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Impact of Moderate Calorie Restriction on the Reproductive Neuroendocrine Axis of Male Rhesus Macaques

Brandon D. Sitzmann[1,2], Julie A. Mattison[3], Donald K. Ingram[3,4], George S. Roth[5], Mary Ann Ottinger[2] and Henryk F. Urbanski*[,1,6]

[1]Department of Neuroscience, Oregon National Primate Research Center, Oregon Health and Science University, Beaverton, Oregon 97006; [2]Department of Animal and Avian Sciences, University of Maryland, College Park, Maryland 20742; [3]Laboratory of Experimental Gerontology, National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224; [4]Nutritional Neuroscience and Aging Laboratory, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, Louisiana 70808; [5]GeroScience Inc., Pylesville, Maryland 21132; and [6]Departments of Behavioral Neuroscience, and Physiology and Pharmacology, Oregon Health and Science University, Portland, Oregon 97239, USA

Abstract: The impact of moderate calorie restriction on reproductive neuroendocrine function was investigated in young adult male rhesus macaques (*Macaca mulatta*). The animals were subjected to either 30% calorie restriction (CR; n=5), or were fed a standard control diet (CON; n=5), starting during their peripubertal period. Plasma LH and testosterone concentrations were examined after 7 years of differential dietary treatment, and were found to be similar in both groups, both during the day and during the night. Microarray profiling of pituitary gland and testicular gene expression was performed after 8 years of treatment, using GeneChip® Rhesus Macaque Genome Arrays (Affymetrix), and showed very little effect of caloric restriction. Using a 1.5-fold difference threshold, our microarray analysis revealed differential expression of only 145 probesets in the pituitary gland and 260 in the testes, out of a total of >54,000. Semi-quantitative RT-PCR performed on pituitary gland mRNA corroborated the microarray findings for selected modulated genes, including TSH receptor (*TSHR*) and sperm-specific antigen 2 (*SSFA2*). Most notably, significantly lower expression of TSH receptor mRNA was observed in the pituitary of CR compared to CON animals. Also, significantly lower expression of the glycoprotein hormone alpha subunit (*CGA*) was observed in CR animals, and this finding was further corroborated using quantitative real-time RT-PCR. No significant diet-induced changes were detected in the testis for genes associated with reproduction, circadian clocks, or oxidative stress. There is mounting evidence that CR may promote health and longevity in a wide range of organisms, including nonhuman primates. Importantly, our data suggest that moderate CR has no obvious lasting detrimental effect on the reproductive neuroendocrine axis of long-lived primates, and has only a modest influence on pituitary and testicular gene expression.

Keywords: Calorie restriction, gene expression, HPG, LH, macaque, reproduction, testosterone.

INTRODUCTION

To date, moderate calorie restriction (CR) is the only known non-genetic method of increasing longevity and attenuating many of the biological changes associated with aging [1-4]. Some characteristic markers of CR include reduced body mass and adiposity, lower body temperature, slowed growth, delayed skeletal maturation, and decreased glucose and fasting plasma insulin levels. In addition, CR can affect gene expression and modify neuroendocrine function by lowering levels of growth hormone (GH), thyroid stimulating hormone (TSH), and thyroid hormones [4-8]. Although the exact mechanisms of action explaining the benefits of CR remain elusive, this dietary paradigm has been effective in attenuating aging processes in diverse organisms including yeast, rotifers, nematodes, flies, fish, birds, rodents, dogs, and nonhuman primates [2, 3, 9-12].

CR exerts beneficial effects on age-associated changes occurring at molecular, cellular, and systemic levels that disrupt homeostatic mechanisms leading to progressive loss of function (including reproductive capacity). A few examples of age-related homeostatic imbalance include decreased stress responses, increased pathology, decline in memory function, and documented alterations in circadian organization, such as changes in hormonal rhythms, core body temperature and sleep/wake cycles [13-15]. In males, the age-related reproductive demise occurs with functional deterioration at several sites along the hypothalamic-pituitary-gonadal (HPG) axis, including reduced testosterone (T) production, decreased hypothalamic gonadotropin-releasing hormone (GnRH), and altered pituitary gland release of gonadotropins [16-19].

The full impact of CR on healthy aging in nonhuman primates is still unknown, although studies in rhesus macaques (*Macaca mulatta*; [20]) have paralleled findings in other species and have shown CR-induced attenuation of age-related changes in plasma triglycerides, oxidative damage, and glucose regulation [9, 21, 22]. Animals on CR have

*Address correspondence to this author at the Departments of Behavioral Neuroscience, and Physiology and Pharmacology, Oregon Health and Science University, Portland, Oregon 97239, USA; E-mail: urbanski@ohsu.edu

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a lower body mass, reduced body fat, and lower body temperatures than age-matched controls. These observations confirm the potential benefit of CR for individuals at risk for diabetes and cardiovascular disease [22-24]. However, the impact of CR on reproductive function in primates is even less clear. In male rhesus macaques, as in rats, CR has been shown to delay the pubertal increase in circulating testosterone concentrations and to postpone skeletal growth [5, 22, 24]. Consequently, there is concern that long-term CR could be detrimental to the reproductive neuroendocrine axis of primates. To address this issue, the present study examined if long-term CR would cause a lasting suppression of the HPG axis of male rhesus macaques, when initiated during the peripubertal period. In addition, we examined if CR would significantly influence pituitary and testicular gene expression, especially genes associated with reproduction, circadian mechanisms, metabolism, and oxidative stress.

MATERIALS AND METHODOLOGY

Animals and Diet

A total of 10 male rhesus macaques (*Macaca mulatta*) were assigned to the study, which was approved by the Institutional Animal Care and Use Committees of the University of Maryland and the Oregon National Primate Research Center (ONPRC). The animals were housed in individual cages in a temperature controlled environment (24°C) under a fixed 12 h light:12 h dark photoperiod (lights on from 7:00 to 19:00 h) with *ad libitum* access to drinking water. They were cared for by the ONPRC in accordance with the *Guide for the Care and Use of Laboratory Animals* [25], which included daily health checks to ensure normal behavior, chow consumption, and waste production. Additionally, routine physical examinations, hematological studies, fecal parasite checks, tuberculin testing and dental cleaning were performed periodically.

