RESEARCH

Sleep deprivation and diet affect human GH gene expression in transgenic mice *in vivo*

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Abstract

Human (h) growth hormone (GH) production studies are largely limited to effects on secretion. How pituitary hGH gene (*hGH-N/GH1*) expression is regulated is important in our understanding of the role hGH plays in physiology and disease. Here we assess for the first time the effect of sleep deprivation (SD) and high-fat diet (HFD) on *hGH-N* expression *in vivo* using partially humanized *171hGH/CS* transgenic (TG) mice, and attempted to elucidate a role for DNA methylation. Activation of *hGH-N* expression requires interactions between promoter and upstream locus control region (LCR) sequences including pituitary-specific hypersensitive site (HS) I/II. Both SD and diet affect hGH secretion, but the effect of SD on *hGH-N* expression is unknown. Mice fed a HFD or regular chow diet for 3 days underwent SD (or no SD) for 6 h at Zeitgeber time (ZT) 3. Serum and pituitaries were assessed over 24 h at 6-h intervals beginning at ZT 14. SD and HFD caused significant changes in serum corticosterone and insulin, as well as *hGH* and circadian clock-related gene RNA levels. No clear association between DNA methylation and the negative effects of SD or diet on hGH RNA levels was observed. However, a correlation with increased methylation at a CpG (cytosine paired with a guanine) in a putative E-box within the hGH LCR HS II was suggested *in situ*. Methylation at this site also increased BMAL1/CLOCK-related nuclear protein binding *in vitro*. These observations support an effect of SD on hGH synthesis at the level of gene expression.

Key Words

- sleep deprivation
- high-fat diet
- DNA methylation
- human growth hormone

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Introduction

Human (h) growth hormone (GH) is synthesized and secreted in a pulsatile manner by somatotrophs located in the anterior pituitary (1). Following the successful isolation and purification of hGH in 1944, GH secretion in humans has been heavily studied (2). Serum levels of hGH vary considerably over the course of the day and during the night as it is dependent on a variety of factors including (and not limited to) the circadian rhythm, sleep–wake cycle, diet, metabolism, age and sex (1, 3, 4). Typically, hGH secretion peaks shortly after sleep onset during the slow-wave sleep stage of non-rapid eye movement sleep. Sleep deprivation (SD) can result in the suppression of hGH nocturnal peaks where increases in GH secretion can occur during the day as a way to compensate (3, 4, 5). Diet and exercise are also known to affect serum hGH levels. Hypersecretion of GH is observed in humans during prolonged reductions in food intake, resulting in dramatic decreases in body weight and fat mass (3), whereas the opposite is observed with excess caloric intake. Reduced hGH serum levels as well as hyperinsulinemia are seen in individuals with obesity (6, 7). During both aerobic and resistance exercise, hGH secretion is increased (1). In addition, increased food intake and decreased physical activity as well as inadequate sleep are considered risk factors for obesity as well as insulin resistance and diabetes in humans (6, 8).

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While the effect of SD on hGH serum levels has been described (4, 9), there is no description of the effect on hGH synthesis and specifically gene expression. This is due to difficulties and confounding variables associated with sampling normal human pituitaries *in vivo*. As such, studies of hGH production are limited largely to measurement of serum levels and thus effects on secretion (protein). This situation has been remedied in part by the development of partially humanized *171hGH/CS* transgenic (TG) CD-1 mice, which include a single copy of the intact hGH gene (*hGH-N*/*GH1)* locus (10, 11). This 171 kilobase (kb) transgene located on mouse chromosome 14 contains *hGH-N* and locus control region (LCR) which is marked by the presence of nuclease hypersensitive sites (HS) I–V, with HS I/II exclusively regulating *hGH-N* expression. The LCR is located 14–32 kb upstream of the *hGH-N* promoter, where interaction between the two regions is critical for pituitary- and cell-specific activation of the gene (11, 12). The *171hGH/CS* TG mice were used previously to assess the effect of an acute 3-day high-fat diet (HFD) on daily rhythmic hGH secretion and synthesis (13). Levels of *hGH-N* RNA displayed a circadian pattern by oscillating over a 24-h time frame that was disrupted by a HFD vs a regular control chow diet (RCD) at Zeitgeber time (ZT) 2. By contrast, mouse GH did not display a clear rhythmic pattern over 24 h or an effect of diet under the conditions and times of assessment (13).

HFD-induced obesity in mice is reported to disrupt circadian rhythms (14, 15). Expression of genes coding for the evolutionarily conserved circadian rhythm-related transcription factors were also assessed in *171hGH/CS* TG mice on an acute 3-day HFD (13). Brain and Muscle ARNT-Like 1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK) gene products heterodimerize (BMAL1/CLOCK) and bind enhancer (E)-box elements to transactivate the Period Circadian Regulator 1-3 (*PER1-3*) and Cryptochrome Circadian Regulator 1 and 2 (*CRY 1* and *2*) genes (16, 17). PER and CRY gene products bind together to form a complex which then binds chromatin to inhibit *BMAL1* and *CLOCK* activity, and subsequently represses PER and CRY gene expression which in turn creates a negative feedback loop (16, 17). BMAL1/CLOCK can also activate the nuclear orphan receptor genes coding for REV-ERBα/β and retinoid-related orphan nuclear receptor alpha/beta (RORα/β), which have opposing actions on *BMAL1* expression in order to ensure rhythmic expression (17). An E-box element containing the core sequence 5′-CACGTG-3′ was identified within the *hGH-N* promoter but is not found within the mouse *Gh* promoter. This core sequence enables binding of BMAL1/CLOCK heterodimer to the *hGH-N* promoter, where association of BMAL1 with this region was reduced as a result of HFD in *171hGH/CS* TG mice (13).

The circadian clock genes are also known to be involved in the homeostatic sleep process. The sleep homeostat keeps track of the time spent awake and asleep, where loss of sleep is typically followed by increased sleep (18). The joint action of the sleep homeostat and circadian processes controls sleep inclination, duration, and quality of wakefulness. Misalignment of both of these processes is experienced by humans during jet-lag and as a result of shift work (18, 19). SD is when an individual does not get enough sleep, which results in wide-spread disturbances in the body and brain. SD has been shown to affect mouse whole brain *Per2* expression (19, 20) as well as reduce DNA binding of BMAL1/CLOCK heterodimer (21). In addition, individuals with obesity typically suffer sleep loss (22).

