Effects of Age and Estradiol on Gene Expression in the Rhesus Macaque Hypothalamus

Dominique H. Eghlidi^{a, b} Henryk F. Urbanski^{a–c}

^aDivision of Neuroscience, Oregon National Primate Research Center, Beaverton, Oreg., and Departments of

^bBehavioral Neuroscience and ^cPhysiology and Pharmacology, Oregon Health and Science University, Portland, Oreg., USA

Key Words

17 β -estradiol · Estrogen receptor- α · Estrogen receptor- β · Progesterone receptor · Androgen receptor · Aging · Menopause · Hypothalamus

Abstract

Background: The hypothalamus plays a key role in mediating the effects of estrogen on many physiological functions, including reproduction, metabolism, and thermoregulation. We have previously observed marked estrogen-dependent gene expression changes within the hypothalamus of rhesus macaques during aging, especially in the KNDy neurons of the arcuate-median eminence (ARC-ME) that produce kisspeptin, neurokinin B, and dynorphin A. Little is known, however, about the mechanisms involved in mediating the feedback from estrogen onto these neurons. **Methods:** We used quantitative real-time PCR to profile age- and estrogen-dependent gene expression changes in the rhesus macaque hypothalamus. Our focus was on genes that encode steroid receptors (*ESR1*, *ESR2*, *PGR*, and *AR*) and on enzymes that contribute to the local synthesis of 17 β -estradiol (*E2*; *STS*, *HSD3B1/2*, *HSD17B5*, and *CYP19A*). In addition, we used RT² ProfilerTM PCR Arrays to profile a larger set of genes that are integral to hypothalamic function. **Results:** *KISS1*, *KISS1R*, *TAC3*, and *NPY2R* mRNA levels increased in surgically meno-

pausal (ovariectomized) old females relative to age-matched ovariectomized animals that received *E2* hormone therapy. In contrast, *PGR*, *HSD17B*, *GNRH2*, *SLC6A3*, *KISS1*, *TAC3*, and *NPY2R* mRNA levels increased after *E2* supplementation. **Conclusion:** The rhesus macaque ARC-ME expresses many genes that are responsive to changes in circulating estrogen levels, even during old age, and these may contribute to causing the normal and pathophysiological changes that occur during menopause.

© 2015 S. Karger AG, Basel

Introduction

Menopause in women and female rhesus macaques is associated with a marked decline in the production and secretion of 17 β -estradiol (*E2*) from the ovaries [1–3]. This attenuation of circulating estrogen levels adversely impacts many physiological processes and is thought to play a major role in the etiology of age-related pathologies such as reproductive quiescence, hot flashes, and disrupted sleep-wake cycles [4, 5]. Little is known, however, about the mechanisms involved in mediating the feedback from *E2* onto neurons in the hypothalamus.

Evidence from human and nonhuman primate studies suggests that loss of circulating estrogen can affect the cy-

to architecture of the arcuate region of the hypothalamus and alter the pattern of gene expression [6–11]. For example, using in situ hybridization, Rance et al. [6] demonstrated a significant increase in the size of estrogen receptor (ER)-expressing neurons of postmenopausal compared to premenopausal women. ER α -expressing neurons in the arcuate-median eminence (ARC-ME) colocalize with kisspeptin (KISS1), neurokinin B (NKB), and dynorphin A (DYN) (referred to as KNDy) and exert a major influence on the neuroendocrine reproductive axis by modulating the secretion of gonadotropin-releasing hormone (GnRH). Moreover, KNDy neurons show marked changes in their pattern of gene expression after menopause or ovariectomy, and these changes can be blocked by exposure to exogenous sex steroids [12–17]. Consequently, it is plausible that the influence of sex steroids on GnRH neuronal function is mediated by ER α , ER β , and the progesterin receptor, associated with the KNDy neural systems [18–25]. Furthermore, the expression of these receptors may change, as animals show the characteristic menopausal decrease in circulating sex steroid levels, and may represent a mechanism by which the hypothalamus undergoes a compensatory increase in its sensitivity to sex steroids. Another possible menopause-associated compensatory mechanism could involve an increased expression of enzymes involved in the local intracrine synthesis of sex steroids from precursor steroids such as dehydroepiandrosterone (DHEA) [26–28]. The circulating levels of DHEA and DHEA sulfate are especially pronounced in adult humans and nonhuman primates, and there is evidence that all of the key enzymes involved in DHEA-to-E₂ conversion are expressed in the primate hypothalamus [29]. This suggests that local synthesis of sex steroids may also be contributing to the hormone milieu of the hypothalamus.

The aim of the present study was to help resolve these issues by examining the effect of age and E₂ treatment on gene expression in the ARC-ME of female rhesus macaques. These nonhuman primates show age-related hormonal changes similar to those in women, though late in their lifespan [2, 30, 31], and thus they represent a pragmatic translational animal model in which to study the neuroendocrine mechanisms that contribute to healthy human aging. Our primary focus was on genes that encode the main sex steroid receptors [32–37]: ER α (encoded by *ESR1*), ER β (encoded by *ESR2*), the progesterin receptor (encoded by *PGR*), and the androgen receptor (encoded by *AR*). In addition, we examined the expression of genes that play a key role in the intracrine conversion of DHEA to testosterone and E₂: steroid sulfatase

(encoded by *STS*), 17 β -hydroxysteroid dehydrogenase type 5 (encoded by *HSD17B5*), 3 β -hydroxysteroid dehydrogenase types 1 and 2 (encoded by *HSD3B1/2*), and aromatase (encoded by *CYP19A1*). Our second goal was to profile the expression of a larger set of genes that contribute to the control of hypothalamus-mediated functions such as reproduction and metabolism. Our prediction was that the expression of hypothalamic genes would increase during aging, ensuring that the hypothalamus remains responsive to sex steroids even when the levels of these hormones in the circulation are attenuated.

