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Effects of age on clock gene expression in the rhesus macaque pituitary gland

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Abstract

Recent studies have shown that circadian clock genes are expressed in various peripheral tissues, raising the possibility that multiple clocks regulate circadian physiology. To study clock gene expression in the rhesus macaque pituitary gland we used gene microarray data and found that the pituitary glands of young and old adult males express several components of the circadian clock (*Per1*, *Per2*, *Cry1*, *Bmal1*, *Clock*, *Rev-erba* and *Csnk1ε*). Semi-quantitative reverse-transcription polymerase chain reaction (sqRT-PCR) confirmed the presence of these core-clock genes and detected significant age-related differences in expression of *Per2*. sqRT-PCR also showed differential expression of core-clock genes at two opposing time-points over the 24 hour day, with greater expression of *Per2* and *Bmal1* ($P < 0.05$) at 1300 h as compared to 0100 h. Immunohistochemistry revealed rhythmic expression of REV-ERBα in the pituitary glands of female macaques. These data provide evidence that the rhesus macaque pituitary gland expresses core-clock genes and their associated protein products in a 24-hour rhythmic pattern, and that their expression is moderately impacted by aging processes.

Keywords

circadian; age; pituitary; rhesus macaque

1. Introduction

Mammals rely on endogenous circadian pacemakers to regulate a wide range of physiological processes including metabolism, stress response, thermoregulation, and reproduction (Takahashi, *et al.* 2001). Although the underlying circadian mechanism is still unclear it is well established that the hypothalamic suprachiasmatic nucleus (SCN) acts as a master circadian pacemaker to synchronize, coordinate and sustain circadian physiology and function (Refinetti 2005; Takahashi *et al.* 2001). The SCN oscillator is itself synchronized or 'entrained' by afferent sensory inputs such as temperature changes, food intake or light-dark cycles (Glossop and Hardin 2002). These signals then drive overt circadian rhythms throughout the body via

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the SCN's own efferent connections and neuroendocrine humoral outputs (Lowrey and Takahashi 2004; Morse and Sassone-Corsi 2002; Stehle, *et al.* 2003).

The molecular clock mechanism of the mammalian SCN consists of highly conserved core-clock genes (Lowrey and Takahashi 2004). As such, interconnected cyclic transcriptional/translational feedback loops autoregulate the expression of both positive and negative oscillator components and their respective output proteins. The positive transcriptional activators are CLOCK and BMAL1, which bind to E-box *cis*-regulatory enhancer sequences in the promoter regions of genes, including those of the negative feedback loop. Period proteins (PER1, PER2) and Cryptochrome proteins (CRY1, CRY2) disrupt activity of the bound CLOCK/BMAL1 transcriptional complex and subsequently generate a circadian rhythm in their own transcription. These positive and negative feedback loops communicate via another core-clock gene product, REV-ERB α . This gene codes for an orphan nuclear receptor, and like the PER and CRY genes, is activated by CLOCK/BMAL1 heterodimers binding to E-box enhancers. REV-ERB α protein inhibits BMAL1 gene transcription by functionally competing with retinoic acid-related orphan receptors at the retinoic acid-related orphan receptor response elements in the promoter region of BMAL1. Thus, BMAL1 activation of REV-ERB α attenuates its own transcription. Finally, phosphorylation of PER proteins (especially PER2) by casein kinase 1 epsilon (CSNK1E) activates their degradation and is the rate-limiting step in their accumulation. Only after there is adequate PER present in the cytoplasm to overwhelm CSNK1E-mediated degradation, does enough PER persist to dimerize with CRY. Casein kinase then phosphorylates the dimer to allow for nuclear translocation and disruption of the positive CLOCK/BMAL1 complex. These inherent transcription, translation, and posttranslational modifications give the clock its own natural rhythmicity approximately equal to one terrestrial day (Glossop and Hardin 2002; Guillaumond, *et al.* 2005; Lowrey and Takahashi 2004; Okamura, *et al.* 2002; Reppert and Weaver 2002).

