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Age-associated gene expression changes in the arcuate nucleus of male rhesus macaques

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Abstract

The hypothalamic arcuate nucleus (ARC) represents a major component of the neuroendocrine reproductive axis, and plays an important role in controlling the onset of puberty as well as age-associated reproductive senescence. Although significant gene expression changes have been observed in the ARC during sexual maturation it is unclear what changes occur during aging, especially in males. Therefore, in the present study we profiled the expression of reproduction-related genes in the ARC of young and old male rhesus macaques, as well as old males that had received 6 months of hormone supplementation (HS) in the form of daily testosterone and dehydroepiandrosterone; we also compared morning versus night ARC gene expression in the old males. Using Affymetrix gene microarrays, we found little evidence for age-associated expression changes for genes associated with the neuroendocrine reproductive axis, whereas using qRT-PCR we detected a similar age-associated decrease in *PGR* (progesterone receptor) that we previously observed in post-menopausal females. We also detected a sex-steroid-dependent and age-associated decrease in *AR* (androgen receptor) expression, with highest AR levels being expressed at night (i.e., coinciding with the natural peak in daily testosterone secretion. Finally, unlike previous observations made in females, we did not find a significant age-associated increase in

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Declaration of Interest

The authors confirm that no actual, or potential, conflicts of interest exist.

Author contributions

D H E performed experiments, analyzed the data, and prepared the manuscript. V T G executed the study and helped with the data analysis. H F U and S G K designed and executed the study and helped with preparation of the manuscript.

KISS1 (Kisspeptin) or *TAC3* (Neurokinin B) expression in the ARC of males, most likely because the attenuation of circulating sex-steroid levels in the males was much less than that in postmenopausal females. Taken together, the data highlight some similarities and differences in ARC gene expression between aged male and female nonhuman primates.

Keywords

Aging; Androgen receptor; Circadian Rhythms; Reproduction; Testosterone

Introduction

Neural circuits located in the hypothalamic arcuate nucleus (ARC) play an important role in regulating homeostatic and endocrine functions. By modulating the secretion of hormones from the anterior pituitary gland, neuropeptides released from the ARC can affect a wide range of physiological functions, including somatic growth, metabolism, lactation, stress, and reproduction. The ARC also serves as an important feedback integration center for hormones produced by peripheral organs, especially sex steroids. During aging, this dynamic relationship between ARC neuropeptides and sex-steroid feedback changes markedly, and in the case of female humans and rhesus macaques this is associated with the onset of menopause (Gilardi *et al.* 1997; Downs & Urbanski 2006). However, little is known about the gene expression changes that occur in the human ARC during aging, in part because of the difficulty in obtaining quality mRNA from postmortem human brain tissue.

Like humans, rhesus macaques are long-lived diurnal primates that show many similar age-associated physiological and hormonal changes. For this reason, rhesus macaque studies represent a valuable translational animal model in which to gain insights about molecular changes that characterize neuroendocrine aging in humans. Some of the most dramatic changes observed in the ARC of aged female primates pertain to the control of the reproductive neuroendocrine axis, and include an increase in the expression of genes that encode kisspeptin (*KISS1*) and tachykinin 3 (*TAC3*), and a decrease in the expression of the progesterone receptor gene (*PGR*) (Rance 2009; Eghlidi *et al.* 2010; Eghlidi & Urbanski 2015). These gene expression changes in the neuroendocrine reproductive axis and steroid receptors have been shown to result from the marked decrease in circulating estradiol concentrations, rather than from aging *per se*, because they can be reversed by administration of exogenous estradiol (Betha *et al.* 1996; Eghlidi *et al.* 2010; Eghlidi & Urbanski 2015). Although age-related hormone changes also occur in males, the decrease in circulating sex-steroid concentrations is more gradual and less complete than in females (Bremner *et al.* 1983; Down & Urbanski 2006; Downs *et al.* 2008; Urbanski & Sorwell 2012; Urbanski *et al.* 2014). Therefore, it is unclear if the ARC of male primates undergoes similar age-related gene expression changes as in females (Hrabovszky *et al.* 2011; 2012; Molnar *et al.* 2012; Hrabovszky 2014). The primary goal of the present study was to profile gene expression in the ARC of aging male rhesus macaques, with a special emphasis on genes associated with the neuroendocrine reproductive axis. To determine the sex-steroid dependence of the age-associated changes, we also profiled gene expression in the ARC of old males supplemented with androgens for 6 months.

Materials and methods

Animals

Six young adult (age range 7–12 years) and 18 old (age range 21–26 years) male 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*. The animals were housed indoors under controlled environmental conditions: 24° C temperature; 12-hour light, 12-hour dark photoperiods with lights on at 0700 h. Meals were provided regularly at 0800 h and 1500 h (ZT1 and ZT8, respectively) (LabDiet High Protein Monkey Chow; LabDiet, Inc., St. Louis, MO, USA), and supplemented with fresh fruit or vegetables; drinking water was available *ad libitum*. Procedures for care and treatment of the animals were in accordance with methods approved by the Institutional Animal Care and Use Committee.