Materials and Methods

Animals

Adult female rhesus macaques (*Macaca mulatta*) were cared for by the Division of Comparative Medicine at the Oregon National Primate Research Center (ONPRC) in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals, and used in a study approved by the Institutional Animal Care and Use Committee. The animals were housed indoors under controlled environmental conditions: 24°C temperature; 12-hour light/12-hour dark photoperiods (lights on at 07.00 h); regular meals at 08.00 and 15.00 h (Purina High Protein Monkey Chow; Purina Mills, Inc., St. Louis, Mo., USA) supplemented with fresh fruit and vegetables, and fresh drinking water available ad libitum.

Design of Experiments

Effect of Aging

Experiment 1 used quantitative real-time (qRT-)PCR to compare mRNA levels between young (~13 years) and old (~26 years) ovary-intact animals.

Effect of E₂

Experiment 2 used qRT-PCR to compare mRNA levels between young (~10 years) bilaterally ovariectomized (OVX) animals and young (~10 years) OVX animals that had been treated with E₂ for ~1 month. This experiment also compared mRNA levels between old OVX controls and old OVX animals that had been treated with E₂ for ~4 years. The two older groups of animals were ~21 and ~22 years of age at the onset of the study, respectively.

Effect of Menopause and Hormone Therapy

Experiment 3 used custom-made RT² Profiler™ PCR Arrays (SABiosciences) to compare gene expression in the ARC-ME of young animals (~12 years) with that of old, surgically menopausal animals (old OVX) from experiment 2, and also with the long-term estradiol-treated animals (old OVX + E₂).

Menstrual Cycle Status and Serum Hormone Concentrations

Experiment 1

The serum levels of E₂ were 36.5 ± 6.3 pg/ml in the young gonad-intact animals and 62 ± 16.5 pg/ml in the old gonad-intact animals. All but 1 of these animals had progesterone (P₄) levels below the assay detection limit. One young animal showed elevat-

ed P₄ concentrations (2.4 ng/ml) and was assumed to still be in the luteal phase of the menstrual cycle. Based on individual animal menstruation records, all 4 young intact animals were normal cyclers, and the old intact animals consisted of 2 cycling animals, 1 irregular cycler, and 1 postmenopausal animal.

Experiment 2

One month following ovariectomy, half of the animals in the young and old OVX groups were implanted subcutaneously with empty Silastic capsules (Dow Corning, Midland, Mich., USA), while the other half received capsules containing crystalline E₂ (Steraloids, Wilton, N.H., USA), as previously described [38]; these were designed to last up to 1 year and to maintain circulating E₂ levels between 100 and 200 pg/ml, which is similar to what is observed during the late follicular phase of the menstrual cycle. In the old OVX + E₂ group, the E₂ capsules were replaced annually to ensure sustained long-term delivery of the steroid for the entire duration of the study (~4 years); the untreated OVX animals maintained the same capsules throughout. Terminal serum E₂ levels were undetectable in the young untreated OVX animals and had a mean level of 117 ± 7.0 pg/ml in the young OVX + E₂ group. Serum E₂ concentrations were measured at various time points across the 4-year experiment in the old OVX animals to confirm that the target hormone concentrations were being maintained. The serum E₂ levels in the old untreated OVX group were <30 pg/ml at all time points and often fell below the limit of assay sensitivity. In the old OVX + E₂ animals, the mean E₂ concentration during the final 2 years of the study was 118.8 ± 15.9 pg/ml, and at the time of tissue collection it was 94.3 ± 20.5 pg/ml.

Experiment 3

At the time of postmortem tissue collection, the young intact animals had a mean serum E₂ level of 58.9 ± 24.5 pg/ml and very low serum P₄ levels (<0.3 ng/ml). These hormone levels are indicative of the follicular phase of the menstrual cycle. In 1 of these animals, however, the E₂ level was considerably higher (128 pg/ml), suggesting that the animal was most likely in the late follicular phase. Note that the surgically menopausal animals used in experiment 3 were the same old OVX and old OVX + E₂ animals from experiment 2.

The E₂ and P₄ assays were performed by the ONPRC Endocrine Technology and Support Core using a chemiluminescence-based automatic clinical platform (Immulin 2000; Siemens Healthcare Diagnostics, Deerfield, Ill., USA). The sensitivity limits of these assays were 20 pg/ml and 0.2 ng/ml for E₂ and P₄, respectively [39], and the intra-assay and inter-assay coefficients of variation were all <15%.

RNA Isolation

All the animals had previously been involved in various cross-sectional aging studies; their hypothalami and terminal blood serum became available for postmortem analysis through the ONPRC Tissue Distribution Program. At the time of necropsy, the mean ($\pm$ SEM) age of the gonad-intact young animals from experiment 1 was 12.8 ± 2.3 years ($n = 4$), and the age of the old animals was 25.8 ± 0.4 years ($n = 4$). The animals from experiment 2 were 9.7 ± 0.3 years old (young OVX; $n = 4$), 9.6 ± 0.8 years old (young OVX + E₂; $n = 4$), 25.0 ± 1.7 years old (old OVX; $n = 4$), and 27.0 ± 0.9 years old (old OVX + E₂; $n = 4$). The age of the young animals from experiment 3 was 12.3 ± 0.9 years ($n = 4$).

After sedation with ketamine (15–25 mg/kg, i.m.) and pentobarbital sodium (25–30 mg/kg, i.v.), a procedure consistent with the recommendations of the American Veterinary Medical Association's Panel on Euthanasia, each brain was flushed with 1 liter of 0.9% saline via a vascular catheter, and the hypothalamus was removed and preserved for ~2 weeks in RNAlater (Ambion, Austin, Tex., USA). A coronal slice encompassing the ARC-ME was dissected and stored frozen at -80°C . The boundaries for this tissue block included the exterior ventral edge of the ME, lateral cuts midway between the third ventricle and the optic nerve, an anterior cut along the posterior edge of the optic chiasma, a posterior cut just anterior to the mammillary bodies, and a cut 1 mm dorsal to the base of the third ventricle (i.e., based on stereotaxic coordinates, this represents the border between the arcuate and the ventromedial hypothalamus). Subsequently, each ARC-ME block was individually homogenized using a PowerGen rotor-stator homogenizer (Fisher Scientific, Pittsburgh, Pa., USA), and RNA was extracted using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, Calif., USA). An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif., USA) was used to determine the quality of the RNA, and a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, Mass., USA) was used to determine the concentration. For each sample, 1 μg of RNA was then converted to cDNA using random hexamers and the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, Calif., USA).