Epigenetics represents the chemical modifications made to chromatin that influence gene expression (23). The methylation of cytosine paired with a guanine (CpG) termed 'DNA methylation' is one of the many epigenetic mechanisms that govern gene expression where approximately 70–80% of CpG dinucleotides are methylated (23). The putative BMAL1/CLOCK binding E-box element located in the *hGH-N* promoter (5′-CACGTG-3′) contains a CpG site, and thus also represents a potential target for DNA methylation as a result of sleep or diet disturbances. Several genome-wide methylation studies have observed changes in methylation in obesity-, SD- and Clock-related genes (24, 25, 26, 27). Whether modification of the *hGH-N* promoter E-box occurs in response to SD or diet, and/or whether DNA methylation alters E-box factor binding, and specifically association related to BMAL1/CLOCK, is not known. Thus, here we investigated for the first time the acute effect of a 6-h period of SD and an acute 3-day HFD on *hGH-N* expression in the daily cycle. This has been pursued for possible involvement of epigenetic changes in CpG methylation status associated with the *hGH-N* promoter and LCR HS I/II.

Methods

Animals and diet

Ethical animal use approval was obtained from Central Animal Care Services (Protocol #19-042 and #15-036/1/2) at the University of Manitoba. Mice were housed in an environmentally controlled room maintained on a 12 h light/12 h darkness cycle (06:00 h/18:00 h; ZT 0/ZT 12).

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Access to tap water and food in the form of palatable pellets was ad libitum. Male 3–4 week old *171hGH/CS* TG CD-1 mice were used since growth hormone levels are at their highest during puberty (onset at days 21–35) (28). Mice were randomly assigned to receive RCD (fat 14 kcal%, carbohydrate 60 kcal%, and protein 25 kcal%; Prolab® RHM 3000 5P00*) or HFD (fat 60 kcal%, carbohydrate 20 kcal%, and protein 20 kcal%; Research Diets D12492). Mice were fed starting in the afternoon of day 1 for 3 full days before being sacrificed every 6 h by cervical dislocation, starting at ZT 14 (20:00 h) on day 3 of diet and then subsequently at ZT 20 (02:00 h), and then ZT 2 (08:00 h), ZT 8 (14:00 h) and ZT 14 on day 4 to represent a full 24-h cycle to assess circadian effects. Combined assessment of ZT 14–20 and ZT 2–8 were used to generate values for the dark/active and light/inactive phases of the daily cycle for mice, respectively. SD was effectuated on the morning of day 3 of receiving the diets for a total of 6 h, before being allowed to settle and sleep. Cages were removed from the holding rack, opened, and animals were disrupted by moving various cage components (nest, food tray) from one side of the cage to the other, as well as being stroked or handled briefly every 30 min starting at ZT 3 (29, 30). Body weights were taken prior to and after diet intervention before sacrificing. The amount of food consumed over the 3-day period was also recorded. Average (mean ± S.E.M.) starting body weight for each group of mice was 23.95 ± 0.27 g for the no sleep deprivation group (n = 140) and 25.54 ± 0.25 g for the sleep-deprived group (n = 90).

Blood and tissue collection

After cervical dislocation and decapitation, trunk blood was collected and allowed to clot for ≥10 min. The cranium was opened and the pituitary gland was removed, flash-frozen immediately, and stored at –80°C. Serum was separated by centrifugation at 9300 ***g*** for 10 min in a microcentrifuge at 4°C, then aliquoted and stored at –80°C. Serum insulin and corticosterone levels were detected by ELISA according to manufacturer instructions (Rat/Mouse Insulin, EZRMI-13K, Millipore; DetectX Corticosterone Enzyme Immunoassay Kit, K014-H5, Arbor Assays).

Quantitative realtime RT-PCR (qPCR)

RNA was extracted from pituitaries using the RNeasy® Plus Mini Kit 250 (REF #74136) with the Qiagen QIA Shredder™ 250 (REF #79656) according to manufacturer instructions. Pituitaries were homogenized by a pestle and mortar for 40–60 s followed by a 5-min incubation. This was repeated three times before continuing with the extraction. Average RNA yield was 4725 ng (S.E.M. ± 319.7) and 4942 ng (S.E.M. ± 368.7) for RCD and HFD (respectively). RNA (1–2 µg) was reverse transcribed into cDNA using the QuantiTect RT Kit (205314; Qiagen) in the MJ Research PTC-100™ Programmable Thermal Controller. RT negative samples contained sterile distilled water instead of RT enzyme. cDNA samples were then diluted to 0.02 µg/µL and amplified in the Applied Biosystems 7500 Fast Real-Time PCR system with specific forward and reverse primers ((0.5 pM); 1 µL each) manufactured by Integrated DNA Technologies (Table 1) and with Power SYBR® Green PCR Master Mix (10 µL; #4367659, Thermo Fisher Scientific). cDNA was denatured at 95ºC (5 min), followed by 40 cycles of denaturing at 95ºC (15 s), annealing at 60ºC (15 s), and 72ºC (30 s). The gene expression level in each sample was calculated from a standard curve and normalized to β-actin as the reference gene. qPCR was run in duplicate and on four to six independent samples per timepoint for each set of primers.

Minus RT samples were also produced and subjected to RT-PCR and qPCR with the same conditions as above with forward and reverse primers for mouse growth hormone (*mGh*) to control for genomic DNA contamination and to assess technique (results not shown).

DNA extraction and targeted bisulfite sequencing

Genomic DNA from pituitaries was extracted using the DNeasy Blood and Tissue Kit (#69506, Qiagen) according

Table 1 List of human (h) and mouse (m) forward and reverse primer sequences used in this study.