Tissue collection

In the main study, all of the postmortem brain tissue was collected in the morning, between 1000–1100 h. The animals were sedated with ketamine (15–25 mg/kg im) followed by pentobarbital sodium (25–30 mg/kg iv), a procedure consistent with the recommendations of the American Veterinary Medical Association's Panel on Euthanasia. Brains were collected after first flushing with 1 liter of 0.9% saline via a cardiac catheter; the hypothalami were then blocked and preserved in RNAlater (Ambion, Austin, TX, USA) for 1 to 2 weeks. Subsequently, the ARC was bilaterally sub-dissected from a coronal slice of each hypothalamic block, by a single investigator, and stored at –80° C. As previously described (Eghlidi *et al.* 2010), the boundaries for this tissue block included the exterior ventral edge of the hypothalamic median eminence, 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 ARC and the ventromedial hypothalamus).

Experimental design

The male monkeys were assigned to one of three groups: Young (n = 6), Old (n = 7), and Old HS (n = 6); in the latter group, the animals were given daily oral hormonal supplements (HS), comprising testosterone (T) and dehydroepiandrosterone (DHEA), for 6 months. T was administered at 1900 h (12 mg/kg body weight) and two oral DHEA administrations were administered at 0700 and 1000 h (0.04 mg/kg body weight); both T and DHEA were obtained from Sigma-Aldrich Corp (St. Louis, MO, USA). Previously, it had been shown that when T is administered orally in oil significant quantities bypass the liver, presumably because of reduced passage into the hepatic portal system and increased uptake by the lymphatic system, which elevate circulating T concentrations (Amory & Bremner 2005). Consequently, as previously described (Urbanski *et al.* 2014; Urbanski 2017), we suspended the T in sesame oil at a concentration of 120 mg/ml and then mixed an appropriate amount depending on the animal's body weight with ~12 g of chocolate or placed it inside a 5-g cookie, based on the animal's preference. Similarly, we suspended the DHEA in sesame oil

(10 mg/ml) and mixed an appropriate amount with chocolate or placed it inside a cookie. This daily androgen supplementation paradigm was previously shown to recapitulate mean youthful circulating levels of DHEA sulfate (DHEAS), estradiol, estrone, 5 α -dihydrotestosterone and T, while preserving their characteristic circadian profiles (although not the underlying pulsatile patterns) (Urbanski *et al.* 2014). The old control animals did not receive control treats or vehicle but were otherwise exposed to the same environmental conditions. Using Affymetrix Rhesus Gene Chip 1.0 ST Arrays we profiled all the genes in the ARC of male rhesus monkeys during aging and following T and DHEA supplementations. Next, we focused on steroid receptor and reproductive genes. Array data was corroborated using quantitative real-time polymerase chain reaction (qRT-PCR) to measure sex-steroid gene expression as well as *KISS1* and *TAC3*, the genes that encode kisspeptin and tachykinin 3 (also known as neurokinin B, NKB), respectively.

There is growing evidence from monkey and rodent studies that many genes show a circadian pattern of expression (Lemos *et al.* 2006; Zhang *et al.* 2014). Consequently, we were concerned that by focusing our gene profiling on only one time-of-day (i.e., 1000–1100 h; ZT3–4), we may have missed significant age-related expression changes in genes that show a peak later in the day. To help address this issue, we also collected postmortem hypothalamic tissue from five old gonad-intact male rhesus macaques at 2200–2300 h (ZT15–16), i.e., 12 hours later in the day. As described above, we similarly profiled gene expression in the ARC of these animals (Old PM) and compared the expression pattern to that observed in the morning in the Old controls (Old AM). The animals in the ZT15–16 group were maintained under the same 12-hour light 12-hour dark photoperiods as described above. During the nighttime tissue collections, the animals' eyes were covered to prevent photic phase resetting via light input through the retina.

RNA extraction and gene expression profiling

RNA was isolated from the whole ARC using the RNeasy Plus Universal Mini Kit (Qiagen) with two QIAcube Automation Systems (Qiagen). An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to determine the quality of the RNA and a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the concentration. Gene expression profiling was performed by the OHSU Gene Profiling Shared Resource. Briefly, target labeling was performed using total RNA samples that were prepared for array hybridization by synthesizing cDNA using the Ovation Pico WTA RNA Amplification System v.2 (NuGEN Technologies). Array hybridization and processing was performed by injecting the hybridization cocktail into a cartridge containing the GeneChip Rhesus Gene 1.0 ST array (Affymetrix) containing 37,375 gene-level probe sets. Arrays were incubated, washed, and stained on a GeneChip Fluidics Station 450 (Affymetrix) using the Hybridization Wash and Stain kit (Affymetrix). They were then scanned using the GeneChip Scanner 3000 7G with an autoloader (Affymetrix). Each array file was then analyzed using the Affymetrix® Command Console™ v.3.1.1 software, and expression analysis was performed using Affymetrix® Expression Console™ Software 1.4. Probe cell intensity files (CEL) for each of the experimental samples were uploaded into the Affymetrix® Expression Console™ Software 1.4, and a multi-array analysis incorporating all the samples of the study group was performed using the Robust Multi-Array Average

(RMA)-Sketch normalization, and a summarization algorithm for all exon transcripts was generated for further comparisons using the Affymetrix® Expression Console™ Software 1.4.