Genetic components of this clock mechanism are also expressed in various peripheral tissues, raising the possibility that circadian physiology is ultimately regulated by a coordinated network of oscillators rather than by the single master circadian clock of the SCN (Balsalobre 2002; Balsalobre, *et al.* 1998; Glossop and Hardin 2002). Gene microarray, RT-PCR, *in situ* hybridization and immunohistochemistry experiments in a number of animal models have shown that circadian transcriptional mechanisms temporally regulate biochemical pathways in various tissues throughout the body, including the hypothalamus, pineal gland, liver, heart, kidney and adrenal gland (Balsalobre 2002; Chappell, *et al.* 2003; Jilg, *et al.* 2005; Lemos, *et al.* 2006; Morse and Sassone-Corsi 2002; Stehle *et al.* 2003; von Gall, *et al.* 2002). These intrinsic peripheral clocks have been shown to regulate the circadian expression of specific sets of genes.

Because multiple-oscillator circadian mechanisms are likely to play a role in regulating human physiology, and may contribute to the etiology of various age-related pathologies, our goal was to investigate circadian clock mechanisms in a peripheral endocrine organ of a primate species. Specifically, our aims were: 1) to examine whether the pituitary gland of the rhesus macaque expresses core-clock genes and their protein products, 2) to determine if core-clock gene expression exhibits a 24-hour rhythmic pattern, and 3) to disclose any age-related changes.

2. Materials and methods

2.1 Animals and diet

Rhesus macaque (*Macaca mulatta*) pituitary glands were collected through the Tissue Distribution Program at Oregon National Primate Research Center (ONPRC), following approved protocols by the Institutional Animal Care and Use Committees at the University of Maryland and the ONPRC; the animals provide postmortem tissues for these and other

unrelated studies. Prior to necropsy, the animals were individually housed under controlled environment with 12 L:12 D photoperiod (lights on at 0700 h), in auditory, visual and olfactory contact with male and female conspecifics. Diet consisted of primate chow (Purina Mills, Inc., St. Louis, MO, USA) supplemented with fresh fruits and vegetables, provided in two meals at 0800 h and 1500 h daily; water was available *ad libitum*.

In Experiment 1, pituitary glands were collected from Juvenile (n=4; 1–2 years; 1.8 kg average body mass), Young Adult (n=4; 7–12 years; 6.9 kg average body mass), and Old Adult (n=4; 18–26 years; 6.8 kg average body mass) male rhesus macaques, sacrificed between 1100 h and 1500 h. The pituitary glands were kept whole or were sectioned sagittally; they were then either placed in RNeasy[®] (Qiagen, Inc., Austin, TX, USA) and frozen, or simply flash frozen in liquid nitrogen. In Experiment 2, pituitary glands were obtained from ovariectomized female rhesus macaques (n=6; 8–11 years; 6.4 kg average body mass), sacrificed at either 0100 h or 1300 h; the pituitary glands were collected and preserved as in Experiment 1. In Experiment 3, pituitary glands were collected from ovariectomized females (n=5; 7–12 years; 5.1 kg average body mass) at 0300, 0700, 1500, 1900, and 2300 h, and were fixed with 4% paraformaldehyde.

2.2 RNA extraction and gene microarrays

The pituitary glands were homogenized with a PowerGen rotor-stator homogenizer (Fisher Scientific, Pittsburgh, PA, USA) and total RNA was isolated by RNeasy Mini Kit, according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The concentration and integrity of RNA in the final samples were assessed by microcapillary electrophoresis using a model 2100 Agilent Bioanalyzer (Santa Clara, CA, USA).

Microarray analysis was performed by the Affymetrix Microarray Core of the OHSU Gene Microarray Shared Resource, in accordance with the manufacturer's instructions (Affymetrix Genechip[®] Analysis Technical Manual; Santa Clara, CA, USA). Labeled target cRNA was hybridized to human arrays (Affymetrix HG_U133A), detecting a total of 18,400 transcripts. Image processing and expression analysis were performed using the GeneSifter software (VizXlabs, Seattle, WA, USA), and differentially expressed mRNAs were disclosed using the GC-RMA algorithm. Comparisons were performed using Student's *t*-test, with a cut off fold change value of 1.5. 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.