Gene Expression Profiling

The ARC-ME expression levels of genes encoding the sex steroid receptors *ESR1*, *ESR2*, *PGR*, and *AR* were determined using qRT-PCR. Similarly, the ARC-ME expression levels of genes encoding the following steroidogenic enzymes were examined: *STS*, *HSD3B1/2*, *HSD17B5*, and *CYP19A1*. Finally, RT² ProfilerTM PCR Arrays were used to profile a more extensive set of genes in the ARC-ME during aging and after treatment with E₂.

Primers and Probes

Table 1 depicts nucleotide sequences of the primers and probes that were used for detection of the steroid receptor genes (*ESR1*, *ESR2*, *PGR*, and *AR*), steroidogenic enzyme genes (*STS*, *HSD3B1/2*, *HSD17B5*, and *CYP19A1*), and also two housekeeping genes (*ALG9* and *RPL13A*) that we have previously shown to be stably expressed in the rhesus ARC-ME across various sex steroid environments [17, 40]. The nucleotide sequences for these genes were designed using Primer Express 2.0 software [Applied Biosystems (ABI), Foster City, Calif., USA] and were based on NCBI rhesus macaque reference sequences. The primers and probes were purchased from Invitrogen Life Technologies and ABI, respectively. Reverse transcriptase-PCR (RT-PCR), using ARC-ME and ovarian RNA samples (as positive controls), was used to validate each of the primer sets by producing amplicons of a predicted size.

TaqMan qRT-PCR

A 7900HT Fast Real-Time PCR thermal cycler and sequence detection system software (version 2.2.1; ABI) was used to obtain qRT-PCR data. Initially, pooled cDNA was used to create standard curves for each gene, and the experimental samples were subsequently diluted accordingly so as to fall within the linear part of the curve. The PCR mixtures contained 5 μl TaqMan Universal PCR Master Mix, 0.3 μl of each specific forward and reverse primer (300 nM final concentration), 0.25 μl of specific probe (250 nM final

Table 1. TaqMan qRT-PCR primer and probe nucleotide sequences (5'–3')

Target genes	Primer sequences	Probe sequences	GenBank accession ID
<i>ESR1</i>	F- ACAAGATCACAGACACTTTGATCCA R- CCTGATGTGGGAGAGGATGAG	AGGCCTGACCCTGCAG	XM_002803858
<i>ESR2</i>	F- GATCGCTAGAACACACCTTACCTGTA R- GGTGCAACGGTTCCCACTAA	AGAGACATTGAAAAGGAAG	NM_001265821
<i>PGR</i>	F- CATGTCACTGGGCAGATGCT R- TGCCACATGGTAAGGCATAATG	CTGAATGAACAGCGGATG	NM_001278456
<i>AR</i>	F- AAAATCCCACATCCTGCTCAA R- CAGGTCAAAAGTGAAGTGAATGCA	TGCAGCCTATTGCGAGAG	NM_1032911
<i>STS</i>	F- CCTTCCTCCGGCCTGTCT R- AGCTTTGCCACATGCATCTG	CCAGTGCGACAGAGAAAAACAGGATAAGAGA	XM_001088752
<i>HSD3B1/2</i>	F- AGGACGTCTCGGTCGTCATC R- GAGCTGGGTACCTTTACATTGA	CATTGATGTCTTTGGTGTCACTCA	XM_001113873
<i>HSD17B5</i>	F- TGGAGGGCTTTGCTGAAGTCT R- GGTCCAGTCACCAGCATAACAGA	CAGAAGCCGTGCGTGTGGATGG	XM_001104543
<i>CYP19A1</i>	F- TAGCAGAAAAAAGACGCAGGATT R- CGTCAGGTCACCTCGTTTCTC	AATGCATGGACTTTGCCACTGAGTTGATTTT	XM_001082665
<i>ALG9</i>	F- AACAGTGCCACAGAGCGAGAA R- CGATACCGCCTGGAGCACTA	ACTGTCTTCCTGTTTCGGG	XM_001106180
<i>RPL13A</i>	F- TCACGAGGTTGGCTGGAAGT R- GATCTTGGCTTTCTCCTTCCTCTT	CCAGGCAGTGACAGCCACCTTGG	XM_001115079

Reference sequences for each of the target genes can be accessed through GenBank accession IDs.

concentration), and 2 µl of cDNA. The reaction sequence included 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at 60°C. Automatic baseline and threshold levels were determined by ABI sequence detection system software (version 2.2.1.), and the final expression values were normalized to the arithmetic mean of two reference housekeeping genes, *ALG9* and *RPL13A*. Individual genes from experiments 1 and 2 were examined together on the same 384-well optical plate. A negative control included the omission of cDNA templates from the reaction mixture.

RT² Profiler™ PCR Array

Total RNA samples (0.5 µg) were reverse transcribed using the RT² First Strand Kit (Qiagen). Each reaction was performed in 25 µl of solution containing cDNA, 2 × RT² SYBR Green Mastermix, and RNase-free water, using custom-made RT² Profiler™ PCR Arrays (Custom PCR Arrays; Qiagen) and a QuantStudio™ 12K Flex thermocycler (Life Technologies, Grand Island, N.Y., USA). The reaction sequence included 10 min of incubation at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, 1 min at 60°C, and 15 s at 95°C. The relative gene expression was calculated using the $\Delta\Delta C_t$ method, and the results are expressed with reference to the arithmetic mean of three reference housekeeping genes (*ALG9*, *GAPDH*, and *RPL13A*).

Statistical Analysis

Paired Student's *t* tests were used to compare differences in *ESR1*, *ESR2*, *PGR*, *AR*, *STS*, *HSD3B1/2*, *HSD17B5*, and *CYP19A1* expression between young and old gonad-intact animals (experi-

ment 1) and between OVX and OVX + E₂ animals (experiment 2). ANOVA followed by the Dunnett multiple-range test was used to assess between-group differences in expression for all other analyses (experiment 3). Significance was considered at *p* < 0.05.