Primer	Forward sequence	Reverse sequence
hGH	5'-CCTAGAGGAAGGCATCCAAA-3'	5'-GCAGCCCGTAGTTCTTGAGTAG-3'
mGH	5'-ACGCGCTGCTCAAAAACTAT-3'	5'-CACAGGAGAGTGCAGCAGAG-3'
mβ-Actin	5'-GAGACCTTCAACACCCCAGCC-3'	5'-GGAGAGCATAGCCCTCGTAG-3'
mBmal1	5'-CCACCTCAGAGCCATTGATACA-3'	5'-GAGCAGGTTTAGTTCCACTTTGTCT-3'
mClock	5'- ACAGTTTCACGAGGGTGGTC-3'	5'-TCCCTACCGTCTCATCAAGG-3'
mPer2	5'-TGTGCGATGATGATTCGTGA-3'	5'-GGTGAAGGTACGTTTGGTTTGC-3'
mCry1	5'-TGAGGCAAGCAGACTGAATATTG-3'	5'-CCTCTGTACCGGGAAAGCTG-3'

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to manufacturer instructions. Briefly, pituitaries were allowed to digest overnight in lysis buffer in a 56°C water bath before subjected to extraction. Average DNA yield was 1187 ng (S.E.M. ± 77.4) and 1156 ng (S.E.M. ± 77.4) for RCD and HFD (respectively). Extracted DNA was used for targeted bisulfite sequencing including primer design, validation, target amplification and sequencing which was performed by Zymo Research Corporation (Irvine, CA).

Primer pairs were designed with Rosefinch in order to target CpG sites in the specified regions of interest (ROI) within the *hGH-N* promotor and LCR hypersensitive site II (HS II). Design parameters were chosen such that PCR amplicons would be ideally >100 to <300 bp, and the primers avoided annealing to CpG sites at the region of interest to the maximum extent possible. In the event that CpG sites were absolutely necessary for target amplification, ordered primers were synthesized with a pyrimidine (C or T) at the CpG cytosine in the forward primer, or a purine (A or G) in the reverse primer to minimize amplification bias to either the methylated or unmethylated allele. All primers in TE solution at (100 µM) were diluted to 2 µM each and tested using Real-Time PCR with 1 ng of bisulfite-converted control DNA (duplicate individual reactions).

Genomic DNA samples from pituitaries were subjected to sodium bisulfite treatment using the EZ DNA Methylation-Lightning™ Kit (Zymo Research Corp., D5030) according to the manufacturer's instructions. Bisulfite-treated samples (5 ng) and validated primer pairs were subjected to targeted amplification, purification (ZR-96 DNA Clean & Concentrator™, D4023), and prepared for sequencing using a MiSeq V2 300 bp Reagent Kit (Illumina) and paired-end sequencing protocol according to the manufacturer's guidelines. Sequence reads from bisulfite-treated libraries were identified using the standard Illumina base-calling software and then analyzed using Zymo Research Corp.'s proprietary analysis pipeline. Sequence reads were aligned to the reference genome assembly hg38 using Bismark (http://www.bioinformatics.babraham.ac.uk/projects/bismark/). The methylation level of each sampled cytosine was estimated as the number of reads reporting a C, divided by the total number of reads reporting a C or T. The data shown (n = 2 samples per group) represents methylation ratios; the measured number of methylated cytosines divided by total number of cytosines covered at that site. Each sample consisted of four individual mouse pituitaries that were combined in order to provide sufficient amounts of genomic DNA for analysis.

Electrophoretic mobility shift assay (EMSA)

EMSA as well as human embryonic kidney (HEK) 293 cell culture, *Bmal1*/*Clock* plasmid transfection, and nuclear extract preparation have been described previously (13, 31). Briefly, HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, 12100-061) supplemented with fetal bovine serum (Gibco; REF #12483-020) and antibiotic cocktail (50 µg/mL streptomycin, 0.5 mM glutamine, 50 units/mL penicillin) and grown accordingly. Trans-IT293 reagent (Mirus Biol Corp., Madison, WI) diluted with serum-free DMEM as well as *Bmal1*/*Clock* expression vectors were used for transient transfection. The cDNA expression vectors for mouse *Clock* (Gal4-Clock/UA256), and *Bmal1* (Gal4-Bmal1/UA255) were kindly provided by Dr. Urs Albrecht (University of Fribourg, Fribourg, Switzerland) (32). EMSA was performed using nuclear extract (Nuclear Extraction kit (Abcam, ab113474)) from HEK293 cells either overexpressing Bmal1 and Clock, or an empty vector as a control together with ^{32}P-labeled oligonucleotide probes which included unmethylated and methylated E-box or putative E-box DNA elements in the *hGH-N* promoter and LCR HS II (Table 2). Briefly, 4 µg of nuclear extract

Table 2 List of forward and reverse oligonucleotide sequence probes used for EMSA in this study.

Target	Forward and reverse sequences
Human growth hormone promoter single site methylation (m) in the E-box	Forward: GGCACCCA^{m}CGTGACCCTTAAA Reverse: TTTAAGGGTCA^{m}CGTGGGTGCC
Hypersensitive site II single site methylation (m) in E-box 1	Forward: CTTGGGGCACA^{m}CGTGTTTGTGGGG Reverse: CCCCACAAACA^{m}CGTGTGCCCCAAG
Hypersensitive site II single site methylation (m) in E-box 2	Forward: TAAGAAGGA^{m}CGTGGGTTTGAGTCC Reverse: GGACTCAAACCCA^{m}CGTCCTTCTTA
Unmethylated human growth hormone promoter E-box	Forward: GGCACCCACGTGACCCTTAAA Reverse: TTTAAGGGTCACGTGGGTGCC
Unmethylated hypersensitive site II E-box 1	Forward: CTTGGGGCACACGTGTTTGTGGGG Reverse: CCCCACAAACACGTGTGCCCCAAG
Unmethylated hypersensitive site II E-box 2	Forward: TAAGAAGGACGTGGGTTTGAGTCC Reverse: GGACTCAAACCCACGTCCTTCTTA

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was incubated with EMSA buffer containing 2 µg of poly(dI-dC) for 5 min. The radiolabeled oligonucleotide probes (1 ng) were then added, and the reactions were incubated for a further 20 min at room temperature. Retardation of DNA–protein complexes were resolved in non-denaturing 5% (w/v) polyacrylamide gels and visualized by autoradiography.

Statistical analysis

All data were subjected to statistical analysis utilizing GraphPad Prism 8 Software (Version 8.4.2 (679)). Any value that was more than ± two standard deviations from the mean was considered an outlier and removed from analysis. A non-parametric *t*-test (Mann–Whitney) was used for single comparisons and *P*-values were adjusted using the Benjamini–Hochberg procedure using the formula ((i/m)*Q) and a false discovery rate (FDR) of 0.1. Adjusted *P*-values are shown and significance is marked by $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$, and trends were marked by $^{\dagger}P < 0.075$. All error bars represent the S.E.M.