2.3 Semi-quantitative RT-PCR

The primers and probes (Table 1) were designed using PrimerExpress[®] software (Applied Biosystems, Foster City, CA, USA), and were subsequently purchased from Invitrogen (Carlsbad, CA, USA). In all cases they targeted sequences common to the rhesus macaque and human transcripts, except for the 3' Per2 primer (human), the 3' Cry1 primer (human), and the 5' Csnk1 primer (macaque); in these instances there was a single base-pair mismatch between the two species. The macaque sequences were obtained from the Human Genome Sequencing Center at Baylor College of Medicine (<http://www.hgsc.bcm.tmc.edu/projects/rmacaque/>).

Total RNA (1 μ g) was used to synthesize cDNA using the Omniscript kit (Qiagen) and oligo d(T)₁₅ primers (Promega Corp, Madison, WI, USA) in 20 μ l at 37°C for 1 h. sqRT-PCR amplifications were performed in duplicate using 1 μ l cDNA, 200 μ M deoxynucleotide triphosphates (Promega), 0.5 μ M of each primer, and 2.5 U of HotStarTaq[®] polymerase (Qiagen) in 25 μ l with the following thermocycle profile: 95°C, 15 min; 94°C, 1 min; specific cycle number and annealing temperature for each primer pair (Table 1), 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 (Bethesda, MD, USA, <http://rsb.info.nih.gov/ni-h-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.

2.4 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 Rev-erbα primers (Invitrogen), 50 nM human β-actin primers (Applied Biosystems), 250 nM Rev-erbα probe (Sigma, St. Louis, MO, 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. Rev-erbα primers and probe (Table 1) were designed using the rhesus macaque sequence available in the GenBank database (accession no. BV208705).

2.5 Immunohistochemistry

Fixed pituitary glands were sectioned (25 µm) horizontally from inferior to superior orientation using a frozen-stage sliding microtome. Three continuous sections from three different areas were subjected to 20-second microwave antigen retrieval (MAR) in 2× Antigen Retrieval Citra (BioGenex, San Ramon, CA, USA) followed by single-label immunohistochemistry (polyclonal antibody against human REV-ERBα; Lifespan Biosciences, Seattle, WA, USA), with visualization by ABC amplification (Vector Laboratories, Burlingame, CA, USA), and DAB chromogen (Sigma). Control tissue was not MAR-treated or exposed to primary antibody. The sections were mounted (Superfrost Plus slides; Fisher Scientific) and coverslipped (DPX mounting medium; Fisher Scientific) for image analysis.

2.6 Statistical analysis

Signal intensity and mRNA expression levels were analyzed by Student's *t*-test or one-way ANOVA. In order to control for experiment-wide false positives while still maintaining statistical power, alpha was adjusted to correct for multiple *P*-value comparisons of the eleven circadian transcripts using sequential Bonferroni with Simes-Hochberg correction (Hochberg 1988). If group differences were revealed by ANOVA, differences between individual groups were determined with Tukey-Kramer post-hoc analysis using GraphPad Prism (GraphPad, San Diego, CA, USA). Power analysis was performed using Statistical Analysis System (SAS Institute, Cary, NC, USA). Data are expressed as mean ± SEM for each parameter measured in each group. Significance was evaluated based upon an experiment-wise type I error rate of 0.05 unless otherwise adjusted by Bonferroni correction.

3. Results

3.1 Gene expression profiling and age characterization

Microarray analyses (Affymetrix GeneChip® HG_U133A) of pituitary glands contained eleven probesets for the seven core-clock genes with all transcripts detected across the three age groups (Table 2). Significant differences were observed in *Per2* expression in Young

Adults compared to other age groups, and *Bmal1* expression between Juvenile and Young Adults.