Starting at ~4 years of age (i.e., the peripubertal period), half of the animals were subjected to a continuous 30% CR diet for ~8 years, as previously described [9, 22, 26]. The other half served as age-matched controls (CON), and were fed approximately *ad libitum*. This level of feeding was originally determined for each individual, and was characterized by a few uneaten biscuits remaining in the animal's cage at the end of each day; calorie-restricted animals received 30% less food than the age- and body weight-matched controls. In both groups, food was provided daily at 8:00 h and 15:00 h, and consisted of specially formulated biscuits (Cargill, Minneapolis, MN, USA) supplemented with daily fresh fruits or vegetables (10 - 40 cal). The composition of the diet was 15% protein, 5% fat and 5% fiber with a caloric content of ~3.7 kcal/g. The biscuits included a vitamin/mineral mix that was 40% higher than the recommended allowance for rhesus macaques by the NRC [27], but were otherwise similar to those used in many laboratory studies of rhesus macaques. This vitamin/mineral supplementation was designed to ensure sufficient availability of essential nutrients to both diet groups. Biochemical assays were performed periodically and with every new shipment to ensure diet content and quality [28, 29]. At the end of the study, the mean body mass of animals in the CR and CON groups was 9.19 and 10.72 kg, respectively.

Measurement of Hormones

Circulating levels of LH and testosterone fluctuate markedly across the day. Therefore, to more accurately determine the impact of CR on the secretion of these two hormones, we collected serial blood samples across the day and night. At ~11 years of age, each animal was surgically fitted with an indwelling subclavian vein catheter connected to a swivel-tether remote blood sampling system, as previously described [26, 30]. Prior to catheterization the animals were allowed at least 2 wk to become accustomed to wearing a protective nylon mesh jacket. Using this system, blood (1 ml) could be sampled remotely, every 30 min over a 24-h period, from an adjacent room without disturbing the animals. The samples were collected into EDTA-coated glass tubes, and after centrifugation at 4°C, the plasma supernatant was stored at -20°C until assay.

Plasma LH was measured using a previously described mouse Leydig cell bioassay that involves RIA for testosterone [31]; the results are expressed in terms of a cynomolgus LH-RP1 standard. Plasma testosterone concentrations were measured by RIA as previously described [32, 33].

RNA Extraction and Gene Microarrays

At ~12 years of age the animals were painlessly euthanized using ketamine sedation followed by sodium pentobarbital overdose, in accordance with the *Guide for the Care and Use of Laboratory Animals* [25]. Subsequently, various postmortem tissues were made available to investigators through the ONPRC Tissue Distribution Program. For the present study, pituitary glands from each animal were bisected sagittally and one half was flash-frozen. Similarly, a portion of one testis from each animal was flash-frozen.

The tissues were homogenized (PowerGen rotor-stator homogenizer; Fisher Scientific, Hampton, NH, USA), and total RNA was isolated (RNeasy Mini Kit; QIAGEN, Valencia, CA, USA). Concentration and integrity of RNA in the final samples were assessed by microcapillary electrophoresis (Agilent Bioanalyzer Model 2100; Agilent Technologies, Santa Clara, CA, USA). Three samples from both treatment groups were processed by microarray analysis by the Affymetrix Microarray Core of the Oregon Health & Science University Gene Microarray Shared Resource. Analysis was performed in accordance with the manufacturer's instructions. Briefly, labeled target cRNA was prepared from total RNA samples and hybridized to GeneChip® Rhesus Macaque Genome Arrays (GeneChip® Analysis Technical Manual; Affymetrix, Santa Clara, CA, USA). Quality control metrics included measures of chip background, chip noise, total fluorescence intensity, genes detected and 3':5' ratio of the housekeeping genes β-actin and GAPDH as measures of probe quality. Image processing and expression analysis were performed using Affymetrix GCOS v1.2 software.

Analysis of Gene Microarray Data

Microarray data filtering was performed using Genesifter™ (Geospiza, Inc., Seattle, WA, USA). Briefly, unscaled array data were normalized and log2 transformed using robust multi-array average (RMA) analysis. Student's *t*-test ($P<0.05$) comparisons with Benjamini-Hochberg post-hoc corrections were performed. Signal quality and fold-change parameters were not set in Genesifter™ in order to

allow for qualitative and quantitative filtering later. Probesets showing significant (P<0.05) change between treatments were then imported into Excel (Microsoft, Redmond, WA, USA) and filtered for a minimum 1.5 fold-change in expression. Genes related to metabolic function or the neuroendocrine axis which met these criteria were then selected for further consideration. Additionally, due to the conservative nature of our filter criteria and the small number of resultant probesets, a keyword search of the entire 52,000+ probesets was also performed to identify similarly expressed probesets that were highly expressed and/or were known to be closely associated with reproduction, circadian biology, or oxidative stress.

Semi-Quantitative RT-PCR

PrimerExpress® software (Applied Biosystems, Carlsbad, CA, USA) was used for all primer and probe design (Table **1**). Specific primers were designed for each transcript using the predicted rhesus macaque mRNA sequences (National Center for Biotechnology Information, Entrez Nucleotide database) and were purchased from Invitrogen (Carlsbad, CA, USA).

