum and cortex (Frankfurt et al., 1990; Gould et al., 1990; Woolley and McEwen, 1992,1993; Murphy et al., 1998; Hao et al., 2006; Sasahara et al., 2007; de Castilhos et al., 2008). These observations emphasize how important the ovarian steroids are for optimal function of multiple brain areas. In the dorsal raphe, we need to confirm that these steroid-responsive spinogenetic proteins reside on serotonin neurons. In addition, it is essential to show that changes observed in spinogenetic proteins translate to changes in spine morphology as demonstrated by Golgi staining of the dorsal raphe.

Conclusion

Altogether, our findings indicate that ovarian steroid treatment of Ovx monkeys for 1 month increases the expression of 3 pivotal spinogenetic proteins in the dorsal raphe in a manner that would promote dendritic spine proliferation and stabilization. Morphological criteria suggest that many of the immunopositive neurons are serotonergic. Increased spinogenesis on serotonin neurons would facilitate excitatory glutamatergic input and improve serotonin neurotransmission throughout the brain. This action could improve serotonin neuronal function in postmenopausal women and thereby improve mood and overall mental health.

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Abbreviations

AMPA

Cdc42

DAB

DPX

E

E+P

GABA-A

GTPase

HCl

HT

H₂O₂

KPBS

NiSO₄

NGS

NMDA

Ovx

PBS

P

PR

PSD-95

Rac

RhoA

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Highlights

- Ovarian steroids increased RhoA protein in cell bodies of the dorsal raphe.
- Ovarian steroids increased Cdc42 protein in fibers in the dorsal raphe.
- Ovarian steroids preliminarily increased PSD-95 protein in dorsal raphe.
- Ovarian steroids increase neuroplasticity of dorsal raphe neurons in macaques.

Figure 1. RhoA immunostaining in the dorsal raphe of a P-treated monkey. (A) Antibody was used at the experimental concentration (1:1,000). Immunostaining was restricted to cells. (B) Immunostaining at a lower concentration of antibody (1:6,000). Fewer RhoA-positive cells were observed. (C) Immunostaining after preabsorption of RhoA antibody (1:6000) with antigen peptide (200 µg). The RhoA signal was significantly reduced by incubation with the antigen peptide. Scale bar = 200 µm.

Figure 2. Representative photomicrograph images of RhoA-positive cells in steroid-treated monkeys. RhoA immunostaining was observed in all groups, but there appeared to be an increase in the number of positive cells in E, P and E+P treatment groups relative to the Ovx-control group. Scale bar = 500 μ m.

RhoA-cells

Figure 3.

Histograms demonstrating the *average* number of RhoA-positive cells and the *average* positive pixel area of the cells in the dorsal raphe of OvX monkeys treated with placebo (OvX), E, P or E+P ($n = 3/\text{group}$). There was a significant difference between groups in average number of cells (ANOVA, $p = 0.02$). E, P and E+P increased average number of cells relative to the OvX group (Newman-Keul's, $p < 0.05$ all). In addition, there was a significant increase in average number of cells in the combined E & E+P group relative to OvX group (t-test, $p = 0.005$). No group differences were observed in average cell pixel area (ANOVA, $p = 0.13$), but there was a significant increase in average cell pixel area in the combined E & E+P group relative to the OvX group (t-test, $p = 0.008$). *Greater than OvX with Newman-Keuls or t-test, $p < 0.05$.

Figure 4.

Histograms demonstrating the *total* number of RhoA-positive cells and the *total* positive pixel area of the cells in the dorsal raphe of OvX monkeys treated with placebo (OvX), E, P or E+P ($n = 3/\text{group}$). No group differences were observed in total number of cells or total cell pixel area (ANOVAs, $p = 0.08$ and 0.16 , respectively), but there was a significant increase in total number of cells and total cell pixel area in the combined E & E+P group relative to OvX group (t-tests, $p = 0.004$ and 0.0012 , respectively). *Greater than OvX with Newman-Keuls or t-test, $p < 0.05$.

Figure 5.

Cdc42 immunostaining in the dorsal raphe of a P-treated monkey. (A) Antibody was used at the experimental concentration (1:2,240). Immunostaining was expressed in cells and in fibers. (B) Immunostaining at lower concentration of antibody (1:4,480). Fewer Cdc42-positive cells and fibers observed. (C) Immunostaining after preabsorption of Cdc42 antibody (1:4,480) with antigen peptide (130 µg). The Cdc42 signal was significantly reduced by incubation with the antigen peptide. Scale bar = 200 µm.

Figure 6.

Representative images of Cdc42-positive cells and fibers as separate binary images and as pseudocolored reassembled images from steroid-treated monkeys. There was robust expression of Cdc42-positive cells and fibers in steroid-treated monkeys, which were subjected to image analysis. To reconstruct the dorsal raphe, the images containing cells and fibers were pseudocolored (cells were red and fibers were green) and reassembled with same representative animals. Scale bar = 500 μ m.

Figure 7.

Histograms demonstrating the *average* and *total* positive pixel area of Cdc42-positive fibers in the dorsal raphe of OvX monkeys treated with placebo (OvX), E, P or E+P ($n = 4/\text{group}$). A significant difference between groups was observed in average and total fiber pixel area (ANOVAs, $p = 0.02$ and 0.02 , respectively). E, P and E+P increased average and total fiber pixel area relative to OvX group (Newman-Keul's, $p < 0.05$ all). In addition, there was a significant increase in average and total fiber pixel area in the combined E & E+P group relative to OvX group (t-tests, $p = 0.006$ and 0.008 , respectively). *Greater than OvX with Newman-Keul's or t-test, $p < 0.05$.

Figure 8. PSD-95 immunostaining in the dorsal raphe of a P-treated monkey. (A) Antibody to PSD-95 was used at the experimental concentration (1:1,000). Dark punctate staining was observed. (B) Immunostaining at lower concentration of antibody (1:2,000). Fewer PSD-95-positive puncta were observed. (C) Immunostaining after preabsorption of PSD-95 antibody (1:2,000) with antigen peptide (100 μ g). The PSD-95 signal was significantly reduced by incubation with the antigen peptide. Scale bar = 100 μ m.

Figure 9. Representative segmented images of PSD-95-positive puncta in steroid-treated monkeys. Images for PSD-95 were captured at a level of the optical disector and processed with Image J. PSD-95 puncta were detected in all groups, but there appeared to be an increase in puncta with steroid treatment relative to Ovx-controls. Scale bar = 20 μ m.

Figure 10.

Histograms demonstrating the *total* number of PSD-95-positive puncta in the dorsal raphe of Ovx monkeys treated with placebo (Ovx), E, P or E+P (preliminary study; $n = 2/\text{group}$). There was a significant difference between groups in the total number of puncta (ANOVA, $p = 0.01$). E, P and E+P increased the total number of puncta relative to Ovx group (Newman-Keul's, $p < 0.05$ all). However, with an $n = 2/\text{group}$ there is insufficient statistical power. In addition, there was a significant increase in total number of puncta in the combined E & E+P group relative to the Ovx group (t-test, $p = 0.009$).

*Greater than Ovx with Newman-Keuls or t-test, $p < 0.05$.