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Ovarian steroids regulate gene expression in the dorsal raphe of old female macaques

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ABSTRACT

With extended life spans in modern humans, menopause has become a significant risk factor for depression, anxiety, loss of cognitive functions, weight gain, metabolic disease, osteoporosis, cardiovascular disease, and neurodegenerative diseases. Clinical studies have found beneficial neural effects of ovarian steroid hormone therapy (HT) during the menopausal transition and data are emerging that it can be continued long term. To further understand molecular underpinnings of the clinical studies, we used quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) to examine gene expression in the serotonergic dorsal raphe of old (>18 years) rhesus macaques, focusing on genes related to depression, cellular resilience, and neurodegenerative diseases. The animals were ovariectomized (Ovx, surgically menopausal) and subjected to either estradiol or estradiol plus progesterone HT, or to placebo, starting 2 months after Ovx and continuing for ~4 years. Significant changes were observed in 36 of 48 genes examined that encode proteins supporting serotonin neurotransmission, synapse assembly, glutamate neurotransmission, DNA repair, chaperones, ubiquinases and transport motors, as well as genes encoding proteins that have potential to delay the onset of neuropathologies. The data reveal important gene targets for chronic HT that contribute to neural health. Alternatively, the loss of ovarian steroids may lead to loss of functions at the gene level that contribute to many of the observable neural deficits after menopause.

1. Introduction

Menopause in women and female rhesus macaques is associated with a marked decline in the production and secretion of 17β-estradiol (E) and progesterone (P) from the ovaries (Burger et al., 2002; Downs and Urbanski, 2006; McKinlay et al., 2008). This attenuation of circulating concentrations of E and P adversely impacts many physiological processes and is thought to play a major role in the etiology of age-related pathologies, such as hot flushes and disrupted sleep-wake cycles (Mittelman-Smith et al., 2012; Sarti et al., 2005). Depression, anxiety, and cognitive loss also accompany the onset of menopause in a significant number of women (Epperson et al., 2012; Gordon et al., 2015; Maki et al., 2010). Bio-identical hormone therapy (HT) during perimenopause, or the menopausal transition, alleviates or attenuates many of the neural dysfunctions that occur during menopause (Epperson et al., 2012; Heikkinen et al., 2006; Schmidt and Rubinow, 2009), but it is not possible to probe the underlying neural mechanisms in living humans.

Ovariectomy of nonhuman primates has provided a reasonable model of abrupt human menopause. Studies have shown that E replacement in surgically menopausal macaques improves or maintains cognitive function over placebo controls, with corresponding improvement in synapses, dendritic spines, and mitochondria in prefrontal cortex (Hao et al., 2006, 2007; Hara et al., 2014; Tang et al., 1996; Tinkler et al., 2005; Voytko et al., 2009). However, clinically relevant, continuous HT protocols failed to improve cognitive function or spinogenesis in prefrontal cortex of old female macaques, raising questions regarding the best method of delivery (Baxter et al., 2013; Ohm et al., 2012).

Many of the neural symptoms of menopause have been linked to serotonin in both human and animal studies (Albert et al., 2014; Amin et al., 2006; Araragi and Lesch, 2013; Diaconescu et al., 2011; Stollstorff et al., 2013). Serotonin neurons in adult ovariectomized (Ovx) female macaques express nuclear estrogen receptor beta

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(ER-β) at the same level, with or without HT (Gundlah et al., 2000, 2001). E, acting through ERβ increased expression of progesterone receptors (PRs) in serotonin neurons (Bethea, 1994). In adult short-term Ovx monkeys (age 6–12 years), 1 month of E treatment, with or without supplemental P, significantly altered gene expression in serotonin neurons in a manner that would lead to increased serotonin neurotransmission (Bethea et al., 2002), increased proliferation of dendritic spines (Bethea and Reddy, 2010; Rivera and Bethea, 2012), increased glutamate receptors (Bethea and Reddy, 2012b), increased synaptogenesis (Bethea and Reddy, 2012a), and increased neuronal resilience (Bethea and Reddy, 2015). These functions have largely been confirmed at the transmitter or protein levels (Lima and Bethea, 2009; Rivera and Bethea, 2012, 2013; Sanchez et al., 2013).

The goal of this study was to determine if old animals responded as well as young animals to HT, albeit in a chronic, but clinically relevant model. Therefore, genes were preselected that responded to E or E + P in serotonin neuron-enriched preparations from young adult animals treated for 1 month with HT (Bethea and Reddy, 2008, 2010, 2012a, 2012b, 2015; Rivera and Bethea, 2012). The serotonin-related genes were selected from our earlier experiments demonstrating their regulation by ovarian steroids at gene and protein levels. Dendritic spines are the elementary structural units of neuronal plasticity, and their proliferation and stabilization involve components of synapse assembly and glutamate neurotransmission. Therefore, genes were selected that related to dendritic spine proliferation, glutamate transmission and synapse assembly, and that had shown regulation by ovarian steroids in young adult females. We showed that serotonin neurons in Ovx animals had more DNA fragmentation (TUNEL positive) than in animals with HT for 1 month. DNA fragmentation and other neurodegenerative mechanisms are controlled by DNA repair enzymes, chaperones (protein folding), ubiquinases, transportation motors, and mutations within specific genes. Hence, we selected genes related to these categories and that showed regulation by ovarian hormones in young adult females. Our E+P group is similar to the "Econtinuous + Pcyclic" treatment group in NHP studies by Ohm et al. (2012) and Baxter et al. (2013).