Total RNA (1 µg) was used to synthesize cDNA using the Omniscript kit (QIAGEN) and oligo d(T)15 primers (Promega Corp., Madison, WI, USA) in 20 µl at 37°C for 1 h. Semi-quantitative RT-PCR amplifications were performed in duplicate using 1 µl cDNA, 200 µM deoxynucleotide triphosphates (Promega), 0.5 µM of each primer (Invitrogen), and 2.5 U of HotStarTaq® polymerase (QIAGEN) in 25 µl with the following thermocycle profile: 95°C, 15 min; 94°C, 1 min; specific cycle number and annealing temperature for each primer pair, 1 min; and 72°C, 1 min. Resulting PCR products were resolved by electrophoresis on 2% agarose gels with ethidium bromide and photographed under ultraviolet light. Subsequent bands were analyzed with NIH Image-J software 1.37v (http://rsb.info.nih.gov/nih-image). A single rectangle was drawn horizontally around all bands in a selected gel image and a plot profile of signal intensities was generated. Area selections were created under the peak for each band using the 'straight lines selection' tool; area under the curves was used for statistical comparisons.

Quantitative Real-Time RT-PCR

cDNA was prepared by random-primed reverse transcription using random hexamer primers (Promega), 200 ng RNA and the Omniscript kit (QIAGEN); reactions were diluted 1:100 for subsequent PCR analysis. PCR mixtures contained 5 µl Taqman® Universal PCR Master Mix (Applied Biosystems), 300 nM CGA primers (Invitrogen), 50 nM human β-

Table 1. Nucleotide Sequences for Rhesus Macaque Gene Primers and Probes, Used for sqRT-PCR and qRT-PCR Along with Expected Amplicon Size, Optimal Number of Cycles for Each Reaction, and Annealing Temperature

sqRT-PCR Primers	Sequence	Size of PCR Product (bp)	PCR Cycles	Temp (°C)
5'CGA	5′-GGAGAGTTTACAATGCAGGATTGC-3′	310	22	62
3'CGA	5′-GTCATCAAAACAGCACTTGGCA-3′			
5'TSHR	5′-CTTCTTTTCTGAAACTGCCAGCTC-3′	302	29	62
3'TSHR	5′-GAGATTCCAGGTGAGTCCAGCA-3′			
5'AGPAT	5′-GCTGACTGTTGGCCAGTTTCA-3′	316	27	62
3'AGPAT	5′-CCTGCAGCATTTCACCAGTACA-3′			
5'UCP2	5′-TGCAAAGCCCTGCTGTTCA-3′	301	26	63
3'UCP2	5′-AGATAGAGGAACTCTGCCGGAATC-3′			
5'SSFA2	5′-GGAAGAAAGTATTCCGAGCATCGG-3′	307	26	65.5
3'SSFA2	5′-ACACCTCCACCAGTGCATATCATTG-3′			
5'CsnK1ε	5′-AAGTATGAGCGGATCAGCGAGA-3′	218	28	65
3'CsnK1ε	5′-CCGAATTTCAGCATGTTCCAGT-3′			
5'COX2	5′-AGAGGCTAGTGCCTCAGAGAG-3′	437	N/A	N/A
3'COX2	5′-GCTAGCACACAGGCCTATCC-3′			
5'β-actin	5′-CATTGCTCCTCCTGAGCGCAAG-3′	~300	23	65
3'β-actin	5′-GGGCCGGACTCGTCATACTCC-3′			
qRT-PCR Primers/Probe				
5'CGA	5′-TGGAGAGTTTACAATGCAGGATTG-3′	101	40	60
3'CGA	5′-AGCAGCAGCCCATACACTGA-3′			
5'CGA	6FAM-AATTCTTCTCCAAGCCGGGTGCCC-TAMRA	N/A	40	60

actin primers (Applied Biosystems), 250 nM CGA probe (IDT, Coralville, IA, USA) and 2 µl cDNA. Reactions were run in triplicate in an ABI/Prism 7700 Sequences Detector System (Applied Biosystems) with the following cycle parameters: 2 min at 50°C, 10 min at 95°C, and then 40 cycles each at 95°C for 15 s and 60°C for 60 s. Human ACTB (β-actin) Endogenous Control (Applied Biosystems) was used to generate a standard curve and convert the critical threshold values (i.e. above background) into relative RNA concentrations for each sample, thus compensating for any differences in reverse transcription efficiency. CGA primers and probe were designed using the predicted rhesus macaque sequence available in the NCBI Entrez Nucleotide database (Table **1**).

Amplicon Sequencing

PCR products were purified (QIAquick Gel Extraction Kit; QIAGEN), and DNA sequencing was performed on an ABI 3130XL Genetic Analyzer using dye terminator sequencing chemistry (Applied Biosystems). Resulting sequences were then BLASTed in the NCBI database (www.ncbi.nlm.nih.gov) to verify primer specificity and proper amplicon production.

Statistical Methods

Overall mean plasma LH and testosterone concentrations were calculated for the CR and CON groups. In addition, mean plasma LH and testosterone concentrations were also calculated separately for samples collected during the day (7:00 – 19:00 h) and night (19:00 – 7:00 h). Between-treatment comparisons and night-day comparisons were both analyzed using Student's *t*-test.

Between-treatment differences in mRNA expression levels (signal intensity) were analyzed by Student's *t*-test. To control for experiment-wide false positives, while still maintaining statistical power, alpha was adjusted to correct for multiple comparisons using sequential Bonferroni with Simes-Hochberg correction [34]. For all analyses, a significant difference was $P<0.05$ unless otherwise adjusted by Bonferroni correction.

RESULTS

Hormone Analysis

Mean (±SEM) plasma LH and T levels for CON and CR animals are shown in Fig. (**1**). In both treatment groups LH levels were generally higher at night (19:00 - 7:00 h) than in the day (7:00 - 19:00 h), but this diurnal difference was not statistically different ($P>0.05$). Likewise, no significant differences were detected between the two treatment groups with regard to daytime, nighttime, or overall mean plasma LH levels ($P>0.05$).

Plasma testosterone levels were significantly higher at night than in the day, both in the CON ($P<0.05$) and CR ($P<0.01$) groups. As with LH, however, no significant differences were detected between the two treatment groups with regards to daytime, nighttime, or overall mean plasma T levels ($P>0.05$).