TaqMan quantitative real-time polymerase chain reaction (qRT-PCR)

As previously described (Eghlidi *et al.* 2010; Eghlidi & Urbanski 2015), a QuantStudio™ 12K Flex thermocycler was used for qRT-PCR (Life Technologies, Grand Island, NY, USA). Initially, 500 ng of ARC RNA was converted to cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA). Next, pooled cDNA was used to create standard curves for each gene, and the experimental samples were subsequently diluted to fall within the linear part of the curve. The PCR mixtures contained 5 µl TaqMan Gene Expression Master Mix, 2.15 µl of water, 0.3 µl of each specific forward and reverse primer (300 nM final concentration), 0.25 µl of specific probe (250 nM final concentration), and 2 µl of cDNA. The qRT-PCR reaction sequence included a 10-min 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. For *AR*, *ESR1*, *ESR2*, *PGR*, *KISS1*, and *TAC3*, the automatic baseline and threshold levels were determined by QuantStudio™ 12K Flex thermocycler detection system software and the final expression values were normalized to *RPL13A*. For each gene, samples were examined on the same 384-well optical plate in triplicate for each animal. A negative control included the omission of cDNA templates from the reaction mixture. The primer and probe base sequences used for the qRT-PCR are listed in Table 1.

Statistics

ANOVAs were used to assess differences between groups. A False Discovery Rate (FDR) correction for multiple comparisons was used for whole genome comparisons (Benjamini & Hochberg 1995). A Bonferroni correction was used when comparing the Young, Old HS, and Old PM groups to the Old AM controls.

Results

First, whole transcriptome expression was compared between Young and Old AM controls, as well as between Old AM controls and Old HS animals, using Affymetrix® Expression Console™ Software 1.4, with additional annotation provided by the ONPRC Collaborative Genetic Resource and Bioinformatics Units. The data were aligned with the Expression Console Output, and to correct for discrepancies in the annotations they were compared to annotations using Ingenuity Pathway Analysis and the NetAffx™ Analysis Center. After applying a FDR correction to the results, none of the 37,375 gene transcripts showed major (>1.5 fold) changes in expression between Young and Old or Old versus Old HS animals (FDR corrected *P*-value > 0.05; data not shown). Importantly, genes associated with the neuroendocrine reproductive axis continued to be constitutively expressed during aging and appeared to be unaffected by the HS (FC < 1.5, *P* > 0.05, Figures 1–2, Table 2). Using qRT-PCR, we confirmed that *ESR1*, *ESR2*, *KISS1* and *TAC3* expression did not change during aging or in response to HS (*P* > 0.05, Table 3), whereas *PGR* and *AR* showed a significant (*P* < 0.05) decrease in the old animals; *AR* expression in the Old HS animals was

significantly higher than in the age-matched Old controls, suggesting that the age-associated decrease may stem from attenuated circulating androgen levels.

Because so few genes in the ARC showed obvious age-related changes, we wondered if we may have missed them because they occur during the night rather than during the day (i.e., when postmortem tissues are usually collected). To help address this concern, we also collected brain tissue from Old males at night (PM) and compared the ARC gene expression profiles with those from Old males in the morning (AM). Although the steroid receptor *AR* and central reproductive genes *GNRHI* and *GNRHR* showed >1.5-fold greater expression in the evening (PM, 2200–2300 h) than in the morning (AM, 1000–1100 h) (Figure 3), only the diurnal difference in *AR* expression was statistically significant ($P < 0.05$, Table 3).