2. Materials and methods

This experiment was approved by the IACUC of the Oregon National Primate Research Center and conducted in accordance with the 2011 Eighth Edition of the National Institute of Health *Guide for the Care and Use of Laboratory Animals*. Most of the animals were born at ONPRC, were aged between 18–22 years, weighed between 5 and 8 kg, and were in good health. The animals were housed indoors under controlled environmental conditions: 24 C temperature; 12-hour light, 12-hour dark photoperiods (lights on at 0700 hours); regular meals at 0800 and 1500 hours and supplemented with fresh fruits and vegetables, and fresh drinking water available ad libitum.

2.1. Animals and treatments

Twelve ovarian intact, old female rhesus monkeys (*Macaca mulatta*) were dedicated to this project from a larger cohort of study animals. The ovarian intact, aged animals were trained in a delayed response task (Rapp et al., 2003; Voytko, 2000) and a Spatial Foodport Maze task (Haley et al., 2009; Voytko, 2002).

Once delayed response training was achieved, the animals were Ovx by ONPRC surgical personnel according to accepted veterinary surgical protocol. After two months of Ovx, subgroups of animals received placebo, E alone or E+P. They were tested for 2 years. The results of the cognitive testing during the first 12 months after Ovx have been published in abstract form (Renner et al., 2010) and are in preparation for publication in a separate article.

The E-treated monkeys were implanted with one 4-cm E-filled Silastic capsules (i.d. 0.132 in.; o.d. 0.183 in.; Dow Corning, Midland, MI, USA). The capsule was filled with crystalline estradiol (1,3,5(10)-estratrien-3, 17-b-diol; Steraloids, Wilton, NH, USA). The E + P–treated animals received an E-filled capsule and were administered micronized progesterone orally for 11 days out of every month to model the menstrual cycle rather than various clinical prescriptions. The capsules were placed in the periscapular area under ketamine anesthesia (ketamine HCl, 10 mg/kg, Fort Dodge Laboratories, Fort Dodge, IA, USA). The treatments were maintained for ~4 years. Placebo controls received one empty Silastic capsule.

The E capsules were designed to last up to 1 year and to maintain serum E concentrations between 100–200 pg/mL, which is similar to concentrations during the mid-to-late follicular phase of the menstrual cycle. They were replaced annually, to ensure sustained long-term delivery of the steroid for the entire duration of the study; the untreated Ovx animals maintained the same empty capsules throughout. Serum E and P concentrations were measured at various times across the 4-year experiment to confirm that the target hormone concentrations were being maintained in the old Ovx animals.

At regular intervals during the treatment period, circadian activity was measured with accelerometers and included day activity, dark:light activity ratio, sleep latency, and wake bouts. At regular intervals, the animals were vaccinated, and the T cell response was determined. MRIs on brain morphology were regularly performed.

2.2. Clinical observations

Biannual physical examinations were conducted by a clinical veterinarian throughout the study. At the beginning of the study, the uterus was noted to be small and difficult to palpate; the cervix was small in all animals. The uterus and cervix remained small throughout the study in the placebo-treated and E + P–treated animals. In the E-treated animals in years 2–4, the uterus was noted to be palpable, enlarged, of firm consistency and no cysts were observed with ultrasound. One uterus was irregularly shaped. At the same time points, the cervix was noted to be enlarged. Menses were irregular and up to 10 days long in 1 animal, but not heavy. The pathology report indicated uterine enlargement with endometrial hyperplasia. One animal had leiomyoma.

2.3. Steroid hormone assays

The E and P assays were performed by the ONPRC Endocrine Technology and Support Core using a chemilluminescence-based automatic clinical platform (Immulite 2000; Siemens Healthcare Diagnostics, Deerfield, IL, USA). The sensitivity limit of the E assay was 20 pg/mL, and the sensitivity limit of the P assay was 0.2 ng/mL. The intra-assay and inter-assay coefficients of variation were all less than 15%. Before these analyses, measurements of E and P on this platform were compared to traditional RIAs as previously reported (Bethea et al., 2005). The E + P treatment regimen has been shown to cause proliferation and differentiation of the uterine endometrium in a manner similar to a normal 28-day menstrual cycle (Brenner and Slayden, 1994).

2.4. Euthanasia

The monkeys were euthanized at the end of the treatment periods according to procedures recommended by the 2013 Edition of the American Veterinary Medical Association *Guidelines for the Euthanasia of Animals*. Each animal was sedated with ketamine,