Gene Microarray and Filtering

Initial Genesifter™ sorting indicated ~26,000 pituitary probesets were upregulated in CR animals compared to CON

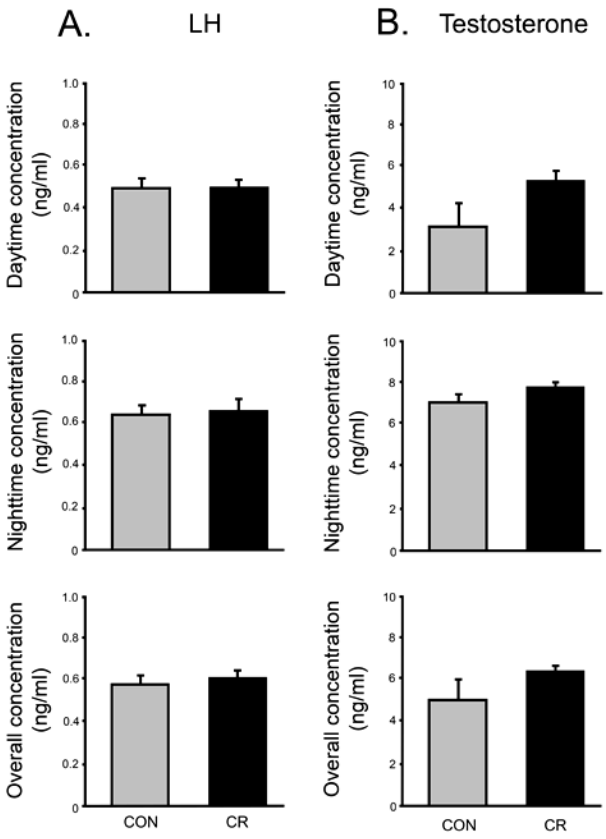

Fig. (1). Effect of calorie restriction on plasma LH (**A**) and testosterone (**B**) concentrations in the male rhesus macaque. Each bar represents the mean plasma hormone level during the daytime (upper panels), nighttime (middle panels) or overall 24-hour period (lower panels); the SEM are shown as vertical lines. For the control (CON, n=5) and calorie-restricted (CR, n=5) groups, mean plasma testosterone concentrations were significantly higher at nighttime than daytime ($P<0.05$ and $P<0.01$, respectively; Student's *t*-test), but mean plasma LH levels showed no significant diurnal difference ($P>0.05$). No significant effect of calorie restriction was observed for any of the endocrine parameters examined ($P>0.05$).

while ~26,700 probesets were downregulated. Based on our filter criteria (*t*-test; $P<0.05$), 1,018 of these probesets were significantly different between the two dietary treatments. When a 1.5 fold-change requirement was applied, 145 differentially expressed probesets were detected (Supplemental Excel File, pituitary worksheet). Filtering of testicular data indicated ~21,400 probesets were upregulated in CR animals compared to CON while ~31,400 probesets were downregulated. Of these probesets, 1,452 were significantly different between the two treatments. When a 1.5 fold-change requirement was again applied, 260 differentially expressed probesets were detected (Supplemental Excel File, testes worksheet).

Three genes corresponding to these probesets were chosen for further investigation: TSH receptor (*TSHR*); 1-acylglycerol-3-phosphate O-acyltransferase 3 (*AGPAT3*), a component of the triacylglycerol synthetic pathway; and sperm-specific antigen 2 (*SSFA2*), a surface antigen respon-

Table 2. Effect of Calorie Restriction on Gene Expression in the Pituitary Glands and Testis of Young Adult Male Rhesus Macaques, as Determined by GeneChip® Analysis (Rhesus Macaque Genome Array)

		Pituitary		Testis	
Probeset	**Gene ID**	**CON**	**CR**	**CON**	**CR**
MmugDNA.14589.1.S1_at	*AGPAT*	27 + 5	17 + 3	315 + 14	319 + 16
MmugDNA.1459.1.S1_s_at		443 + 54	412 + 36	205 + 26	182 + 21
MmugDNA.16471.1.S1_at		104 + 11	79 + 13	508 + 34	625 + 31
MmugDNA.24842.1.S1_at		263 + 17	195 + 27	192 + 18	93 + 18
MmugDNA.24844.1.S1_at		455 + 36	559 + 40	214 + 44	189 + 12
MmugDNA.39097.1.S1_at		0596 + 34*	0450 + 34*	0439 + 47*	0285 + 25*
MmugDNA.6884.1.S1_at		326 + 16	352 + 26	275 + 37	253 + 19
MmugDNA.13477.1.S1_at	*TSHR*	14 + 6	12 + 2	15 + 4	12 + 2
MmugDNA.32655.1.S1_at		042 + 7*	018 + 3*	36 + 8	46 + 5
MmugDNA.796.1.S1_at		14 + 3	11 + 7	12 + 4	4 + 1
MmugDNA.19270.1.S1_at	*SSFA2*	22 + 1	24 + 6	09 + 4	7 + 3
MmugDNA.26522.1.S1_at		337 + 16	212 + 83	0170 + 22*	056 + 5*
MmugDNA.14233.1.S1_at		969 + 43	01049 + 47	579 + 34	0434 + 16
MmugDNA.4648.1.S1_at	*CGA*	0639 + 154	154 + 76	01 + 1	5 + 3
MmugDNA.29263.1.S1_at		13899 + 270	13894 + 1158	600 + 11	0641 + 9
MmugDNA.16174.1.S1_at	*UCP*	553 + 70	568 + 39	243 + 28	340 + 27
MmugDNA.1485.1.S1_at		428 + 72	436 + 35	219 + 28	232 + 25
MmugDNA.4494.1.S1_at	*COX2*	17 + 7	20 + 1	11 + 3	17 + 10
MmugDNA.17389.1.S1_at	*CsnK1ε*	21 + 4	24 + 1	593 + 67	531 + 88
MmugDNA.29650.1.S1_at		36 + 5	46 + 18	53 + 9	55 + 6
MmugDNA.34870.1.S1_at		274 + 31	248 + 36	98 + 11	103 + 13
MmugDNA.4033.1.S1_at		66 + 7	59 + 6	46 + 10	35 + 4
MmuSTS.1889.1.S1_at		242 + 14	234 + 14	79 + 20	76 + 3

Values represent mean probeset signal intensity values (±SEM). CON = control (n = 3), CR = calorie-restricted (n = 3). *P<0.05.

sible for zona pellucida binding. The Supplemental Excel File has been provided for the benefit of investigators with other research interests who may be more familiar with some of the remaining probesets and their functions.