Discussion

In contrast to female primates, males (humans and monkeys) show a gradual and incomplete attenuation of their circulating sex-steroids concentrations during aging (Vermeulen *et al.* 1972; Marrama *et al.* 1982; Brenner *et al.* 1983; Tenover *et al.* 1988; Cooke *et al.* 1993; Harman *et al.* 2001; Feldman *et al.* 2002; Hardy & Schlegel 2004; Sitzmann *et al.* 2014; Urbanski *et al.* 2014). Therefore, it is plausible that age-related sex-differences exist in brain areas that integrate the positive and negative feedback actions of sex steroids on the reproductive neuroendocrine axis. To help resolve this issue, we used rhesus macaque-specific gene microarrays and qRT-PCR, to assess changes in the ARC transcriptome of males during aging and in response to HS. Although all of the genomic sex-steroid receptors were clearly expressed in the ARC of males, only *AR* and *PGR* showed evidence of age-associated change. Using qRT-PCR (Eghlidi & Urbanski 2015) and *in-situ* hybridization (Bethea *et al.* 1996), we previously demonstrated sex-steroid-dependent regulation of *PGR* expression in the ARC of young and old female rhesus macaques, and so were expecting to observe a similar age-related decrease in males. On the other hand, the circulating estradiol levels in all of our males were between 15–30 pg/ml (Urbanski *et al.* 2014), which is similar to the estradiol levels that we typically observed in females during the early follicular phase of the menstrual cycle, but lower than what is observed during the late follicular phase, and much higher than what is observed after menopause (Downs & Urbanski 2006).

Consequently, the relatively subtle differences in circulating estradiol levels seen between the Young, Old and Old HS males precluded any major sex-steroid dependent differences in *PGR* gene expression. It should be emphasized, however, that the current methodologies did not distinguish between the two different *PGR* isoforms (*PGR-A* and *PGR-B*); which have different expression patterns and functions. (Vegeto *et al.* 1993; Duffy *et al.* 1997; Mulac-Jericevic *et al.* 2000; Richer *et al.* 2002; Stouffer 2003). Therefore, we cannot rule out the possibility of compensatory alterations in the expression of *PGR-A* or *PGR-B* during aging.

In a previous study, a ribonuclease protection assay (using the same *AR* target sequence that we used in our qRT-PCR) showed no effect of testosterone on the hypothalamic expression of *AR* in male young rhesus macaques (Abdelgadir *et al.* 1999). Our results, however, suggest that there may be hormonal regulation of *AR*, at least during aging. The reason for this discrepancy is unclear but may stem from differences in baseline androgen levels of young and old males, or in differences in the hormonal supplementation paradigms. Because

testosterone and DHEA both show circadian patterns of release as well as a significant age-associated decline in circulating levels, our androgen supplementation paradigm was designed to keep the circulating levels of these two hormones in a physiological range without perturbing their characteristic 24-hour patterns (Urbanski *et al.* 2014). We also show that *AR* expression is significantly higher at night, i.e., a time when circulating testosterone levels are at their peak, even in aged primates (Urbanski 2011; Urbanski & Sorwell 2012; Sitzmann *et al.* 2014; Urbanski *et al.* 2014). Taken together, our findings show that *AR* expression in the male ARC undergoes similar steroid-dependent age-associated changes as does *PGR* in the ARC of females, and may serve a similar physiological role as a primary mediator of negative gonadal feedback during aging.

Lastly, an important finding from the present study was the insignificant difference in *KISS1* and *TAC3* expression between the Young and Old and between the Old versus the Old HS animals. Previous immunohistochemical examination of the kisspeptin and tachykinin 3 (a.k.a., neurokinin B, NKB) neuronal system in the ARC of men showed an age-associated increase in *KISS1* and *TAC3* fiber density and increased contacts with GnRH neurons in the median eminence (Hrabovszky *et al.* 2011; Molnar *et al.* 2012). In addition, quantitative analysis of *KISS1* and *TAC3* expression in the ARC revealed sex-steroid dependent increases in old ovariectomized females (Kim *et al.* 2009; Rance 2009; Eghlidi *et al.* 2010; Eghlidi & Urbanski 2015). Both of these findings suggest that the attenuated circulating androgen levels of healthy old males may be too modest to stimulate a significant rise in *KISS1* and *TAC3* at the mRNA level, although more physiologically-relevant changes at the translational level cannot be ruled out. *GNRH1*, *GNRH2* and *GNRHR* showed no obvious age-related change, underscoring our previous observations that in healthy rhesus macaques testicular function continues well into old age (Sitzmann *et al.* 2014), and circulating sex-steroid concentrations do not show such an abrupt or complete decrease as do menopausal females (Downs & Urbanski 2006; Urbanski 2011; Urbanski & Sorwell 2012; Sitzmann *et al.* 2014; Urbanski *et al.* 2014).

It is plausible that the negative gene array results reflect relative insensitivity of the methodology at detecting subtle gene expression changes, compared to qRT-PCR. Overall, however, the data from the present study show that gene expression in the ARC of male rhesus macaques is largely resistant to aging, especially genes that are associated with the neuroendocrine reproductive axis. Furthermore, the results largely agree with recent observations made in female rhesus macaques showing that the overall cytoarchitecture and sex-steroid receptor expression in the hypothalamus undergo relatively few changes with age and hormone supplementation (Naugle *et al.* 2014; 2016).