Results

Acute SD affects weight gain and food intake

The effect of acute SD on *hGH-N* RNA levels in dark/active and light/inactive phases of the daily cycle, as well as the possible influence of diet (HFD vs RCD), was assessed beginning at ZT 14–20 (dark/active phase) and finishing at ZT 2–8 (light/inactive phase). There was no significant difference in weight gain ($P = 0.2188$) or food intake ($P = 0.1731$) for mice fed a HFD vs RCD for 3 days (Table 3A and B).

During the light/inactive phase at ZT 3, mice were subjected to 6 h of gentle handling to cause acute SD. Sleep-deprived mice displayed characteristic behavior during the 30-min intervals of maintained wakefulness. At the start, males would resist handling, continuously explore their cage with vigor, hide in their nest, and sample their food. After 2 h, mice started to display signs of SD. There was less resistance when being handled. Mice began sleeping out of the nest, and at times not huddled together as normally observed. Acute SD had significant effects on weight (decrease) and food intake (increase) among both diets (Table 3A and B). Total serum corticosterone levels increased 2.1-fold in both diets in the active phase which was ~4 to 10 h after SD ended (Fig. 1A). This increase was only significant in RCD mice

Table 3 (A) Average body weight gain and food intake over the course of 3–4 days in grams (g) per mouse by acute sleep deprivation (SD) and diet (regular chow (RCD) or high-fat (HFD)). (B) Single *t*-test comparisons of diet (first two columns) by acute sleep status (no SD vs SD), as well as acute sleep status by diet (RCD vs HFD) (last two columns).

(A)	No SD						SD					
	RCD			HFD			RCD			HFD		
Weight (g) per mouse	*n*	Mean	S.E.M.	*n*	Mean	S.E.M.	*n*	Mean	S.E.M.	*n*	Mean	S.E.M.
Average body weight gain	55	2.72	±0.22	55	2.94	±0.20	34	1.00	±0.11	34	1.48	±0.10
Average food intake	15	19.30	±0.70	18	23.21	±3.90	14	22.15	±1.00	14	36.26	±5.20
(B)	**HFD compared to RCD**			**SD compared to No SD**								
Weight (g) per mouse	**No SD**	**With SD**		**RCD**	**HFD**							
Average body weight gain	0.2188	↑ 0.0332[a]		↓ 0.0008[b]	↓ 0.0004[b]							
Average food intake	0.1731	↑ 0.0249[a]		↑ 0.0229[a]	↑ 0.0112[a]							

Mean values and S.E.M. are shown. Food intake in grams per mouse was calculated by taking the starting weight of the food in the cage on the first day and subtracting it from the weight taken on the third/fourth day and dividing that weight by the number of mice in the cage. Arrows represent the direction of change for HFD (when compared to RCD) or SD when compared to no SD. Adjusted *P*-values are shown; [a]$P < 0.05$, [b]$P < 0.001$.

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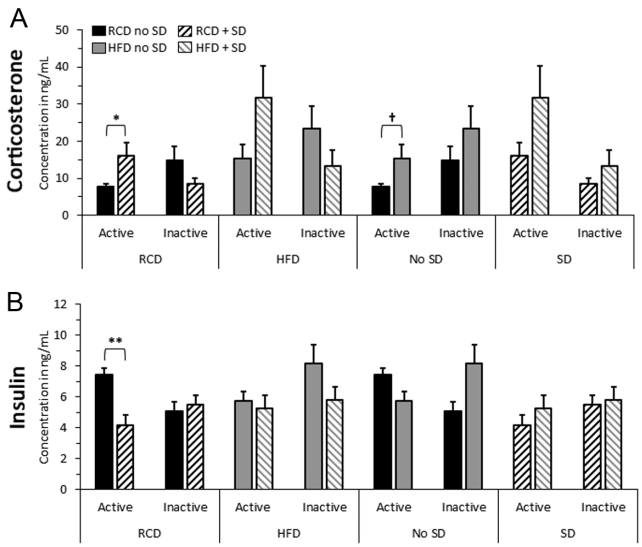

Figure 1
Serum concentrations in ng/mL of (A) corticosterone and (B) insulin by diet and acute sleep deprivation (SD) during the dark/active (Zeitgeber time (ZT) 14 + 20) and light/inactive (ZT 2 + 8) phases. Active phase represents serum sampling at ~4 to 10 h after SD ended, and inactive phase represents serum sampling at ~16 to 20 h after SD ended. Solid-filled bars represent no SD and diagonal pattern fill represents with SD. Black represents regular chow diet (RCD) while gray represents high-fat diet (HFD). n = 7–10 per group. Mean values and S.E.M. are shown. Eight single t-test comparisons were effectuated to compare no SD vs SD and RCD vs HFD. A trend is represented by †$P < 0.075$ and significance is represented by *$P < 0.05$, **$P < 0.01$.

(P = 0.0468, n = 8, 10) and was accompanied by a 44.3% decrease in serum insulin levels (P = 0.0048, n = 9) (Fig. 1B). These effects were no longer detectable in the inactive phase which was ~16 to 22 h after SD ended (Fig. 1A and B). By contrast, the apparent 2.1-fold increase in serum corticosterone levels in the active phase in HFD mice was not significant (P = 0.2648, n = 7, 8) (Fig. 1A). No effect of acute SD in HFD mice on serum insulin was detected in either the active or inactive phases after SD (Fig. 1B).

Acute SD and diet affect *Bmal1, Per2* and *hGH-N* RNA levels

Expression of circadian rhythm-related transcription factor genes, including *Bmal1, Clock, Cry1* and *Per2*, were assessed over 24 h, beginning at ZT 14 in mice fed a RCD and HFD (Fig. 2). Opposing oscillations of *Bmal1* and *Per2* RNA levels were seen with a peak and trough in the light/inactive phase (respectively) with both diets (Fig. 2), consistent with the presence of a daily cycle in the mouse pituitary. Under the time and conditions of assessment, no obvious changes were seen in clock-related gene RNA levels (Fig. 2).