Four other genes not meeting our filter conditions, but which are closely associated with reproductive, circadian, and oxidative stress pathways potentially affected by CR, were also investigated. These included glycoprotein hormone common alpha subunit (*CGA*); uncoupling protein 2 (*UCP2*); casein kinase 1 epsilon (*CsnK1ε*), a core-clock gene; and cyclooxygenase 2 (*COX2*), a cellular stress marker. Table **2** lists the probesets and the statistical comparison of gene expression in CON and CR animals based on microarray results. These seven pituitary gland and testes genes were then subjected to sqRT-PCR and qRT-PCR analyses.

sqRT-PCR and qRT-PCR

By including all 10 animals in the subsequent sqRT-PCR analysis, instead of just 3 per group as in the microarray analysis, we hoped to more accurately disclose differentially expressed genes. Gel images for the six selected genes are depicted in Fig. (**2**). Amplicons were detected between 22 – 29 cycles at 62 – 65.5°C, as shown in Table **1**.

The sqRT-PCR expression levels for the genes within the pituitary glands of the two treatment groups are shown in Fig. (**3A**). In agreement with the gene microarray data (Table **2**), glycoprotein hormone alpha subunit (CGA) mRNA expression was 25% lower (P<0.05) in CR compared to CON males, and TSH receptor mRNA expression decreased 45% (P<0.05) in CR males. Other genes associated with oxidative stress, circadian mechanisms, and reproduction showed no significant diet-induced changes (P>0.05). These included *AGPAT3*, *UCP2*, *SSFA2*, and *CsnK1ε*. Expression of these same genes in the testes showed no significant diet-induced changes (Fig. **3B**). This is in contrast to the filtered microarray data which disclosed a significant difference in AGPAT3 and SSFA2 mRNA expression between CON and CR groups (Table **2**). Consistent with the microarray data, COX2

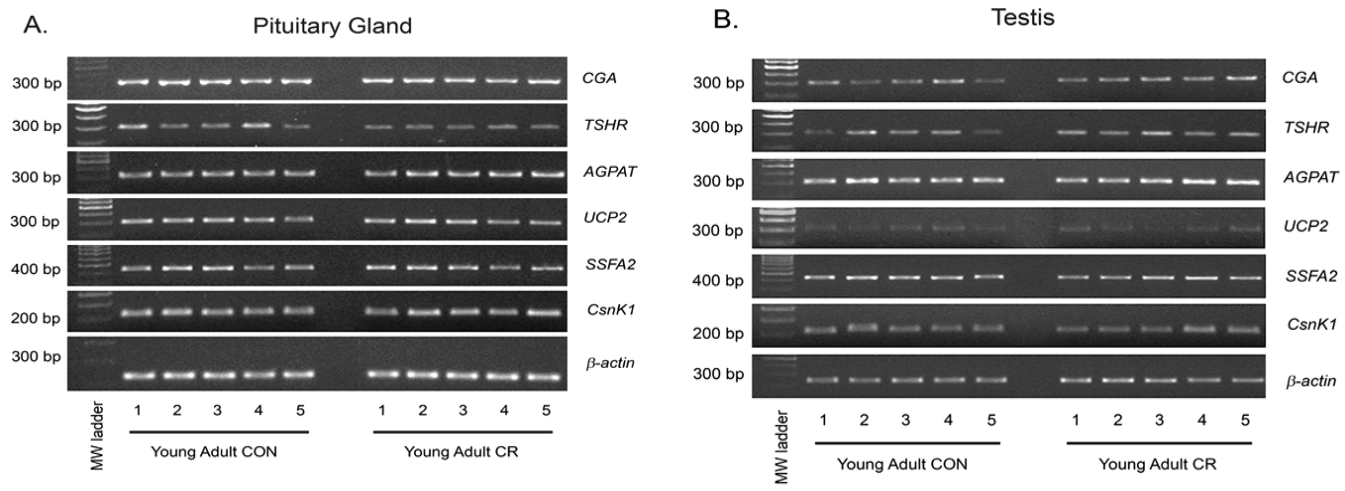

Fig. (2). Effect of calorie restriction on selected gene expression in the rhesus macaque pituitary gland (**A**) and testis (**B**). sqRT-PCR gel images from five control and five calorie-restricted animals is depicted. The housekeeping gene *β*-actin was used as a positive control and for normalizing images for analysis.