Results

The ovary-intact animals showed no detectable effect of age on the expression of genes encoding steroid receptors or steroidogenic enzymes in the ARC-ME (table 2). In both young and old OVX animals, however, *PGR* expression was significantly enhanced by E₂ supplementation (fig. 1). Similarly, *ESR1* and *ESR2* expression was enhanced by E₂ supplementation in the young (fig. 1a) but not in the old (fig. 1b) animals. In the OVX animals, there was no detectable effect of E₂ supplementation on the expression of *AR* (fig. 1) or on genes encoding steroidogenic enzymes (fig. 2), except for a significant (*p* < 0.05) decrease in *HSD17B5* expression in the old OVX + E₂ animals relative to the untreated age-matched controls.

To corroborate and extend the findings from the qRT-PCR experiments, we also performed gene expression analyses on RNA extracts obtained from young go-

Fig. 1. Effect of E₂ treatment on steroid receptor gene expression in the ARC-ME of OVX rhesus macaques. The animals either served as controls or were treated with E₂ for ~1 month (young animals) or for ~4 years (old animals). Each bar represents the mean ($\pm$ SEM) mRNA levels of 4 animals, normalized to the arithmetic mean of two housekeeping genes, *ALG9* and *RPL13A*. In the

young animals (a), *ESR1*, *ESR2*, and *PGR* mRNA levels were significantly higher in the OVX + E₂ group than in the OVX group, whereas *AR* mRNA levels were not significantly different. In the old animals (b), only *PGR* mRNA levels were significantly higher in the OVX + E₂ group relative to the OVX group. * $p < 0.05$, ** $p < 0.01$ (paired Student's *t* test).

Table 2. Effect of age on gene expression in the ARC-ME of female rhesus macaques

Gene symbol	Young intact (n = 4)	Old intact (n = 4)
<i>ESR1</i>	0.81 $\pm$ 0.25	1.32 $\pm$ 0.17
<i>ESR2</i>	0.86 $\pm$ 0.10	1.32 $\pm$ 0.20
<i>PGR</i>	0.44 $\pm$ 0.12	1.25 $\pm$ 0.43
<i>AR</i>	0.89 $\pm$ 0.17	1.48 $\pm$ 0.12
<i>STS</i>	1.39 $\pm$ 0.27	1.96 $\pm$ 0.26
<i>HSD3B1/2</i>	0.35 $\pm$ 0.15	0.53 $\pm$ 0.16
<i>HSD17B5</i>	1.28 $\pm$ 0.26	1.87 $\pm$ 0.20
<i>CYP19A1</i>	0.58 $\pm$ 0.36	1.57 $\pm$ 0.62

Values represent mean ($\pm$ SEM) mRNA levels relative to the arithmetic mean of *ALG9* and *RPL13A*. No significant differences were detected between the two age groups (Student's *t* test, $p > 0.05$).

nad-intact, old OVX, and old OVX + E₂ animals, using custom-made RT² Profiler™ PCR arrays. These three groups were selected to provide insights into ARC-ME gene expression during sexual maturity, after menopause, and after subsequent hormone therapy (HT). Our focus

was on genes that encode steroid receptors, as well as genes associated with the control of the reproductive system, metabolism, and neurotransmission (table 3). As expected, the array analysis revealed a significant decrease ($p < 0.01$) in *PGR* expression in old OVX animals and showed that this decrease could be blocked by long-term HT. The expression of *GNRH2* and *SLC6A3* was also significantly enhanced by E₂ ($p < 0.05$ and $p < 0.01$, respectively). In contrast, a significant increase in expression levels was observed for several genes associated with the KNDy neuronal system or metabolism (*KISS1*, $p < 0.01$; *KISS1R*, $p < 0.05$; *TAC3*, $p < 0.01$; *NPY2R*, $p < 0.05$), and in most cases the increase was reversed by E₂.

Discussion

Previous studies of the rhesus macaque hypothalamus relied on RT-PCR and in situ hybridization to examine the expression of *ESR1*, *ESR2*, and *PGR* [41, 42]. Although these studies were essentially qualitative or semiquantitative, taken together their findings suggested that *ESR1* and *ESR2* expression is unaffected by circulating E₂ levels, whereas *PGR* expression is highly stimulated by E₂. In the

Fig. 2. Effect of E₂ treatment on steroidogenic enzyme gene expression in the ARC-ME of OVX rhesus macaques. Each bar represents the mean ($\pm$ SEM) mRNA levels of 4 animals, normalized to the arithmetic mean of two housekeeping genes, *ALG9* and *RPL13A*.

In the young animals (a), there was no significant effect of E₂ on any of the mRNA levels examined. In the old animals (b), HSD17B5 mRNA levels were significantly higher in the OVX + E₂ group relative to the OVX group. * $p < 0.05$ (paired Student's *t* test).

present study, we used qRT-PCR, a more quantitative molecular approach, to corroborate these previous observations and to examine if these genes show age-associated changes in their expression. First of all, *ESR1*, *ESR2*, and *PGR* were all expressed in the ARC-ME region of the hypothalamus, thus confirming the results from previous studies. Despite their old age, however, we failed to detect a significant change in the expression of these genes in our old ovary-intact animals. This most likely stems from the fact that most of these animals had not completely entered menopause and their circulating E₂ levels had not yet fallen to a sustained basal level. Consequently, to more closely mimic the human postmenopausal condition, we also examined age-related differences in the ARC-ME of young and old OVX animals and exposed some of them to supplementary E₂ in order to gain insights into the influence of HT.