Sleep loss is reported to reduce DNA binding of BMAL1/CLOCK in mouse brain (19) and, thus, may negatively affect *Cry/Per* RNA transcript levels. Given the opposing rhythmic patterns of *Bmal1* and *Per2* RNA levels observed (Fig. 2), the effect of SD during the dark/active and light/inactive phases among mice fed a RCD vs HFD was assessed (Fig. 3). For RCD, no significant decrease in *Bmal1* RNA levels was detected with acute SD. However, a 37.7% decrease was observed in HFD mice after acute SD during the dark/active phase, but this was not significant (P = 0.2552; n = 9, 10). A decrease in *Per2* RNA levels was suggested in RCD mice after acute SD during the dark/active phase, while a significant decrease was detected in the light/inactive phase (52.6%, P = 0.0076). Significant reductions in *Per2* transcript levels were also seen in HFD mice after acute SD in the dark/active phase (43.8%, P = 0.0106) and light/inactive phase (54.4%, P = 0.0420) of the daily cycle (Fig. 3).

GH synthesis and secretion also follow a circadian and diurnal rhythm, and levels are known to be affected by diet (6, 13). A significant decrease in relative RNA levels of *hGH-N* but not *mGh* transcript levels was observed during the dark/active phase (ZT 14+20; 74.6%, P = 0.0284, n = 9,

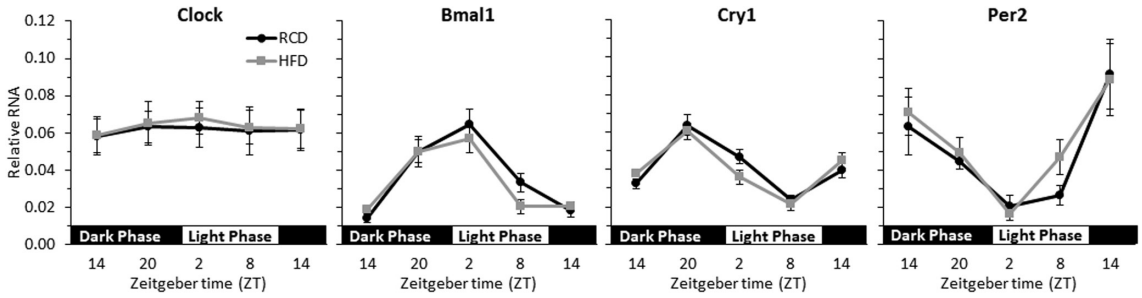

Figure 2
The effect of diet on mouse circadian rhythm transcription factors. *Clock, Bmal1, Cry1* and *Per2* RNA transcript levels normalized to *β-actin* RNA and shown as relative RNA across a 24-h cycle beginning 2 h into the dark/active phase (Zeitgeber time (ZT) 14 = 20:00 h). Black line represents regular chow diet (RCD) and gray line represents high-fat diet (HFD). n = 4, 5 per timepoint. Mean values and S.E.M. are shown.

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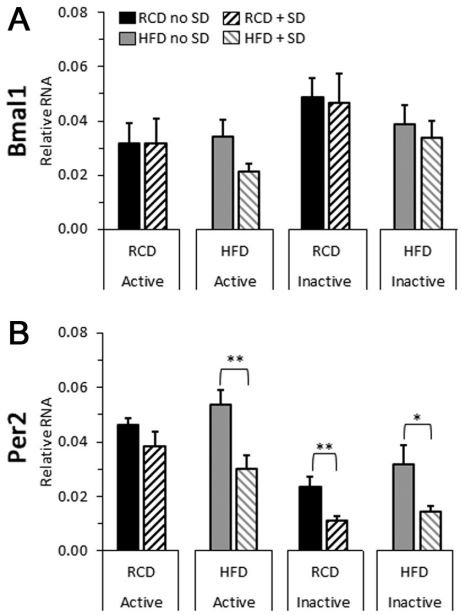

Figure 3
The effect of acute sleep deprivation (SD) on mouse circadian rhythm-related transcriptions factors (A) *Bmal1* and (B) *Per2* RNA levels during the dark/active (Zeitgeber time (ZT) 14–20) and light/inactive (ZT 2–8) phases in both regular control chow (RCD) and high-fat (HFD) diets. *Bmal1* and *Per2* RNA transcript levels normalized to *β-actin* RNA and shown as relative RNA. $n = 9, 10$ per timepoint. Mean values and S.E.M. are shown. Four single *t*-test comparisons were effectuated to compare no SD vs SD. Significance is represented by $*P < 0.05$, $**P < 0.01$.

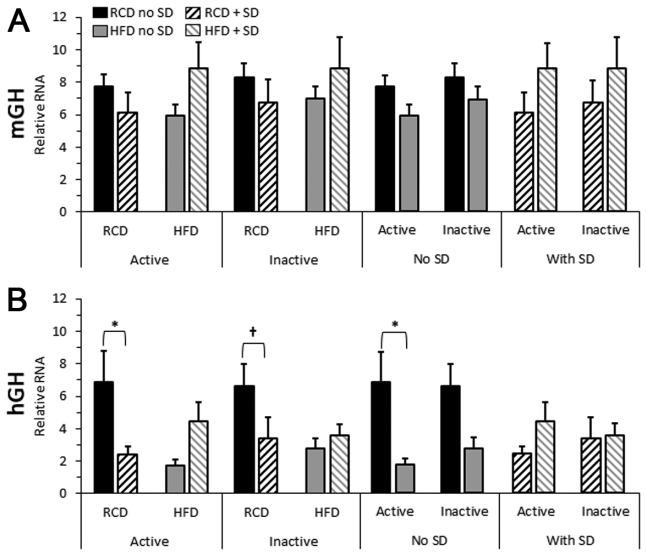

Figure 4
The effect of acute sleep deprivation (SD) on (A) mouse (m) and (B) human (h) growth hormone (GH) during the dark/active (Zeitgeber time (ZT) 14 + 20) and light/inactive (ZT 2 + 8) phases in both regular control chow (RCD) and high-fat (HFD) diets. RNA transcript levels for *mGh* and *hGH-N* normalized to *β-actin* RNA and shown as relative RNA. $n = 9, 10$ per timepoint. Mean values and S.E.M. are shown. Eight single *t*-test comparisons were effectuated to compare no SD vs SD and RCD vs HFD. A trend is represented by $^{\dagger}P < 0.075$ and significance is represented by $*P < 0.05$.