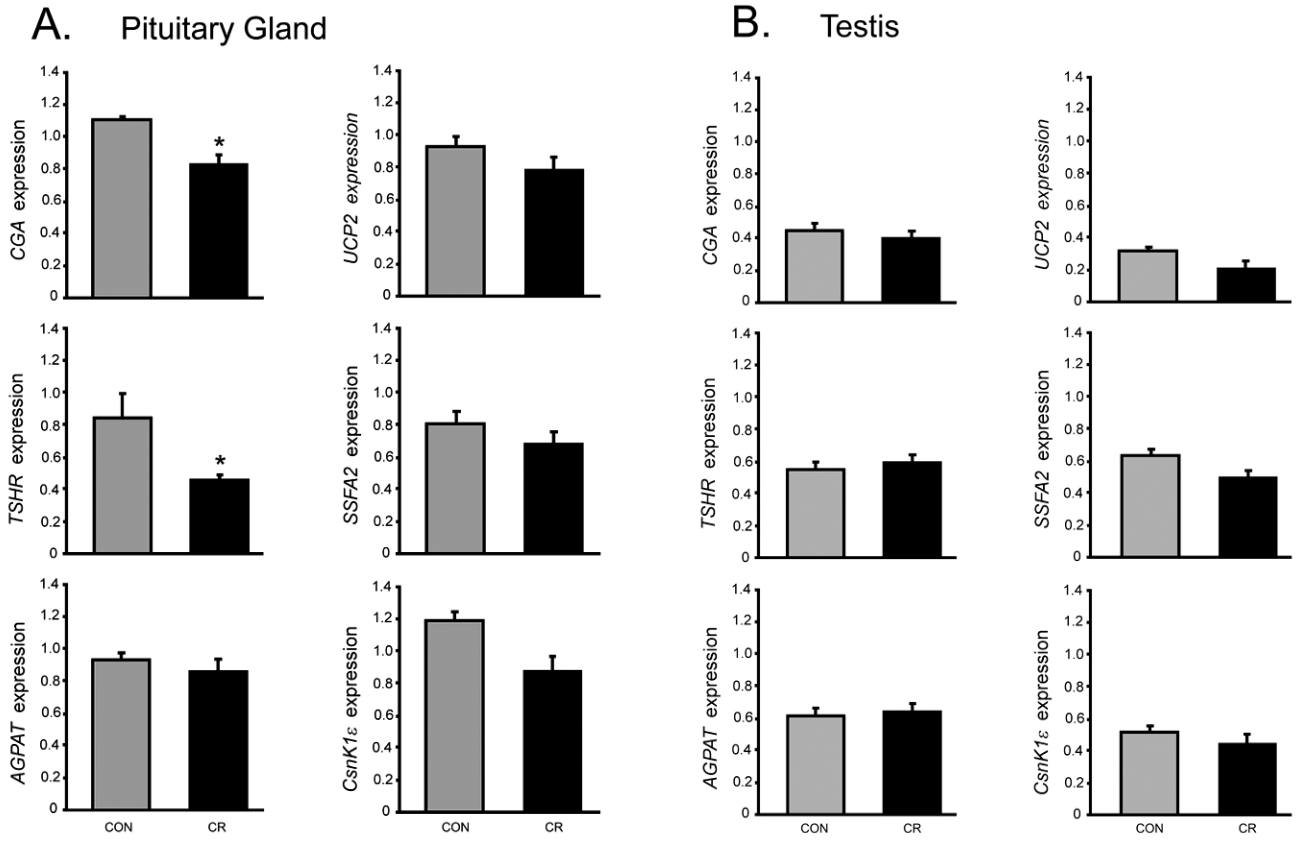

Fig. (3). Analysis of gene expression in the rhesus macaque pituitary gland (**A**) and testis (**B**), using sqRT-PCR. Each bar, along with SEM, represents mean, normalized fluorescence data (in relative units) from five animals, as depicted in Fig. (**2**). Significant differences were observed in *CGA* expression and *TSHR* expression between treatment groups. In both instances, calorie-restricted (CR) animals demonstrated a negative fold-change in mRNA expression of 0.75 (± 0.06) and 0.55 (± 0.04), respectively, relative to the controls (CON). The other transcripts were not significantly different between treatments. None of the selected transcripts showed differential expression in the testis. *, $P<0.05$ (Student's *t*-test).

mRNA expression was undetectable in any of the samples (data not shown), regardless of tissue type.

In agreement with sqRT-PCR results, quantitative real-time RT-PCR measurements of *CGA* showed CR animals had a significant (P<0.01) negative fold-change of 0.57 (±0.10) in pituitary expression (Fig. **4**). Quantitative RT-PCR calculations were based on a sample size of four in both treatments as compared to five animals for sqRT-PCR. During analysis one animal from each category was removed as an outlier because their average detection signal was more than two standard deviations away from the group mean.

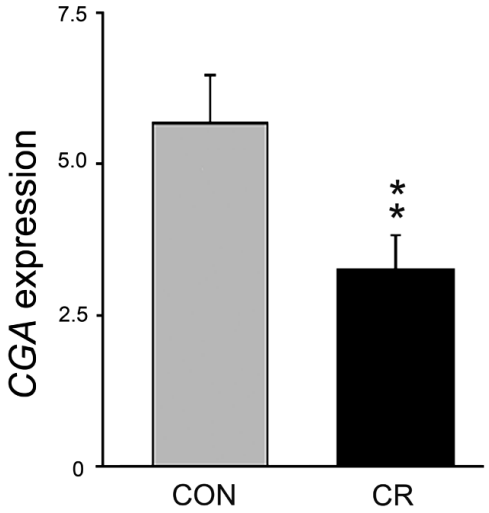

Fig. (4). Quantitative real-time PCR analysis of *CGA* in the rhesus macaque pituitary gland. Each bar, along with SEM, represents mean, normalized fluorescence data (in relative units) for four animals. The result corroborates sqRT-PCR findings from Figs. (**2** and **3**), by demonstrating a significant negative fold-change of 0.57 (± 0.10) in pituitary gland *CGA* expression in the calorie-restricted (CR) animals relative to the controls (CON). **, P<0.01 (Student's *t*-test).

DNA Sequencing

Amplicon sequences were BLASTed in the NCBI database to determine the sequence of best fit. In each instance the experimentally-derived sequence returned a best fit for at least one of the primer pairs for the predicted rhesus macaque mRNA sequence, which had been used for primer design (data not shown).