E₂ HT had an age-related effect on *ESR1* and *ESR2* expression in OVX animals. While young OVX + E₂-treated animals expressed more *ESR1* and *ESR2* mRNA than did untreated age-matched controls, the old animals did not respond to E₂ supplementation. Although the reason for this difference is unclear, one possibility is that the hypothalamus of older animals is itself less responsive to exogenous E₂. Another possibility is that the stimulatory effect of E₂ on *ESR1* and *ESR2* mRNA is transitory and that it

gradually decreases during long-term HT. In contrast to *ESR1* and *ESR2*, *PGR* expression was highly stimulated by E₂ regardless of age. It should be emphasized that the two major isoforms of the progesterin receptor (*PGR-A* and *PGR-B*) show different expression patterns [43, 44] and independent functions [45–47]. In the present study, however, we amplified both isoforms of *PGR* and thus cannot establish which form plays the dominant physiological role in the primate hypothalamus. Expression of the androgen receptor in the female rhesus macaque hypothalamus has not previously been described, but in the present study we found *AR* to be highly expressed in the female hypothalamus and to be unaffected by circulating E₂ levels. This result is consistent with, and complements, the finding from a previous study in which a ribonuclease protection assay showed no effect of testosterone on the hypothalamic expression of *AR* in male rhesus macaques [48]. Taken together, the quantitative data from the present study clearly establish that gonadal nuclear steroid receptors are highly expressed in the female rhesus macaque hypothalamus, and that circulating E₂ levels can significantly modulate progesterin receptor gene expression even in old animals.

Recently, Naugle et al. [49] used immunohistochemistry to examine the effects of long-term E₂ treatment on *ER α* and *PGR* expression in the rhesus macaque ARC-

Table 3. Gene expression in the ARC-ME of female rhesus macaques during reproductive aging

Gene symbol	Gene name	Young intact	Old OVX	Old OVX + E ₂
<i>Sex steroid receptors</i>				
<i>AR</i>	androgen receptor	1.00±0.13	0.91±0.10	1.02±0.07
<i>ESR1</i>	estrogen receptor 1	1.00±0.11	1.19±0.06	0.90±0.07
<i>ESR2</i>	estrogen receptor 2	1.00±0.18	1.22±0.13	1.08±0.14
<i>PGR</i>	progesterone receptor	1.00±0.10**	0.33±0.13	1.82±0.14**
<i>Reproduction</i>				
<i>GNRH1</i>	gonadotropin-releasing hormone 1	1.00±0.17	1.09±0.33	0.89±0.20
<i>GNRH2</i>	gonadotropin-releasing hormone 2	1.00±0.15	0.59±0.07	3.31±1.01*
<i>KISS1</i>	KiSS-1 metastasis suppressor	1.00±0.32**	11.72±2.20	0.25±0.08**
<i>KISS1R</i>	KISS1 receptor	1.00±0.11*	1.99±0.15	2.18±0.38
<i>PDYN</i>	prodynorphin	1.00±0.15	0.89±0.06	1.19±0.07
<i>TAC3</i>	tachykinin 3	1.00±0.09**	3.21±0.64	0.49±0.05**
<i>Metabolism</i>				
<i>AGRP</i>	agouti-related protein homolog	1.00±0.25	1.48±0.24	1.00±0.07
<i>INSR</i>	insulin receptor	1.00±0.16	1.27±0.25	1.12±0.07
<i>LEPR</i>	leptin receptor	1.00±0.09	1.23±0.17	1.12±0.27
<i>MC2R</i>	melanocortin 2 receptor (adrenocorticotrophic hormone)	1.00±0.32	0.37±0.11	0.69±0.43
<i>MC4R</i>	melanocortin 4 receptor	1.00±0.29	0.62±0.07	0.71±0.11
<i>MCHR1</i>	melanin-concentrating hormone receptor 1	1.00±0.05	1.22±0.17	1.71±0.34
<i>NPY</i>	neuropeptide Y	1.00±0.53	0.81±0.40	0.40±0.05
<i>NPY1R</i>	neuropeptide Y receptor Y ₁	1.00±0.19	1.08±0.28	1.05±0.16
<i>NPY2R</i>	neuropeptide Y receptor Y ₂	1.00±0.15*	1.53±0.12	1.04±0.12*
<i>NPY5R</i>	neuropeptide Y receptor Y ₅	1.00±0.12	1.05±0.17	1.31±0.17
<i>POMC</i>	proopiomelanocortin	1.00±0.19	1.17±0.27	0.83±0.11
<i>Neurotransmission</i>				
<i>ACHE</i>	acetylcholinesterase	1.00±0.10	1.17±0.04	1.13±0.10
<i>ADRA1A</i>	adrenergic, alpha-1A, receptor	1.00±0.14	0.80±0.03	0.73±0.21
<i>DRD1</i>	dopamine receptor D ₁	1.00±0.10	0.89±0.08	0.82±0.09
<i>DRD2</i>	dopamine receptor D ₂	1.00±0.21	0.82±0.11	1.02±0.22
<i>SLC18A2</i>	solute carrier family 18, member 2 (vesicular monoamine)	1.00±0.22	0.88±0.08	1.07±0.26
<i>SLC6A3</i>	solute carrier family 6, member 3 (dopamine transporter)	1.00±0.34	0.62±0.06	4.52±0.74**
<i>SLC6A4</i>	member 3, solute carrier family 6, member 4 (serotonin transporter)	1.00±0.57	0.67±0.62	0.55±0.25
<i>GABBR1</i>	gamma-aminobutyric acid B receptor, 1	1.00±0.06	0.99±0.02	1.08±0.10
<i>GABBR2</i>	gamma-aminobutyric acid B receptor, 2	1.00±0.27	0.69±0.04	0.77±0.05
<i>GABRA1</i>	gamma-aminobutyric acid A receptor, alpha 1	1.00±0.23	0.75±0.03	1.01±0.07
<i>GABRA5</i>	gamma-aminobutyric acid A receptor, alpha 5	1.00±0.12	0.82±0.06	0.87±0.16
<i>HTR1A</i>	5-hydroxytryptamine (serotonin) receptor 1A	1.00±0.14	0.71±0.06	0.86±0.15
<i>HTR2A</i>	5-hydroxytryptamine (serotonin) receptor 2A	1.00±0.04	0.81±0.12	0.63±0.07
<i>HTR2C</i>	5-hydroxytryptamine (serotonin) receptor 2C	1.00±0.12	1.35±0.15	1.20±0.06

RT² Profiler™ PCR Arrays were used to profile gene expression in young intact, old OVX, and old OVX + E₂-treated female macaques. The values (means ± SEM) are expressed relative to the arithmetic mean of *ALG9*, *GAPDH*, and *RPL13A*, and normalized against the young intact group. * p < 0.05, ** p < 0.01 relative to the old OVX group (ANOVA followed by the Dunnett test).