10) in *171hGH/CS* TG mice fed a HFD vs RCD. A 57.9% decrease was observed during the light/inactive phase (ZT 2+8) but this was not significant ($P = 0.1128$, $n = 9, 10$) (Fig. 4A and B). This is similar to a previous report, which found a significant decrease in *hGH-N* RNA levels during (ZT 2) but not throughout (ZT 6 or 10) the light/inactive phase, when comparing control diet to HFD (13). Acute SD was also associated with comparable reductions in *hGH-N* RNA transcript levels in mice fed a RCD but not HFD, during both dark/active (64.5%, $P = 0.0284$, $n = 9$) and light/inactive (49.0%, $P = 0.0699$, $n = 9, 10$) phases of the daily cycle (Fig. 4B).

Human *GH* RNA levels were also assessed over 24 h, beginning at ZT 14 in mice fed a RCD and HFD (Fig. 5). Different diurnal oscillations are observed among both diets and acute SD status. Significant changes were observed with acute SD among RCD mice, particularly at ZT 14 ($P=0.0278$) and ZT 8 ($P=0.0278$). Among HFD mice, there is no obvious diurnal cycle and no significant differences with acute SD (Fig. 5), suggesting a disruption as a result of a HFD vs RCD with acute SD.

DNA methylation of E-box-related elements in the hGH-*N* promoter and LCR are associated with both increased and decreased clock-related transcription factor binding

Interaction between *hGH-N* promoter and LCR HS I and II is required for efficient *hGH-N* expression (10, 33). Binding of members of the E-box transcription factor family to a putative E-box element (underlined) within the *hGH-N* proximal promoter region −264/−259 (*hGH-N* promoter E-box, 5′-GCACCCA**CG**TGACCCT-3′), has been suggested to modify this interaction (Fig. 6A) (13, 34). Specifically, changes in insulin-responsive hypoxia-inducible factor α (HIF-1α) binding *in vitro* and BMAL1/CLOCK heterodimer binding *in situ* have been linked to negative effects of HFD and excess insulin on *hGH-N* expression (13, 34). Examination of LCR HS I/II identified two putative E-box-like DNA elements specifically in HS II and outside the sequences encompassing HS I (35). Like the *hGH-N* promoter E-box element, both 5′-GGGCACA**CG**TGTTTGTG-3′ (HS II E-box 1) and 5′-AGAAGGA**CG**TGGGTTTG-3′ (HS II E-box 2) contain a CpG dinucleotide (**bold**) in their core sequences (Fig. 6B).

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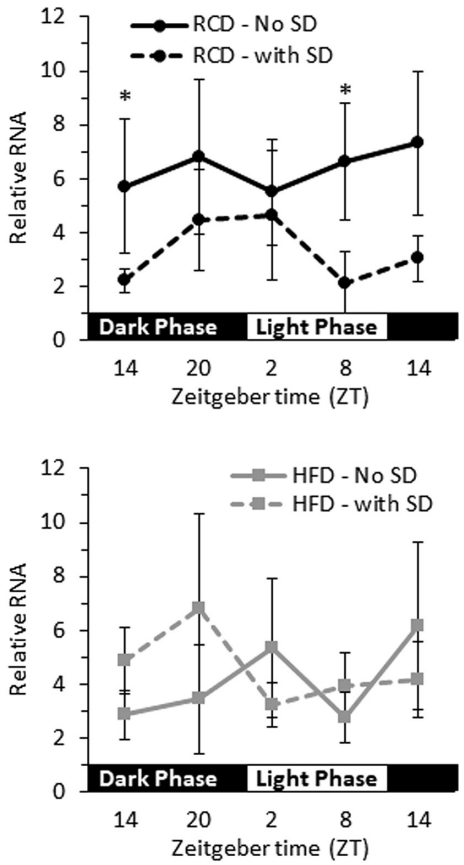

Figure 5
The effect of acute sleep deprivation (SD) on human (h) growth hormone (GH) RNA levels. Transcript levels were normalized to *β-actin* RNA and shown as relative RNA across a 24-h cycle beginning 2 h into the dark/active phase (Zeitgeber time (ZT) 14 = 20:00 h). Black solid line represents regular chow diet (RCD) and gray solid line represents high-fat diet (HFD). Dashed lines represent ± acute SD. $n = 5$ per timepoint. Mean values and S.E.M. are shown. Ten single *t*-test comparisons were effectuated to compare no SD vs SD among the timepoints. Significance is represented by $*P < 0.05$.

Bisulfite conversion and Illumina sequencing were used to assess the methylation status of CpG sites within the *hGH-N* promoter and LCR HS II sequences in the *171hGH/CS* TG mouse pituitary *in situ*, which include putative *hGH* promoter E-box, and HS II E-box 1 and E-box 2 (Fig. 6). Ten CpG sites were identified within the *hGH-N* promoter region −494/+1, including promoter E-box that corresponds to CpG 8 (Fig. 7A). CpG 1 and CpG 8 were relatively hypomethylated compared to the other eight CpG sites under conditions of acute SD and diet (Fig. 7A). Among CpG 8, no considerable effect was observed for acute SD, however, a 1.8-fold decrease in CpG methylation in HFD vs RCD was observed during the light/inactive phase (Fig. 8A). Ten CpG sites were identified in the LCR HS II sequence where CpG 1 is found in putative HS II E-box 1 and CpG 4 is found in HS II E-box 2 (Figs 6 and 7B). CpG 4 was relatively hypomethylated under all conditions of acute SD and diet, relative to other sites including CpG 1 (Fig. 7b). Among CpG 4, no clear effect was observed for acute SD in mice fed a HFD, however acute SD was associated with 1.3-fold and 1.7-fold increases in methylation in the dark/active and light/inactive phases (respectively) in mice fed a RCD (Fig. 8B). A 1.6-fold and 1.3-fold increase in methylation in the dark/active and light/inactive phases (respectively) was also observed in mice fed a HFD vs RCD (Fig. 8B).