DISCUSSION AND CONCLUSION

A growing body of evidence from rhesus macaque studies suggests that moderate CR may delay the onset of age-related pathologies even in long-lived species [12, 19, 22]. On the other hand, the mechanisms underlying CR's health-promoting properties are poorly understood. Moreover, it is unclear if there are any long-term negative consequences of being subjected to a 30% reduction in caloric intake. Because food restriction is known to exert a significant delay on the onset of puberty in many species, including primates, there is concern that CR may be detrimental to the function of the reproductive neuroendocrine axis. To shed light on both of these issues, we subjected young male macaques to a 30% CR paradigm, starting at ~4 years of age (i.e., during the peripubertal phase of development) [35]. After ~7 years of this dietary manipulation, plasma LH and testosterone concentrations were examined across the day and night and were found to be identical in the CR and CON animals, both in terms of overall mean and in terms of daytime-nighttime hormone profiles. This suggests that CR is unlikely to cause any lasting negative influence on the activity of the hypothalamic-pituitary-gonadal (HPG) axis of long-lived male primates. Moreover, because circulating testosterone levels appeared to be normal, it is unlikely that any beneficial effects of CR on primate physiology are mediated by alterations in sex-steroid output. Overall, our hormonal data are in agreement with those previously reported in young male rats, which initially showed slight impairment of fertility with CR, but then showed a return to normalcy as they became older [2]. In addition, our data complement those previously obtained from 7-27-year-old female rhesus macaques, which showed no adverse effects of long-term CR on reproductive hormones [22, 28]. Similarly, ovarian cyclicity remained uninterrupted, suggesting that long-term energy restriction does not negatively affect the animals [22, 36]. Subsequent studies in these same females showed that they continued to cycle normally under moderate calorie restriction [37].

After ~8 years of CR, gene expression in the pituitary gland and testes showed very little difference between the CR and CON animals in our study. Out of >54,000 transcripts, only 145 (<0.3%) appeared to be differentially expressed in the pituitary gland and 260 (<0.5%) in the testes (Supplemental Excel File). These results suggest, in male rhesus macaques at least, that moderate CR has an extremely limited impact on gene expression in these two HPG axis tissues. However, because the necropsies were performed exclusively in the daytime, within a few hours of each other, we cannot exclude the possibility that some gene expression changes may have escaped detection because they occurred only at night. We have previously shown that >10% of the rhesus macaque adrenal gland transcriptome has a 24-hour expression pattern [38], and so a similar 24-hour transcriptome expression pattern in the pituitary gland and testis cannot be ruled out. Nevertheless, our data emphasize that by far the majority of pituitary and testicular genes were equally expressed regardless of dietary manipulation. For our gene array data analysis, we adopted the robust multi-array average (RMA) method [39] and used stringent filter criteria (*t*-test; P<0.05), which included a 1.5 fold-change threshold. It is possible that adoption of less stringent criteria might have disclosed more differentially expressed genes, and a less conservative conclusion.

The subsequent sqRT-PCR study served to corroborate some of the key gene microarray findings and to validate use of the Rhesus Macaque GeneChip®. The genes that we selected for analysis were highly expressed and/or related to neuroendocrine function, metabolism, circadian biology, or oxidative stress. These included three differentially-expressed genes: 1) thyroid-stimulating hormone receptor (*TSHR*), 2) 1-acylglycerol-3-phosphate O-acyltransferase 3 (*AGPAT3*), 3) sperm-specific antigen 2 (*SSFA2*), a surface antigen responsible for zona pellucida binding; and also three similarly-expressed genes: 1) glycoprotein hormone common alpha subunit (*CGA*), 2) uncoupling protein 2 (*UCP2*), 3) casein kinase 1 epsilon (*CsnK1ε*), a core-clock gene. *AGPAT3*, a component of the triacylglycerol (triglyc-

eride) biosynthesis pathway, was chosen because CR has been shown to lower triglyceride levels [21]. Although expected in the testis, the function of SSFA2 in pituitary gland tissue is unclear, and it is intriguing that the microarray data showed high expression levels in our samples. Uncoupling proteins such as UCP2 are known to be expressed in the rhesus pituitary gland [40] and are thought to regulate the efficacy of oxidation and suppress the generation of reactive oxygen species; thus, they may play a critical role in protecting cells against degeneration that stems from aging or pathological conditions. Casein kinase 1 epsilon, is a core component of the circadian clock mechanism, and has previously been detected in peripheral organs of the rhesus macaque, including the adrenal and pituitary glands [38, 41]. Cyclooxygenase 2 (*COX2*), which showed no significant expression on the microarray data, was also included as a negative control measure of cellular stress in the animals.

From the perspective of neuroendocrine function, the most interesting sqRT-PCR finding was differential expression of CGA and TSH receptor mRNA in the pituitary gland. Although the microarray analysis showed *CGA* to be highly expressed, with a large fold-change difference, this change was not statistically significant, most likely due to large intra-group variation. Nevertheless, in the subsequent RT-PCR analysis, which was performed using 5 animals instead of 3 per group, 25% lower *CGA* expression was detected in the CR compared to CON animals. This finding was further corroborated using real-time RT-PCR results, which showed an even lower (43%) level of *CGA* gene expression in the CR animals. Taken together, these findings raise the possibility that our microarray analysis might have disclosed a few more differentially expressed genes had we included larger group sizes (i.e. 5 animals per group instead of 3). On the other hand, the other genes examined using sqRT-PCR yielded essentially similar results to those from the microarray, even though the group sizes were larger in the former. For example, TSH receptor expression decreased in CR males compared to CON with a 45% decline. These sqRT-PCR data were consistent with the microarray probeset values, which also indicated a significant decline in expression in the CR-treated animals. The biological relevance of the observed alterations in pituitary *CGA* expression is unclear, due to the lack of available β-subunit information. Preliminary attempts were made to identify expression levels for the specific beta subunits for each of the glycoprotein hormones, *LH*, *FSH*, *CG*, and *TSH*, but we were unsuccessful in our attempts to design sqRT-PCR primers able to amplify these sequences. Unfortunately, without further analysis of the unique beta subunits, it is impossible to determine if the change in *CGA* is related to FSH/LH levels in the reproductive axis or, alternatively, to TSH levels in the metabolic pathway. The lack of an obvious change in plasma LH concentrations, combined with the observed changes in *TSHR* expression, would seem to indicate, however, that differential expression of *CGA* is most likely associated with changes in metabolic function rather than changes within the reproductive neuroendocrine axis.