ME. Semiquantitative analysis of the immunoreactive cells revealed no effect of E₂ on the number and density of ER α -positive cells in old OVX females, which is in agreement with the mRNA findings from the present study. On the other hand, E₂ also failed to significantly affect the number and density of PGR-positive cells, which does not agree with our mRNA findings or those previously reported by Bethea et al. [33]. It is unclear whether this discrepancy is a reflection of the relative insensitivity of the semiquantitative immunohistochemistry methodology or whether it indicates that E₂-induced PGR expression changes are not reflected at the translation level. Western blot analysis would help to resolve this issue in the future.

Given that the brain has an intrinsic capacity to synthesize sex steroids de novo [26–29], it is plausible that the hypothalamus can also compensate for the menopausal loss of sex steroids by increasing its local production of these steroids. There are several key enzymes involved in the synthesis of testosterone and E₂ in the brain, using DHEA as a precursor. In the present study, we examined the hypothalamic expression of *STS*, *HSD3B1/2*, *HSD17B5*, and *CYP19A1*. These genes encode key enzymes in the conversion of DHEA to testosterone and E₂ and are expressed in the rhesus macaque hypothalamus [29]. The findings from the current study corroborate these earlier observations. Moreover, they show that the expression of *HSD17B5* becomes enhanced in old animals when circulating levels of E₂ are low, which may result in a compensatory increase in hypothalamic E₂ levels due to enhanced intracrine synthesis from DHEA. On the other hand, circulating levels of DHEA (particularly in the sulfated form as DHEA sulfate) show a significant age-related decline [29, 50]. Consequently, it is unclear if the expression of *STS*, *HSD3B1/2*, *HSD17B5*, and *CYP19A1* alone is sufficient to maintain high hypothalamic sex steroid levels during aging. Nevertheless, the results suggest that local steroid synthesis in the hypothalamus is likely to be preserved in older animals, provided that there is a sufficient amount of DHEA precursor available.

Other significant age-related gene expression changes have previously been observed in the human and nonhuman primate ARC-ME, especially in the KNDy neurons [12–17]. We observed the same neuronal hypertrophy-related increase in *KISS1* and *TAC3* gene expression observed in postmenopausal women, and this increase could be blocked by the administration of exogenous E₂ [17]. This suggests that the observed gene expression changes stemmed from an age-associated decline in cir-

culating E₂ levels, rather than from a sex steroid-independent aging mechanism. Although the physiological significance of the E₂-dependent changes in *KISS1*, *KISS1R*, and *TAC3* expression is unclear, these changes may serve to maintain homeostatic control of the reproductive neuroendocrine axis after secretion of sex steroids from the ovaries has declined [2]. In addition, we found that the expression of *NPY2R*, a central mediator of food intake, was also suppressed following chronic E₂ supplementation in old OVX animals [51]. In postmenopausal women, therefore, *NPY2R* of the arcuate region may help to mediate the beneficial effects of estrogen HT in the treatment of disorders such as diabetes, obesity, and metabolic syndrome [52].

Analysis of the RT² ProfilerTM PCR arrays also established that most of the selected genes are stably expressed during reproductive aging and after HT. On the other hand, E₂ supplementation was found to significantly stimulate the expression of *GNRH2*. We previously reported that E₂ exerts a stimulatory action on *GNRH2* gene expression in the hypothalamus of young OVX animals [53–55]. The finding that *GNRH2* expression in old OVX animals also responds to E₂ suggests that this second form of primate GnRH may play an important role in mediating E₂-positive feedback to the reproductive neuroendocrine axis, as previously suggested [54, 55]. E₂ supplementation was also found to stimulate the expression of *SLC6A3*, the gene that encodes the dopamine transporter. Voytko et al. [56] reported that chronic E₂ therapy did not have an effect on dopaminergic activity in the ventral tegmentum and dorsolateral prefrontal cortex. However, the present results show that chronic E₂ supplementation was able to significantly increase the expression of the dopamine transporter in arcuate neurons of aged OVX animals. The influence of estrogen on the dopaminergic system is less well understood, but our results suggest that chronic E₂ treatment may facilitate reuptake of dopamine by presynaptic neurons in the arcuate region.

Menopause in women and female rhesus macaques is associated with attenuated circulating E₂ concentrations [1–3], which is thought to contribute to the development of many age-related pathologies. On the other hand, the results from the present study suggest that the hypothalamus retains its capacity to respond to sex steroids even in old age, and that the sensitivity of the hypothalamus to E₂ shows some compensatory changes that may help to maintain local homeostasis.

Acknowledgements

This work was supported by NIH grants AG-019100, AG-023477, AG-029612, AG-036670, and OD-011092. We wish to thank Laurie Renner for managing the in vivo component of the study, and Dr. Steven Kohama for collecting and processing the postmortem tissues. We also wish to thank the ONPRC Endocrine Technology and Support Core for conducting the hormone assays, and the ONPRC Molecular and Cell Biology Core for assistance with the mRNA quantitation.

Disclosure Statement

The authors have no conflicts of interest to declare.