The effect of CpG methylation in the core sequences of HS II E-box 1 and E-box 2 as well as *hGH-N* promoter E-box, on clock-related E-box factor binding was assessed by EMSA. This was done using unmethylated vs methylated radiolabeled oligonucleotide probes (Table 2) with nuclear extract enriched in BMAL1/CLOCK proteins (Fig. 9). A low mobility complex that increased in intensity with BMAL1/CLOCK overexpression was detected using unmethylated HS II E-box 1 oligonucleotide. A series of at least four low to high mobility complexes were detected using unmethylated *hGH-N* promoter E-box (thick arrow, Fig. 9, Lane 3), including a major complex with a mobility comparable to the predominant low mobility complex seen using unmethylated HS II E-box 1 as a probe (thick arrow, Fig. 9, Lane 9). However, these low mobility complexes related to *Bmal1/Clock* overexpression were decreased in intensity (Fig. 9, Lane 6) or absent (Fig. 9 Lane 12) when methylated *hGH-N* promoter E-box and HS II E-box 1 were used as probes. By contrast, there was little evidence of binding to unmethylated HS II E-box 2 (thin arrow, Fig. 9, Lanes 14 and 15), however, a low mobility complex was detected with greater intensity when methylated HS II E-box 2 was used as a probe (thin arrow, Fig. 9, Lanes 17 and 18).

Discussion

Based on studies using partially humanized *171 hGH/CS* TG mice, evidence is presented that supports extending a negative effect of acute SD on hGH production at the level of synthesis, and more specifically *hGH-N* RNA accumulation. For the first time, these mice expressing *hGH-N* in a pituitary-specific and rhythmic manner were used to assess the effect of acute SD on *hGH-N* RNA levels in active and inactive phases of the daily cycle, based on conservation of clock-related transcription factors and thus circadian rhythm machinery in mammals (36).

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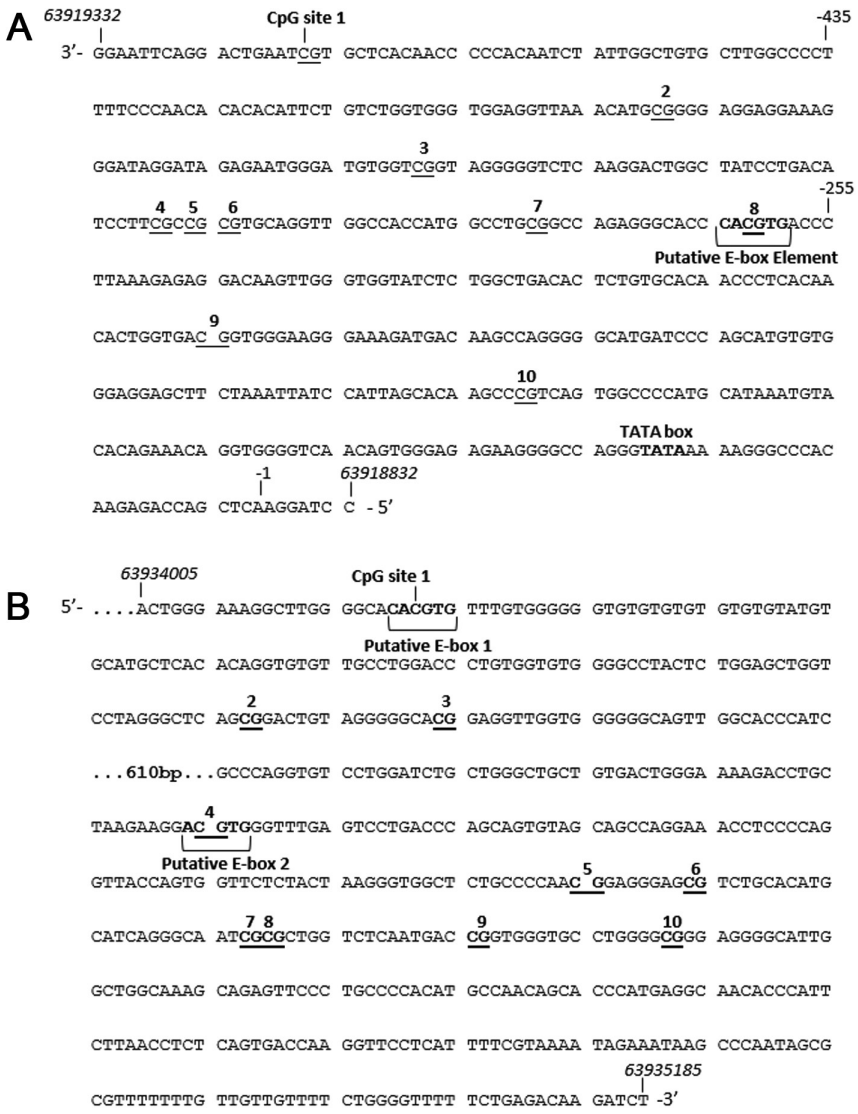

Figure 6
(A) Human growth hormone gene promoter region sequence (negative strand; 501bp; chr17: 63918832–63919332), and (B) locus control region hypersensitive site II sequence (positive strand; 1181 bp; chr17: 63934005–63935185). Identified CpG (cytosine next to guanine) sites with methylation status are underlined. Putative E-box DNA elements are in bold and identified. TATA box is also in **bold**.

The possible influence of HFD was also explored as well as the potential for DNA methylation to affect clock-related transcription factor binding. To our knowledge, this is the first study to use a murine system to observe the effect of SD on hGH synthesis, as studies on *hGH-N* RNA levels in the pituitary are not possible in living humans. As expected, the consumption of a HFD for 3–4 days did not cause significant weight gain (12, 13), but there was evidence of stress due to acute SD that was independent of diet. SD was associated with significant weight reduction and stimulated food intake. A clear trend toward increases in serum corticosterone levels as well as decreased insulin were also detected. Stress is expected to increase glucocorticoid levels in mice (37), and lower insulin presumably contributes to maintenance of glucose or energy levels under the stress of SD. Glucocorticoids have been shown to increase GH levels (38, 39, 40), as well as in the context of HFD *in vitro* (41). Glucocorticoids are also involved in circadian rhythms by activating transcription factors through their binding at glucocorticoid response elements within genes, and are themselves subject to circadian control (42, 43).