Reverse-transcription polymerase chain reaction (RT-PCR) corroborated the microarray data by showing expression of core-clock genes in the pituitary gland (Fig. 1). Comparison of expression levels by image analysis showed a statistically significant expression difference between Juveniles and older males in *Per2* expression, with a 37% decline in Young Adult mRNA expression and a 42% decline in Old Adults as compared to Juvenile animals (Fig. 2). Due to large variation within some groups, differences in *Csnk1ε* and *Bmal1* expression (Fig. 2) were not statistically significant across ages but showed trends similar to those revealed by the microarrays. In general, very few other pituitary genes appeared to change significantly during aging (Supplementary Tables 1-3).

3.2 Expression of clock genes at 0100h vs. 1300h

RT-PCR performed using pituitaries collected 12 hours apart showed significant changes in the expression of rhythmically expressed core-clock genes, relative to the housekeeping gene β -actin at 0100h and 1300h (Fig. 3); *Csnk1ε* was not measured because it is constitutively expressed throughout the 24-hour cycle. As shown in Fig. 4, significant differences were detected in *Per2* (1.33 fold) and *Bmal1* (1.82 fold) expression at the opposing time-points, with increased expression at 1300 h. To confirm the semi-quantitative findings, we subsequently performed quantitative real-time RT-PCR (qRT-PCR) for *Rev-erba*, using the same RNA samples, and confirmed the absence of a significant change in *Rev-erba* expression (Fig. 5).

3.3 24-hour oscillation of REV-ERB α

Circadian changes in REV-ERB α were examined immunohistochemically in pituitary glands collected at 4-hour intervals over 24 hours from ovariectomized females. Immunostaining was detected in the anterior and intermediate pituitary but not the posterior pituitary gland, demonstrating regional specificity in distribution of the nuclear protein (data not shown). Representative changes in circadian expression for REV-ERB α in the anterior pituitary gland of the rhesus macaque are shown in Fig. 6. These data clearly show regional and temporal differences in immunostaining intensity across the 24-hour cycle, providing evidence of rhythmic expression of a core-clock gene.

4. Discussion

The release of hormones from the anterior pituitary gland plays a major role in coordinating various physiological functions. Throughout life and during specific life stages the hypothalamic-pituitary-gonadal (HPG) axis is responsible for a number of circadian activities in mammals including rhythmic release of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), growth hormone (GH) and adrenocorticotrophic hormone (Griffin and Ojeda 2000; Knobil, *et al.* 1994). Indeed, the levels of most reproductive hormones, such as testosterone, are regulated in a circadian fashion in mammals (Jilg *et al.* 2005; Sehgal 2004). Early work investigated the possibility that the pituitary gland functions as an autonomous clock capable of generating rhythmic LH release independent of hypothalamic control (Lewy, *et al.* 1996). The results, from mice, indicated that pituitary gland gonadotrophs are capable of producing rhythms of LH release for a long duration *in vitro*. More recent data from rats demonstrated that *in vitro* release patterns of prolactin were affected by photoperiod and age. In this model, both mean levels and rhythmic prolactin release are determined by the age of the animal, the circadian time of pituitary gland isolation, and the photoperiodic conditions in which the animal was housed (Lewy, *et al.* 2005). In addition, at least some of the molecular clock components have been shown to exist in the rat pituitary gland. *In situ* hybridization experiments mapped *Per1*, *Per2* and *Cry1* mRNA to areas of the anterior

pituitary, pars tuberalis/median eminence and intermediate pituitary gland. No signals for mRNA expression were detected in the posterior pituitary gland (Shieh 2003).

In the present study, microarray analyses, semi-quantitative and quantitative RT-PCR analyses, and immunohistochemistry provided a multifaceted examination of the presence of a peripheral circadian clock mechanism within the rhesus macaque pituitary gland. Our data build on the findings from other mammalian species and demonstrate that mRNA and protein core-clock components are also expressed in the pituitary gland of a nonhuman primate, the rhesus macaque. Furthermore, the finding that mRNA levels are significantly different at two specific, opposing time points, that protein levels of at least one component oscillated with a 24-hour rhythmic pattern, and that at least one mRNA component of the clock mechanism was affected by age, all give support to the view that a functional circadian clock mechanism exists within the primate pituitary gland.