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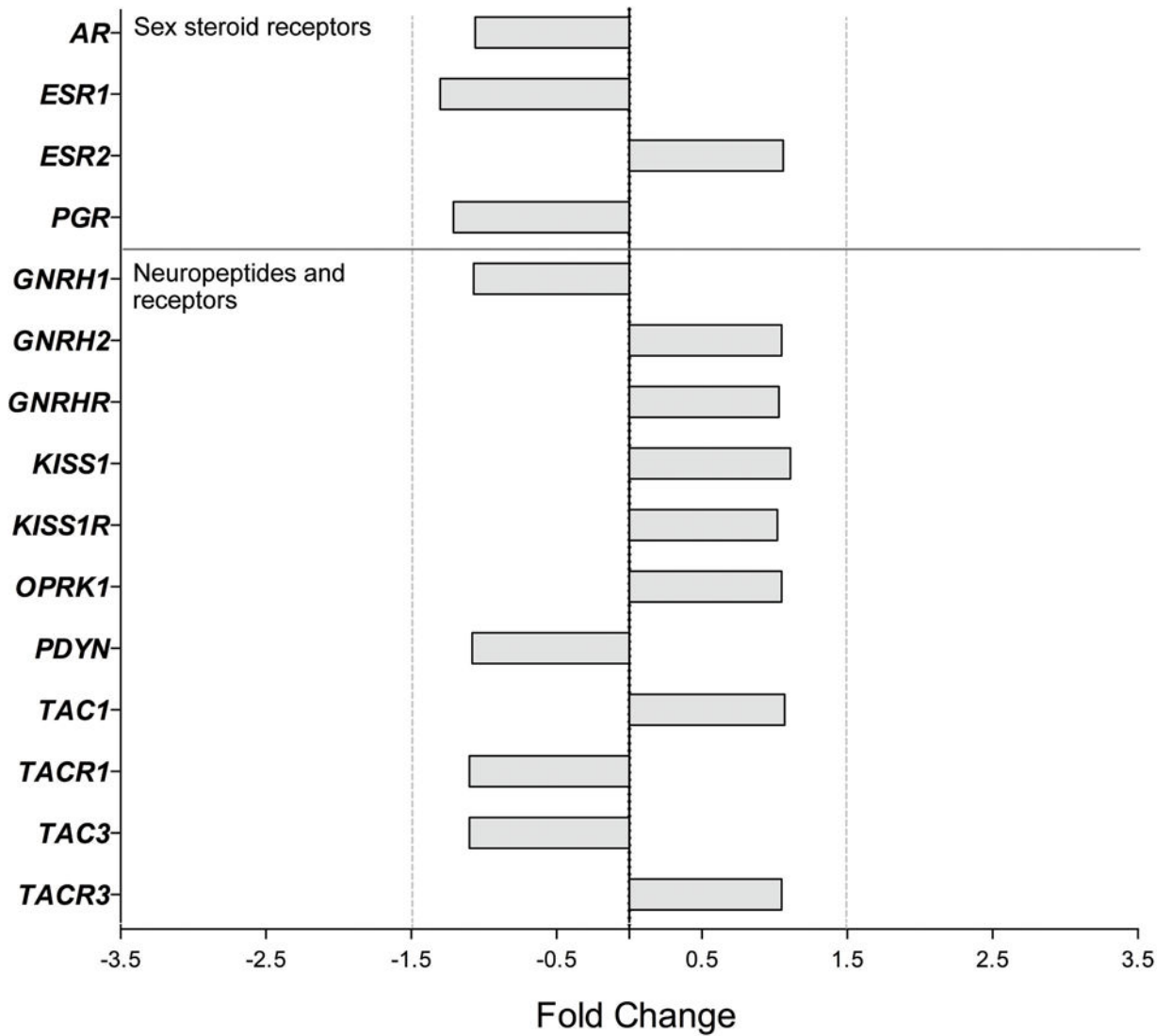


Figure 1.

Gene expression in the ARC of male rhesus macaques: effect of age on genes encoding sex-steroid receptors and reproduction-related neuropeptide systems. Fold change (FC) differences in expression are shown for the Old AM controls relative to the Young animals. No significant differences were detected ($P > 0.05$).