Several hypotheses related to the mechanisms of the biological effects of CR focus on reduced (or more efficient) processing of energy [7, 22, 42]. Two manifestations of CR-induced metabolic adjustments that have been well documented in rodents, non-human primates, and humans are lower core-body temperature [2, 22, 24, 43, 44] and suppression of the thyroid axis as measured by lower TSH and thyroid hormone levels [4, 7-8, 45, 46]. Because thyroid hormones help regulate energy metabolism and body temperature, any modification of upstream components of their signaling pathway could influence their actions. These changes would be consistent with the observed reduction in pituitary CGA and TSHR mRNA expression in our CR males, again suggesting a CR-related modification of metabolic pathways rather than reproductive neuroendocrine pathways.

TSHR may also be involved at the pituitary gland level in humans, where the receptors are found on folliculo-stellate cells, which are known to influence neighboring endocrine cells. Here an ultra-short-loop pituitary gland feedback system may be involved in the pulsatile generation of pituitary gland hormone secretion and fine-tuning of their release by attenuating the oscillation in their serum levels [47]. Again, the changes in *CGA* and *TSHR* expression measured in our present study may be a result of such feedback regulation.

Other genes that showed no expression changes in the pituitary, based on sqRT-PCR results, included 1-acylglycerol-3-phosphate O-acyltransferase 3, uncoupling protein 2, sperm-specific antigen 2, and casein kinase 1 epsilon (Fig. **3**). Although at least one *AGPAT3* probeset had been detected as significantly changing in both the pituitary gland and testis based on our microarray filtering parameters, there were conflicting expression changes in these probesets with some showing decreased expression in CR animals while others showed increased mRNA expression. Because our sqRT-PCR was unable to detect significant differences between groups, it is difficult to say with any certainty what is occurring with regards to expression of this gene.

It is generally accepted that moderate CR slows the accrual of oxidative stress markers which appear with aging. Data further suggests that a decrease in the buildup of oxidative damage is the most probable mechanism for CR's attenuation of the aging process rather than an up-regulation of scavenging and repair enzymes, such as *UCP2* [8, 48]. Providing support for this theory, we report no demonstrably significant changes to *UCP2* in the pituitary gland or testes. Alternatively, our findings could indicate that our study animals were simply too young to be impacted by significant levels of oxidative stress. Such an argument is reinforced by the absence of *COX2* which was undetectable in either tissue type for both groups. Additional age and CR microarray studies which report effects on uncoupling protein mRNA expression are lacking, however [48]. Furthermore, since heterogeneity exists within and between tissue types with regard to oxidative stress response, further studies are needed to elucidate CR's effect on the rate at which different tissue and cell types accumulate oxidative damage with age [49].

In marked contrast to the pituitary gland, sqRT-PCR revealed no differential expression in the testis of any of the selected genes. This would suggest that CR has minimal lasting impact on the testicular component of the HPG axis. Biologically this makes sense; full reactivation of spermatogenesis is very slow and so it would be very inefficient to switch spermatogenesis on and off in response to moderate fluctuations in caloric availability. Further, transcriptome

stability in the pituitary gland and testes is not necessarily unexpected as it would be disconcerting for components of a primary axis, such as the HPG, to fluctuate too broadly under anything less than pathological conditions. With CR, however, there may be a few key components that are carefully modified in such a way as to affect multiple physiological responses simultaneously.

Finally, even though our microarray data revealed very few CR-induced gene expression changes in the rhesus macaque pituitary gland and no changes in testicular expression, there are some caveats. Without further analysis it is unknown whether or not morphological changes may be occurring. Even though both organs may appear to be functioning normally under calorie-restricted conditions, there may be changes occurring in cell location, number, and size. Both organs are composed of multiple regions and cell types, which lie in very close apposition, often invaginating or migrating into one another. Therefore, gross dissection of tissue and region-specific gene expression analysis may result in erroneous conclusions. We chose to use all regions of the pituitary gland and testes for our microarray analysis, even though this may have led to some loss of sensitivity due to normalization of the intensity of each probeset to an average chip intensity value. Also, it is possible that some genes oscillate in one cell type, but not in others, or the same genes may have different phases of expression in different cell types, resulting in a net cancellation of gene expression detection. As a consequence, it is possible that in our analysis some genes fell below the detection threshold for expression.

Ultimately, appreciating the dynamic processes by which CR retards aging will involve whole organism analyses from the molecular to behavioral levels. As greater understanding is gained to the exact mechanisms underlying CR's beneficial properties, it may eventually be possibly to deploy CR mimetics to improve human health [50-52]. Mimetics, agents or strategies that can mimic the beneficial health-promoting effects of CR, would be a critical step in implementing the model as a functional anti-aging intervention within average human populations where longer-term CR may be extremely difficult to employ. Such interventions would only be acceptable, however, if potential side effects were limited, particularly with regard to reproductive function.

The present study is among the first to address the potential impact of moderate CR on the HPG axis of male rhesus macaque, both at the endocrine and gene expression level. Overall, the data suggest that moderate CR has no obvious lasting negative impact on the reproductive neuroendocrine axis and has only a modest influence on pituitary and testicular gene expression. Previously-reported advantageous health benefits of CR [12, 19, 21] and eventual mimetic exploitation may then be achievable without causing significant long-term impairment of the HPG axis. It remains to be elucidated how the observed changes in pituitary *TSHR* and *CGA* expression levels are manifested physiologically under a 30% CR nutritional paradigm, but most likely they are related to altered metabolic function rather than any type of reproductive neuroendocrine function.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

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