The microarray analysis revealed the expression of seven core-clock genes: *Per1*, *Per2*, *Bmal1*, *Cry1*, *Clock*, *Rev-Erba* and *Csnk1ε*, and interestingly showed small but significant age-related changes in mRNA expression of some of these genes. Specifically, *Per2* expression in Young Adults was significantly less than in the other two age groups, while *Bmal1* mRNA expression levels were higher in Young Adults compared to Juvenile animals. At the time of the experiments the rhesus macaque GeneChip[®] was unavailable, so the human chip (Affymetrix HG_U133A) was utilized. Given the high similarity between non-human primate (NHP) and human genomes [e.g., 98.77% similarity between chimpanzee and human (Dillman and Phillips 2005); 97.5% between rhesus macaque and human (Gibbs, *et al.* 2007)], it was reasonable to expect that the human GeneChip[®] could successfully hybridize with NHP samples. This has in fact been determined in multiple studies (Dillman and Phillips 2005; Wang *et al.* 2004).

Overall, few age-related changes in pituitary gland gene expression were detected utilizing the 18,400 transcripts on the Affymetrix HG_U133A human array (Supplementary Tables 1-3). This finding correlates well with previous microarray results that revealed few hypothalamic and pituitary gland changes in gene expression in 3, 15, and 24 month old Sprague-Dawley male rats (Kappeler, *et al.* 2003). Altogether, these data support the idea that the pituitary gland exhibits a high consistency of mRNA expression with aging, so that age-associated modifications in gene expression appear to be restricted to a rather modest number of genes.

Our microarray data clearly show that the main components of the circadian clock mechanism are intrinsically expressed in the rhesus macaque pituitary gland. Furthermore, this conclusion was validated using RT-PCR, which showed that all six of the investigated genes (*Clock* was not analyzed) were present. Based on the microarray data, one of the *Per2* transcripts showed a significant pubertal decrease in expression, and one of the *Bmal1* transcripts showed an increase. The developmental *Per2* change was corroborated by the RT-PCR study, although no detectable differences were detected between the Young and Old adults; the RT-PCR study benefited from having larger group sizes than the microarray study. Although *Csnk1ε* and *Bmal1* just fell short of showing statistically significant age-related differences, in the RT-PCR analysis, both of these transcripts showed a trend in the same direction as the microarray data (i.e., with *Csnk1ε* mRNA expression declining with age and *Bmal1* increasing). Taken together, these observations suggest that some components of the molecular clock circuitry may change across the life span and/or be influenced by circulating sex-steroid concentrations, which rise significantly after puberty and subsequently decline during aging. Although all of the observed changes were subtle, they could potentially lead to temporal dysregulation of gene expression, a principal factor in cellular malfunction and disease in aging. It is worth noting, too, that the pituitary gland is also a very heterogeneous tissue consisting of multiple cell types (gonadotrophs, lactotrophs, somatotrophs) and each may have its own circadian rhythm with

a specific phase. Hence the difference in phases may result in the dampening of the overall rhythm of the tissue, consequently confounding our ability to detect expression changes.

In order to ascertain the functionality of the clock mechanism, we examined RNA in pituitary glands from ovariectomized females obtained at 0100 h and 1300 h. These tissues provided opposing times in the circadian cycle and therefore were useful in verifying circadian changes. Because hormone variation can influence gene expression, the availability of pituitary glands from gonadectomized animals enabled disclosure of universal circadian changes across gender, in the absence of a fluctuating sex-steroid environment. Five rhythmically expressed core-clock genes were measured using sqRT-PCR. Expression of *Per2* and *Bmal1* levels were significantly different ($P<0.05$), with *Per2* displaying a fold-change of $1.33 (\pm 0.05)$ from 0100 h to 1300 h and *Bmal1* showing a similar shift in direction with a $1.82 (\pm 0.34)$ fold-change. No differences were observed for Rev-erb α mRNA levels at 0100 h vs 1300 h using quantitative real-time RT-PCR.