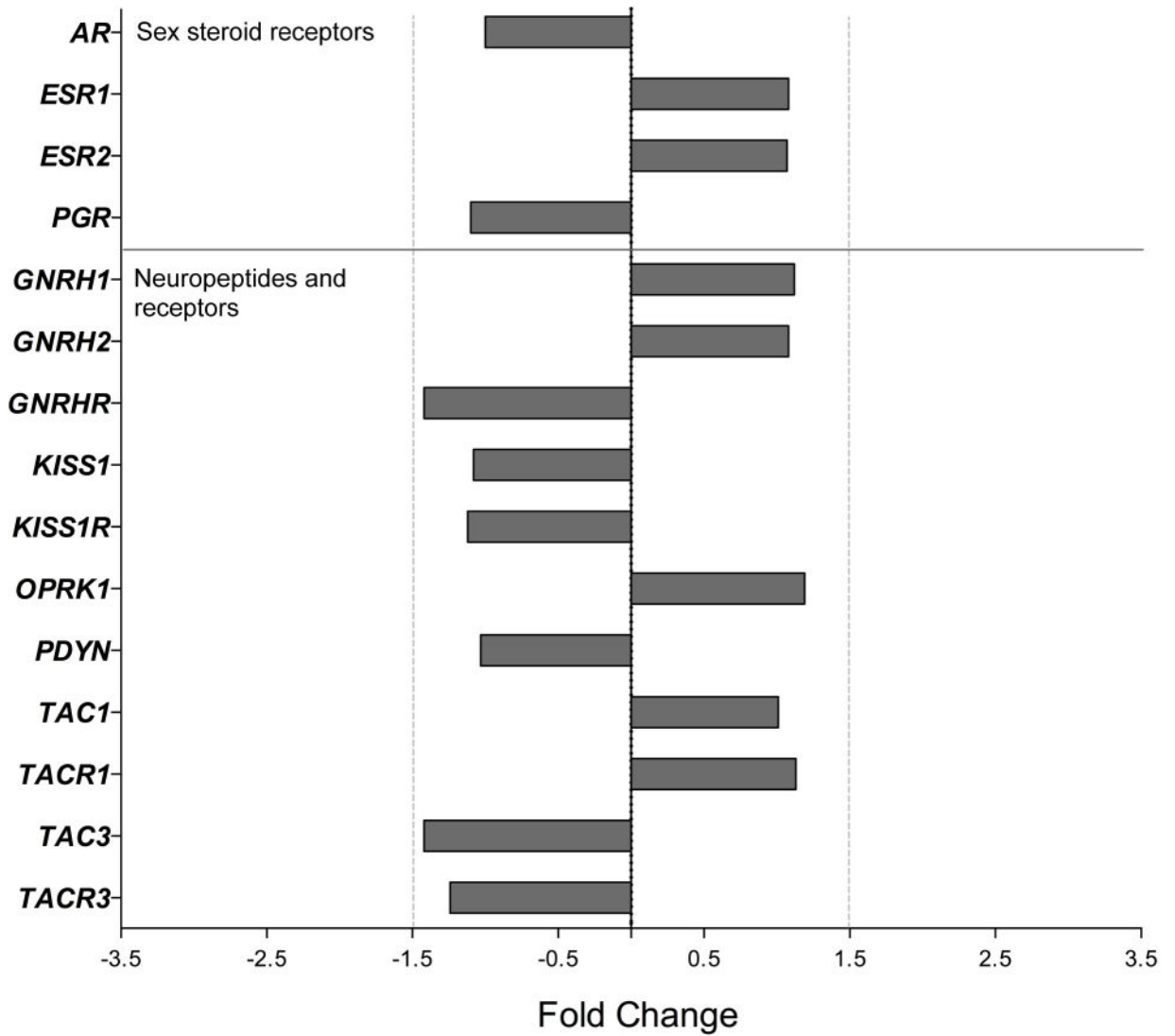


Figure 2. Gene expression in the ARC of male rhesus macaques: effect of androgen supplementation on genes encoding sex-steroid receptors and reproduction-related neuropeptide systems. Fold change (FC) differences in expression are shown for the Old HS animals relative to the Old AM controls. No significant differences were detected ($P > 0.05$).