References

- 1 Burger HG, Dudley EC, Robertson DM, Dennerstein L: Hormonal changes in the menopause transition. *Rec Prog Horm Res* 2002;57:257–275.
- 2 Downs JL, Urbanski HF: Neuroendocrine changes in the aging reproductive axis of female rhesus macaques (*Macaca mulatta*). *Biol Reprod* 2006;75:539–546.
- 3 McKinlay SM, Brambilla DJ, Posner JG: The normal menopause transition. *Maturitas* 2008;61:4–16.
- 4 Mittelman-Smith MA, Williams H, Krajewski-Hall SJ, McMullen NT, Rance NE: Role for kisspeptin/neurokinin B/dynorphin (KNDy) neurons in cutaneous vasodilatation and the estrogen modulation of body temperature. *Proc Natl Acad Sci USA* 2012;109:19846–19851.
- 5 Sarti CD, Chiantera A, Graziottin A, Ognisanti F, Sidoli C, Mincigrucci M, Parazzini F: Hormone therapy and sleep quality in women around menopause. *Menopause* 2005;12:545–551.
- 6 Rance NE, McMullen NT, Smialek JE, Price DL, Young WS 3rd: Postmenopausal hypertrophy of neurons expressing the estrogen receptor gene in the human hypothalamus. *J Clin Endocrinol Metab* 1990;71:79–85.
- 7 Rance NE, Uswandi SV: Gonadotropin-releasing hormone gene expression is increased in the medial basal hypothalamus of postmenopausal women. *J Clin Endocrinol Metab* 1996;81:3540–3546.
- 8 Abel TW, Rance NE: Proopiomelanocortin gene expression is decreased in the infundibular nucleus of postmenopausal women. *Brain Res Mol Brain Res* 1999;69:202–208.
- 9 Abel TW, Voytko ML, Rance NE: The effects of hormone replacement therapy on hypothalamic neuropeptide gene expression in a primate model of menopause. *J Clin Endocrinol Metab* 1999;84:2111–2118.
- 10 Krajewski SJ, Abel TW, Voytko ML, Rance NE: Ovarian steroids differentially modulate the gene expression of gonadotropin-releasing hormone neuronal subtypes in the ovariectomized cynomolgus monkey. *J Clin Endocrinol Metab* 2003;88:655–662.
- 11 Escobar CM, Krajewski SJ, Sandoval-Guzman T, Voytko ML, Rance NE: Neuropeptide Y gene expression is increased in the hypothalamus of older women. *J Clin Endocrinol Metab* 2004;89:2338–2343.
- 12 Rance NE, Young WS 3rd: Hypertrophy and increased gene expression of neurons containing neurokinin-B and substance-P messenger ribonucleic acids in the hypothalamus of postmenopausal women. *Endocrinology* 1991;128:2239–2247.
- 13 Rometo AM, Krajewski SJ, Voytko ML, Rance NE: Hypertrophy and increased kisspeptin gene expression in the hypothalamic infundibular nucleus of postmenopausal women and ovariectomized monkeys. *J Clin Endocrinol Metab* 2007;92:2744–2750.
- 14 Rometo AM, Rance NE: Changes in prodynorphin gene expression and neuronal morphology in the hypothalamus of postmenopausal women. *J Neuroendocrinol* 2008;20:1376–1381.
- 15 Rance NE: Menopause and the human hypothalamus: evidence for the role of kisspeptin/neurokinin B neurons in the regulation of estrogen negative feedback. *Peptides* 2009;30:111–122.
- 16 Rance NE, Krajewski SJ, Smith MA, Cholanian M, Dacks PA: Neurokinin B and the hypothalamic regulation of reproduction. *Brain Res* 2010;1364:116–128.
- 17 Eghlidi DH, Haley GE, Noriega NC, Kohama SG, Urbanski HF: Influence of age and 17 β -estradiol on kisspeptin, neurokinin B, and prodynorphin gene expression in the arcuate-medial eminence of female rhesus macaques. *Endocrinology* 2010;151:3783–3794.
- 18 Van Look PF, Lothian H, Hunter WM, Michie EA, Baird DT: Hypothalamic-pituitary-ovarian function in perimenopausal women. *Clin Endocrinol* 1977;7:13–31.
- 19 Dorling AA, Todman MG, Korach KS, Herbison AE: Critical role for estrogen receptor alpha in negative feedback regulation of gonadotropin-releasing hormone mRNA expression in the female mouse. *Neuroendocrinol* 2003;78:204–209.
- 20 Glidewell-Kennedy C, Hurley LA, Pfaff L, Weiss J, Levine JE, Jameson JL: Nonclassical estrogen receptor alpha signaling mediates negative feedback in the female mouse reproductive axis. *Proc Natl Acad Sci USA* 2007;104:8173–8177.
- 21 Sullivan KA, Witkin JW, Ferin M, Silverman AJ: Gonadotropin-releasing hormone neurons in the rhesus macaque are not immunoreactive for the estrogen receptor. *Brain Res* 1995;685:198–200.
- 22 Terasawa E: Control of luteinizing hormone-releasing hormone pulse generation in non-human primates. *Cell Mol Neurobiol* 1995;15:141–164.
- 23 Petersen SL, Ottem EN, Carpenter CD: Direct and indirect regulation of gonadotropin-releasing hormone neurons by estradiol. *Biol Reprod* 2003;69:1771–1778.
- 24 Skinner DC, Evans NP, Delaleu B, Goodman RL, Bouchard P, Caraty A: The negative feedback actions of progesterone on gonadotropin-releasing hormone secretion are transduced by the classical progesterone receptor. *Proc Natl Acad Sci USA* 1998;95:10978–10983.
- 25 Wilson ME, Rosewell KL, Kashon ML, Shughrue PJ, Merchenthaler I, Wise PM: Age differentially influences estrogen receptor- α (ER α) and estrogen receptor- β (ER β) gene expression in specific regions of the rat brain. *Mech Ageing Dev* 2002;123:593–601.
- 26 Labrie F: Intracrinology. *Mol Cell Endocrinol* 1991;78:C113–C118.
- 27 Sorwell KG, Urbanski HF: Dehydroepiandrosterone and age-related cognitive decline. *Age (Dordr)* 2010;32:61–67.
- 28 Labrie F: All sex steroids are made intracellularly in peripheral tissues by the mechanisms of intracrinology after menopause. *J Steroid Biochem Mol Biol* 2015;145:133–138.
- 29 Sorwell KG, Kohama SG, Urbanski HF: Perimenopausal regulation of steroidogenesis in the nonhuman primate. *Neurobiol Aging* 2012;33:1487.e1–e13.
- 30 Gilardi KV, Shideler SE, Valverde CR, Roberts JA, Lasley BL: Characterization of the onset of menopause in the rhesus macaque. *Biol Reprod* 1997;57:335–340.
- 31 Walker ML, Herndon JG: Menopause in non-human primates? *Biol Reprod* 2008;79:398–406.
- 32 Pfaff DW, Gerlach JL, McEwen BS, Ferin M, Carmel P, Zimmerman EA: Autoradiographic localization of hormone-concentrating cells in the brain of the female rhesus monkey. *J Comp Neurol* 1976;170:279–293.