The fact that *Per2* and *Bmal1* both increased at 1300 h compared to 0100 h was somewhat surprising. In the SCN these two transcripts would be in antiphase to one another at these sampling times. While *Per2* should be high during circadian day (1300 h) and low at circadian night (0100 h), *Bmal1* would be the exact opposite. Our findings in the pituitary gland are likely attributed to the nature of peripheral tissues and their relationship to the SCN. There are reports that peripheral tissues exhibit a 3-9 hour phase shift in mRNA core-clock oscillation compared to the SCN, suggesting that peripheral tissues might be receiving timing cues from the master oscillator in a delayed fashion (Morse and Sassone-Corsi 2002). Lemos *et al.* (2006) previously examined adrenal glands from some of the same animals that were the source of pituitary glands in the present study, and found clear-cut circadian rhythms in the expression of core clock within this peripheral organ. These rhythms, however, were not in synchrony with the pituitary gland. Consequently, our findings support the contention that each peripheral tissue, although receiving signals from the SCN, may regulate its own circadian expression of genes within the limits of its physiological constraints and demands.

In our final experiment, staining for REV-ERB α protein revealed a temporal change in expression within the anterior and intermediate pituitary gland of the rhesus macaque. As with mRNA expression, there may be a noticeable phase shift in protein expression levels compared to the SCN as the peripheral tissues receive timing cues from the master oscillator in a delayed fashion. Future work involving double-staining of the pituitary gland for REV-ERB α and FSH/LH is particularly appealing. DAB immunohistochemistry identified the nuclear REV-ERB α and combined with NiCl staining for cytoplasmic FSH/LH could potentially demonstrate co-localization of the proteins. This would give added weight to the theory that core-clock gene expression is of physiological relevance in the pituitary gland, particularly with regard to daily hormonal rhythms.

There are well-documented alterations in circadian organization during aging, including changes in hormonal rhythms, core body temperature, sleep/wake cycles, activity and locomotor patterns, behavioral responses, and response to the phase-shifting effects of light (Asai, *et al.* 2001; Hofman and Swaab 2006; Oster, *et al.* 2003). Despite these overt signs, however, the physiological underpinnings for the circadian dysregulation remain unclear. There is evidence that the amplitude of the central circadian pacemaker decreases in older animals. For example, it has recently been reported that *Clock* and *Bmal1* expression in the SCN decreased with age in hamsters, but that there was no difference between young and old hamsters in the expression of either *Per1* or *Per2* (Kolker, *et al.* 2003). The change in *Clock/Bmal1* expression was not enough to disrupt the overall cycle as the circadian mechanism continued to exhibit a normal phase profile (Hofman and Swaab 2006; Kolker, *et al.* 2004). Similar results were found in rats where *Per* and *Cry* expression levels in the SCN were not

dampened or disrupted by aging (Asai *et al.* 2001; Hofman and Swaab 2006; Yamazaki, *et al.* 2002). It appears then that the daily rhythm of expression of core-clock genes is similar in the SCN of young and old rodents and that oscillation of the master pacemaker remains remarkably stable.

Within peripheral tissues there appears to be more variation in aging impaired circadian expression of core-clock genes. While some findings have shown deterioration of transcription and rhythmicity in peripheral tissues, including lung and aortic/cardiac muscle cells (Kunieda, *et al.* 2006; Yamazaki *et al.* 2002), others have failed to show age-related changes in rhythmicity in the liver, pineal gland, kidney or pituitary gland (Hofman and Swaab 2006; Yamazaki *et al.* 2002). It may be that some peripheral tissues, like the pituitary gland, are more resilient with regard to circadian changes brought about by cellular senescence. In this way they may mirror the stability seen in the suprachiasmatic nucleus.