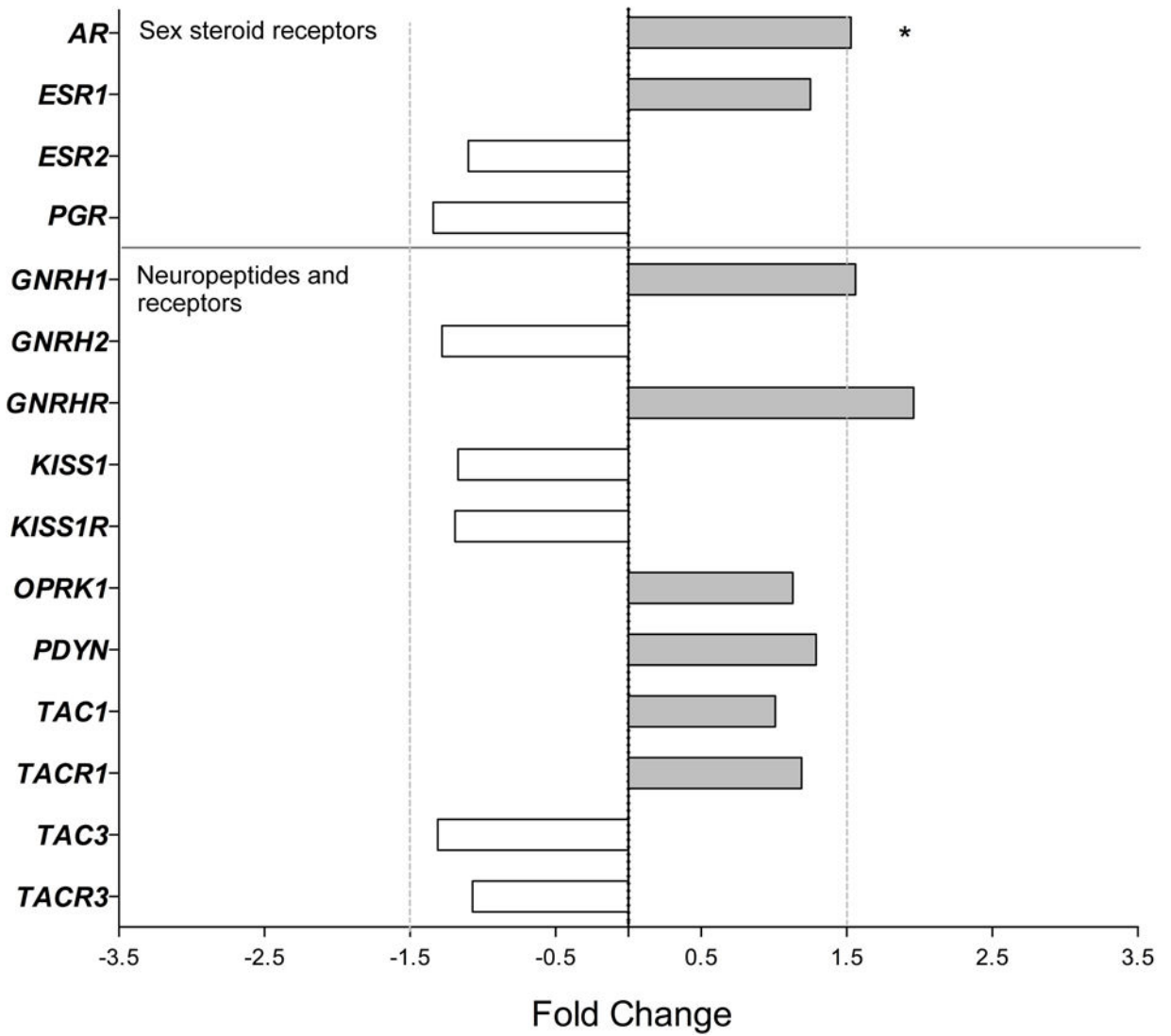


Figure 3.

Gene expression in the ARC of male rhesus macaques: effect of time of day on genes encoding sex-steroid receptors and reproduction-related neuropeptide systems. Fold change (FC) differences in expression are shown for the Old PM animals (at 2200–2300 h) relative to the Old AM controls (at 1000–1100 h). * $P < 0.05$.

TABLE 1

TaqMan qRT-PCR Primer and Probe nucleotide sequences (5' – 3')

Gene name	Primer Sequences	Probe Sequences	GenBank Accession ID.
<i>AR</i>	(F) AAAATCCCACATCCTGCTCAA (R) CAGGTCAAAGTGAAGTACTGATGCA	6FAM-TGCAGCCTATTGCGAGAG-MGB	AF092930
<i>ESR1</i>	(F) ACAAGATCACAGACACTTTGATCCA (R) CCTGATGTGGGAGAGGATGAG	6FAM-AGGCCTGACCCTGCAG-MGB	S71040
<i>ESR2</i>	(F) GATCACTAGAACACACCTTACCTGTA (R) GGTGCAACGGTTCCCACTAA	6FAM-AGAGACATTGAAAAGGAAG-MGB	NM_001265821
<i>PGR</i>	(F) CATGTCAGTGGGCAGATGCT (R) TGCCACATGGTAAGGCATAATG	6FAM-CTGAATGAACAGCGGATG-MGB	XM_001095317
<i>KISS1</i>	(F) AGAAAAGGTGGCCTCTGTGGA (R) AGGCTCTGCTCCACGG	6FAM-CAGGCCAGCAGCTGGAATCCCTG-MGB	AY823262
<i>TAC3</i>	(F) GAGCCACAGGAGGAGATGGTT (R) TCCAGAGATGAGTGGCTTTTGA	6FAM-ACAGCAAGAGGGATCT-MGB	XM_001115535
<i>RPL13A</i>	(F) TCACGAGGTTGGCTGGAAGT (R) GATCTTGGCTTTCTCCTTCCTCTT	6FAM-CCAGGCAGTGACAGCCACCTTGG-MGB	XM_001115079

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

TABLE 2
GeneChip Evaluation of Gene Expression in the Male Rhesus Macaque Arcuate Nucleus