- 33 Bethea CL, Brown NA, Kohama SG: Steroid regulation of estrogen and progesterin receptor messenger ribonucleic acid in monkey hypothalamus and pituitary. *Endocrinology* 1996; 137:4372–4383.
- 34 Blurton-Jones MM, Roberts JA, Tuszyński MH: Estrogen receptor immunoreactivity in the adult primate brain: neuronal distribution and association with p75, *trkA*, and choline acetyltransferase. *J Comp Neurol* 1999;405: 529–542.
- 35 Mills RH, Romeo HE, Lu JK, Micevych PE: Site-specific decrease of progesterone receptor mRNA expression in the hypothalamus of middle-aged persistently estrus rats. *Brain Res* 2002;955:200–206.
- 36 Simerly RB, Chang C, Muramatsu M, Swanson LW: Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. *J Comp Neurol* 1990;294:76–95.
- 37 Fernandez-Guasti A, Kruijver FP, Fodor M, Swaab DF: Sex differences in the distribution of androgen receptors in the human hypothalamus. *J Comp Neurol* 2000;425:422–435.
- 38 Kohama SG, Bethea CL: Steroid regulation of tyrosine hydroxylase messenger ribonucleic acid in dopaminergic subpopulations of monkey hypothalamus. *Endocrinology* 1995;136: 1790–1800.
- 39 Jensen JT, Zelinski MB, Stanley JE, Fanton JW, Stouffer RL: The phosphodiesterase 3 inhibitor ORG 9935 inhibits oocyte maturation in the naturally selected dominant follicle in rhesus macaques. *Contraception* 2008;77: 303–307.
- 40 Noriega NC, Kohama SG, Urbanski HF: Microarray analysis of relative gene expression stability for selection of internal reference genes in the rhesus macaque brain. *BMC Mol Biol* 2010;11:47.
- 41 Pau CY, Pau KY, Spies HG: Putative estrogen receptor beta and alpha mRNA expression in male and female rhesus macaques. *Mol Cell Endocrinol* 1998;146:59–68.
- 42 Gundlach C, Kohama SG, Mirkes SJ, Garyfalou VT, Urbanski HF, Bethea CL: Distribution of estrogen receptor beta (ER β) mRNA in hypothalamus, midbrain and temporal lobe of spayed macaque: continued expression with hormone replacement. *Brain Res Mol Brain Res* 2000;76:191–204.
- 43 Vegeto E, Shahbaz MM, Wen DX, Goldman ME, O'Malley BW, McDonnell DP: Human progesterone receptor α form is a cell- and promoter-specific repressor of human progesterone receptor B function. *Mol Endocrinol* 1993;7:1244–1255.
- 44 Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM, Horwitz KB: Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. *J Biol Chem* 2002;277:5209–5218.
- 45 Duffy DM, Wells TR, Haluska GJ, Stouffer RL: The ratio of progesterone receptor isoforms changes in the monkey corpus luteum during the luteal phase of the menstrual cycle. *Biol Reprod* 1997;57:693–699.
- 46 Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP, Conneely OM: Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. *Science* 2000;289:1751–1754.
- 47 Stouffer RL: Progesterone as a mediator of gonadotrophin action in the corpus luteum: beyond steroidogenesis. *Hum Reprod Update* 2003;9:99–117.
- 48 Abdelgadir SE, Roselli CE, Choate JV, Resko JA: Androgen receptor messenger ribonucleic acid in brains and pituitaries of male rhesus monkeys: studies on distribution, hormonal control, and relationship to luteinizing hormone secretion. *Biol Reprod* 1999;60:1251–1256.
- 49 Naugle MM, Nguyen LT, Merceron TK, Firlardo E, Janssen WG, Morrison JH, Rapp PR, Gore AC: G-protein coupled estrogen receptor, estrogen receptor α , and progesterone receptor immunohistochemistry in the hypothalamus of aging female rhesus macaques given long-term estradiol treatment. *J Exp Zool A Ecol Genet Physiol* 2014;321:399–414.
- 50 Sorwell KG, Kohama SG, Urbanski HF: Testosterone increases circulating dehydroepiandrosterone sulfate levels in the male rhesus macaque. *Front Endocrinol (Lausanne)* 2014; 5:101.
- 51 Koegler FH, Enriori PJ, Billes SK, Takahashi DL, Martin MS, Clark RL, Evans AE, Grove KL, Cameron JL, Cowley MA: Peptide YY_(3–36) inhibits morning, but not evening, food intake and decreases body weight in rhesus macaques. *Diabetes* 2005;54:3198–3204.
- 52 Salpeter SR, Walsh JM, Ormiston TM, Greyber E, Buckley NS, Salpeter EE: Meta-analysis: effect of hormone-replacement therapy on components of the metabolic syndrome in postmenopausal women. *Diabetes Obes Metab* 2006;8:538–554.
- 53 Densmore VS, Urbanski HF: Effect of 17 β -estradiol on hypothalamic GnRH-II gene expression in the female rhesus macaque. *J Mol Endocrinol* 2004;33:145–153.
- 54 Urbanski HF: Differential roles of GnRH-I and GnRH-II neurons in the control of the primate reproductive axis. *Front Endocrinol (Lausanne)* 2012;3:20.
- 55 Urbanski HF: Selective targeting of GnRH-II neurons to block ovulation. *Contraception* 2014, Epub ahead of print.
- 56 Voytko ML, Tinkler GP, Browne C, Tobin JR: Neuroprotective effects of estrogen therapy for cognitive and neurobiological profiles of monkey models of menopause. *Am J Primatol* 2009;71:794–801.