The functional significance of our observation, that *Per2* expression declines with age in the rhesus macaque pituitary, remains to be determined. However, it is known that in addition to their role within the circadian clock, PER homologues also exhibit tumor suppression activity (Fu *et al.* 2002; Lee 2006). Furthermore, desynchronization of the circadian cycle has been shown to increase the incidence of cancer and reduce life expectancy (Filipski *et al.* 2003; Fu *et al.* 2002; Penev *et al.* 1998) while resetting of circadian rhythms can increase longevity (Hurd and Ralph 1998). Also, calorie restriction (CR) and CR models such as the α MUPA transgenic mouse, which show a more robust biological clock than wild-type mice (Froy *et al.* 2006), appear to slow down the aging process (Mattison *et al.* 2003; Merry 2002; Weindrich and Walford 1988). Interestingly, comparative analysis of murine DNA microarray datasets for 10 different tissue types found 28 genes for which the response to CR is most commonly shared (Swindell 2008). Among the consistent changes was an increase in *Per2* expression, which was upregulated in seven of the tissues. Taken together, such findings suggest that CR may be eliciting its beneficial effects by restoring the circadian clock mechanism, and specifically *Per2*.

Finally, there may be a biological significance to the trend observed in the decline of *Csnk1e* in the pituitary gland with age. Hamsters harboring the naturally occurring *tau* mutation possess a mutated *Csnk1e* that is unable to fully phosphorylate its physiologically relevant substrate, *Per2* (Eide and Virshup 2001). Because limited phosphorylation leads to less *Per2* degradation it is able to build up quicker in the cytoplasm. With higher *Per2* content the Per/Cry dimer forms faster causing the negative loop of the cycle to progress more rapidly. That is to say, a shorter circadian cycle persists which is closer to 20-22 hours in the *tau* mutant (Eide and Virshup 2001; Morse and Sassone-Corsi 2002).

In summary, our data support the presence of an intrinsic clock mechanism, which may contribute to the rhythmic physiology of the pituitary gland. In addition, they suggest that some components of the underlying circadian clock circuitry may change during aging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Validation of pituitary gland core-clock gene expression in the rhesus macaque as determined by reverse-transcription polymerase chain reaction (RT-PCR). Pituitaries were collected across three age categories (n=4) of rhesus macaques (Juvenile = 1 - 2 years, Young Adult = 7 - 12 years, and Old Adult = 18 - 26 years). Composite gel image is representative of RT-PCR results and clearly demonstrates expression of clock genes in each age category. The housekeeping gene *β-actin* was used as a positive control and for normalizing images for semi-quantitative analysis. MW = molecular weight ladder.

Figure 2.

Semi-quantitative RT-PCR expression levels of core-clock genes across three age categories in the rhesus macaque pituitary gland. Each bar, along with SEM, represents relative mean, normalized fluorescence data from four animals. Statistical comparisons were made using one-way ANOVA and Tukey-Kramer post-hoc analysis ($P < 0.05$). Significant differences were observed in *Per2* expression between Juvenile animals and the other two groups, resulting in a negative fold-change of $0.63 (\pm 0.05)$ and $0.58 (\pm 0.06)$, respectively. *Csnk1ε* and *Bmal1* expression did not reach significant levels due to large variation within some groups, but trends were observed that correlated with microarray data. The other transcripts were not significantly different across age categories.

Figure 3.

Expression of core-clock genes in the rhesus macaque pituitary gland at 0100 h vs. 1300 h. Composite gel image is representative of RT-PCR results demonstrating expression of core-clock genes at 0100 h (lanes 1-3) and 1300 h (lanes 4-6). The housekeeping gene β -actin was used as a positive control and for normalizing images for semi-quantitative analysis. *Csnk1ε* was not measured because it is constitutively expressed across the 24-hour cycle.

Figure 4.

Semi-quantitative RT-PCR expression levels of core-clock genes at 0100 h and 1300 h in the rhesus macaque pituitary gland. Each bar, along with SEM, represents relative mean, normalized fluorescence data from three animals. Statistical comparisons were made using Student's *t*-test (*, $P < 0.05$). Significant differences were observed in expression levels between time points for *Per2* and *Bmal1*, resulting in a fold-change of $1.33 (\pm 0.03)$ and $1.82 (\pm 0.17)$, respectively.

Figure 5.

Quantitative real-time RT-PCR of *Rev-erba* in the rhesus macaque pituitary gland. Each bar, along with SEM, represents mean, normalized fluorescence data from three animals. Statistical comparisons were made using Student's *t*-test ($P < 0.05$). As with sqRT-PCR, we found no difference in mRNA expression at these sampling times.