Gene Symbol	Description	ID	AM			PM		P value
			Old (Control)	Young	Old HS	Old	PM	
Steroid receptors								
<i>AR</i>	Androgen receptor	13806207	5.81 ± 0.10	5.89 ± 0.05	5.81 ± 0.08	6.42 ± 0.08*	0.0001	
<i>ESR1</i>	Estrogen receptor 1	13827480	5.54 ± 0.09	5.92 ± 0.10	5.65 ± 0.06	5.86 ± 0.28	0.2026	
<i>ESR2</i>	Estrogen receptor 2	13784393	4.64 ± 0.13	4.56 ± 0.06	4.74 ± 0.11	4.50 ± 0.06	0.3874	
<i>PGR</i>	Progesterone receptor	13658473	6.89 ± 0.09	7.17 ± 0.08	6.75 ± 0.05	6.46 ± 0.25	0.0090	
Neuropeptides and receptors								
<i>GNRHI</i>	Gonadotropin-releasing hormone 1	13790870	6.94 ± 0.15	7.03 ± 0.16	7.10 ± 0.28	7.58 ± 0.35	0.2997	
<i>GNRH2</i>	Gonadotropin-releasing hormone 2	13606683	5.46 ± 0.11	5.38 ± 0.15	5.57 ± 0.09	5.10 ± 0.16	0.1015	
<i>GNRHR</i>	Gonadotropin-releasing hormone receptor	13752156	4.31 ± 0.40	4.27 ± 0.43	3.81 ± 0.41	5.28 ± 0.75	0.2657	
<i>KISS1</i>	Kisspeptin	13589384	5.28 ± 0.07	5.14 ± 0.15	5.18 ± 0.11	5.06 ± 0.19	0.6768	
<i>KISS1R</i>	Kisspeptin receptor	13820906	6.18 ± 0.13	6.14 ± 0.09	6.01 ± 0.14	5.93 ± 0.11	0.4600	
<i>OPRK1</i>	Opioid receptor, kappa 1	13791718	6.56 ± 0.11	6.50 ± 0.12	6.82 ± 0.09	6.74 ± 0.09	0.1592	
<i>PDYN</i>	Prodynorphin	13612278	6.38 ± 0.10	6.50 ± 0.13	6.35 ± 0.18	6.74 ± 0.19	0.3140	
<i>TAC1</i>	Tachykinin 1	13735408	7.63 ± 0.19	7.54 ± 0.20	7.65 ± 0.09	7.64 ± 0.34	0.9802	
<i>TACR1</i>	Tachykinin receptor 1	13644855	4.49 ± 0.20	4.62 ± 0.12	4.67 ± 0.23	4.74 ± 0.18	0.8168	
<i>TAC3</i>	Tachykinin 3	13625903	6.40 ± 0.28	6.53 ± 0.21	5.89 ± 0.15	6.01 ± 0.47	0.3154	
<i>TACR3</i>	Tachykinin receptor 3	13757467	4.48 ± 0.10	4.41 ± 0.16	4.17 ± 0.08	4.38 ± 0.32	0.5849	

Affymetrix GeneChip Rhesus 1.0 ST Arrays were used to profile gene expression in the arcuate nucleus. Values represent means ± SEMs of bi-weighted average signal intensity. AM = morning necropsy; PM = evening necropsy; HS = 6 months of T and DHEA hormone supplementation.

* $P < 0.05$, ANOVA with post hoc Bonferroni adjustment, relative to the Old (Control) group.

TABLE 3
qRT-PCR Evaluation of Gene Expression in the Male Rhesus Macaque Arcuate Nucleus

Gene Symbol	Description	GenBank ID	AM		PM		P value
			Old (Control)	Young	Old HS	Old	
Steroid receptors							
<i>AR</i>	Androgen receptor	AF092930	0.87 ± 0.10	1.26 ± 0.05 *	1.15 ± 0.04 *	1.24 ± 0.10 *	0.0052
<i>ESR1</i>	Estrogen receptor 1	S71040	1.16 ± 0.25	0.98 ± 0.18	0.84 ± 0.08	0.84 ± 0.07	0.5705
<i>ESR2</i>	Estrogen receptor 2	NM_001265821	0.86 ± 0.13	1.07 ± 0.09	0.94 ± 0.09	1.06 ± 0.06	0.4096
<i>PGR</i>	Progesterone receptor	XM_001095317	0.89 ± 0.10	1.51 ± 0.08 *	1.09 ± 0.09	1.07 ± 0.25	0.0211
Neuropeptides							
<i>KISS1</i>	Kisspeptin	AY823262	1.21 ± 0.18	1.94 ± 0.37	1.00 ± 0.20	1.83 ± 0.87	0.3570
<i>TAC3</i>	Tachykinin 3	XM_001115535	0.44 ± 0.06	0.66 ± 0.11	0.36 ± 0.07	0.59 ± 0.25	0.3220

GeneChip array data were corroborated by qRT-PCR expression analysis. Values represent means ± SEMs. AM = morning necropsy; PM = evening necropsy; HS = 6 months of T and DHEA hormone supplementation.

* $P < 0.05$, ANOVA with post hoc Bonferroni adjustment, relative to the Old (Control) group.