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OVARIAN STEROIDS INCREASE SPINOGENETIC PROTEINS IN THE MACAQUE DORSAL RAPHE

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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 [Ras homolog gene family member A (RhoA), cell division control protein 42 homolog (Cdc42), and ras-related C3 botulinum toxin substrate (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, postsynaptic density-95 (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/group). The number and positive pixel area of RhoA-positive cells and the positive pixel area of Cdc42-positive fibers were determined. On 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 two 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/group), and the total number of PSD-95-positive puncta was determined with stereology across four 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.

Key words: estradiol, immunocytochemistry, PSD-95, progesterone, RhoGTPase, serotonin.

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, 2009). In addition, we have shown that ovariectomized macaques in a semifree ranging troop exhibit an increase in anxiety-related behaviors compared with 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

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Abbreviations: ANOVA, analysis of variance; Cdc42, cell division control protein 42 homolog; DAB, diaminobenzidine; DPX, dibutyl phthalate and xylene; E, estradiol; E+P, estradiol+progesterone; GTPases, guanosine triphosphatases; KPBS, potassium phosphate-buffered saline; NGS, normal goat serum; NMDA, *N*-methyl-D-aspartate; ONPRC, Oregon National Primate Research Center; Ovx, ovariectomized; P, progesterone; PBS, sodium phosphate-buffered saline; PR, progesterone receptor; PSD-95, postsynaptic density-95; Rac, ras-related C3 botulinum toxin substrate; RhoA, Ras homolog gene family member A.

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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 owing 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 three 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 use a cost-effective model of surgical menopause and HT in which adult rhesus macaques are ovariectomized (Ovx) for a relatively short period of time, that is, 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 h after spine protrusion in the hippocampus (de Roo et al., 2008). We examined the effect of E, P, and E+P on three 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 (ONPRC) approved this study, which followed the guidelines of the Animal Welfare Act. The minimum number of animals to obtain sufficient statistical power was used. Pain or suffering were eliminated with anesthetics and analgesics as dictated by the American Veterinary Association and 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 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 laporoscopy 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, USA) filled with crystalline E [1,3,5(10)-estratrien-3, 17B-diol; Steraloids, Wilton, NH, USA] 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, USA). 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, USA), and exsanguinated by severance of the descending aorta.