Figure 6.

Rhythmic expression of REV-ERBα in the rhesus macaque pituitary gland. REV-ERBα nuclear immunostaining (black arrows) in the anterior pituitary gland showed temporal changes in intensity across a 24-hour cycle. The horizontal white and black bar represent day and nighttime; scale bar = 40 μm.

Table 1

Oligonucleotide sequences used for sqRT-PCR and qRT-PCR of core-clock genes in the rhesus macaque pituitary gland.

sqRT-PCR primers	Sequence	Size of PCR product (bp)	PCR Cycles	Temp (°C)
5'Per1	5 -GCCAGCATCACTCGCAGCAGC-3	~400	27	65
3'Per1	5 -GTGGTTCATCAGGGTGACACAGG-3			
5'Per2	5 -CATCCACTGGTGGACCTCGCG-3	~325	27	65
3'Per2	5 -GCTCACTGGGCTGCGAGCG-3			
5'Cry1	5 -GCCTGTCTTAAGAGGCTTCCCTG-3	~375	28	65
3'Cry1	5 -ACTGAGACCAGTCCCCATGGAGC-3			
5'Bmal1	5 -CACAGCATGGACAGCATGCTGC-3	~450	26	65
3'Bmal1	5 -GCCACCCAGTCCAGCATCTGC-3			
5'Rev-erba	5 -TGGCGCTTACCGAGGAGGAGC-3	225	26	66
3'Rev-erba	5 -TCCACCGGAAGGACAGCAGC-3			
5'Csnk1ε	5 -AAGTATGAGCGGATCAGCGAGA-3	218	28	65
3'Csnk1ε	5 -CCGAATTCAGCATGTTCCAGT-3			
5'β-actin	5 -CATTGCTCCTCCTGAGCGCAAG-3	~300	22	65
3'β-actin	5 -GGGCCGGACTCGTCATAC'TCC-3			
qRT-PCR primers/probe				
5'Rev-erba	5 ACCCTGAACAACATGCATTCC-3	~100	40	60
3'Rev-erba	5 -GGAGAGAGAAAGTGCAGAGTTCGA-3			
5'Rev-erba	6FAM-CTGGCGCTGCCCCCTTG'TACA-TAMRA	N/A	40	60

Table 2
Age-related changes in core-clock gene expression in the rhesus macaque pituitary gland.

Probeset	Gene	Age Category		
		Juvenile (n=3; 1-2 years)	Young Adult (n=3; 7-12 years)	Old Adult (n=3; 18-26 years)
202861_at	<i>Per1</i>	765 ± 185	553 ± 44	735 ± 103
36829_at	<i>Per1</i>	1301 ± 254	1037 ± 115	1038 ± 46
205251_at	<i>Per2</i>	1881 ± 57	1283 ± 327	1460 ± 101
208518_s_at	<i>Per2</i>	311 ± 27 ^A	186 ± 15 ^B	317 ± 25 ^A
209674_at	<i>Cry1</i>	314 ± 62	309 ± 63	360 ± 124
209824_s_at	<i>Bmal1</i>	500 ± 30 ^A	792 ± 71 ^B	630 ± 69 ^{A,B}
210971_s_at	<i>Bmal1</i>	298 ± 31	355 ± 80	444 ± 123
31637_s_at	<i>Rev-Erba</i>	1989 ± 232	2617 ± 203	2188 ± 282
202332_at	<i>Csnk1ε</i>	207 ± 13	184 ± 16	148 ± 18
204980_at	<i>Clock</i>	280 ± 16	365 ± 38	357 ± 41
217563_at	<i>Clock</i>	142 ± 25	172 ± 18	142 ± 15

Gene expression was determined by Affymetrix GeneChip[®] microarray analysis. Values represent mean signal intensity (±SEM). Significant differences were observed in *Per2* and *Bmal1* expression using one-way ANOVA and Tukey-Kramer post-hoc analysis (letter notations, *P*<0.05).