Expression of circadian rhythm transcription factor genes, *Bmal1, Clock, Cry 1,* and *Per 2*, were assessed every 6 h over 24 h, beginning 2 h into the dark/active phase at ZT 14 in both HFD and RCD mice. Opposing oscillations of *Bmal1* and *Per2* RNA levels were seen with a peak and trough in the light/inactive period, respectively, consistent with the presence of a daily cycle in the mouse pituitary. Mouse brain *Per2* expression is known to be sensitive to SD and time of day (19, 20). This response appears to extend to the pituitary, as SD caused significant decreases in *Per2* RNA levels, which was independent of diet. There was also an observed (but not significant)

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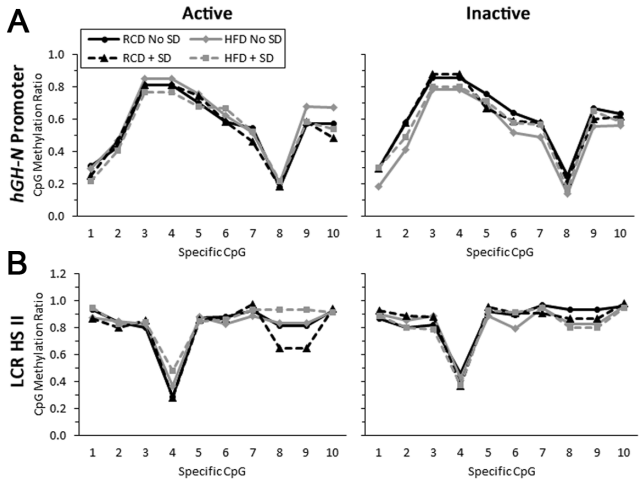

Figure 7
Bisulfite conversion and Illumina sequencing of DNA extracted from mouse pituitary samples by acute sleep deprivation (SD) as well as by regular control chow diet (RCD) or high-fat diet (HFD). The methylation status of ten CpG dinucleotides was identified in (A) the *hGH-N* promoter sequence as well as in (B) the locus control region (LCR) hypersensitive site II (HS II). *hGH-N* promoter CpG 1 and CpG 8 as well as LCR HS II CpG 4 demonstrated hypomethylation. Mean CpG methylation ratios are shown which represent the number of methylated CpGs over total CpGs. $n = 2$ per CpG.

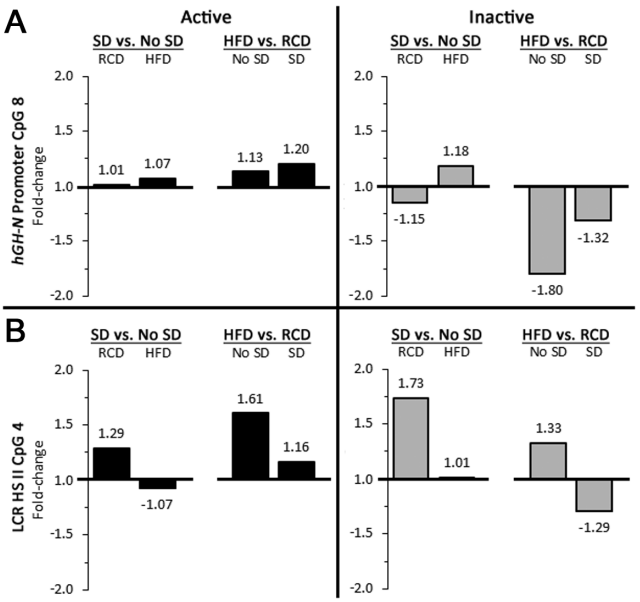

Figure 8
Fold-effects of acute sleep deprivation (SD) and diet on (A) the *hGH-N* promoter CpG 8, as well as (B) the locus control region (LCR) hypersensitive site II (HS II) CpG 4 which are located within the BMAL1/CLOCK E-box binding element. CpG methylation fold-change is shown by acute SD (SD vs No SD) as well as by high-fat diet (HFD) (HFD vs Regular Chow Diet (RCD)) in both the dark/active and light/inactive phases (respectively). A fold increase is represented by a positive number (black fill) while a fold decrease is represented by a negative number (white fill).

decrease of *Bmal1* RNA levels among HFD mice only in the dark/active phase, which is consistent with a negative effect of BMAL1/CLOCK heterodimer availability reported previously in mouse brain following SD (21). A decrease in BMAL1 availability would be expected to result in decreased *hGH-N* promoter activity (13).

Acute SD resulted in significant decreases in *hGH-N* but not *mGh* RNA accumulation in RCD mice, which was independent of assessment in the light/inactive or dark/active phase of the daily cycle. This is consistent with a negative effect of acute SD on hGH synthesis as well as secretion (4). A comparable decrease in *hGH-N* RNA levels was also seen with HFD vs RCD in the dark/active phase of the daily cycle. These effects of acute SD and HFD do not appear to be additive as there was no further decrease in *hGH-N* RNA levels with SD for HFD mice. This would also be consistent with both SD and HFD impacting *hGH-N* expression through a common pathway. Previously, the lack of response of endogenous *mGh* to HFD was used to support the possible involvement of an E-box element that is present at nucleotides -264/-259 in the promoter region of *hGH-N* but not *mGh* (13, 34). Similarly, *mGh* RNA levels were not affected significantly by acute SD. Thus, given the similarity of response to SD and HFD and no evidence of an additive effects, it is possible that the *hGH-N* promoter E-box element represents a target of both SD and HFD. The presence of this E-box element may explain the difference in the response of hGH and mGH genes observed, in spite of the common pituitary (nuclear transcription factor) environment (44). The presence of this E-box element would also explain why both *hGH-N* and *mGh* gene expression are negatively regulated by insulin in the same *171 hGH/CS* TG mouse pituitary cells, but *hGH-N* appears to be more sensitive to insulin levels possibly via an additional independent mechanism (34).

Human *GH-N* RNA levels demonstrated variability between mice when assessed over a 24-h period. RCD mice did not demonstrate a *hGH* RNA peak and trough at ZT 2 and ZT 14, respectively, as previously reported (13); however, a similar oscillation in *hGH* synthesis was suggested with acute SD in RCD but not HFD mice. This may reflect a synchronizing effect of SD on the diurnal cycle in mice on RCD that is also disrupted by HFD.

Bisulfite conversion and Illumina sequencing identified methylated CpG dinucleotides located within the *hGH-N* promoter E-box element at nucleotides

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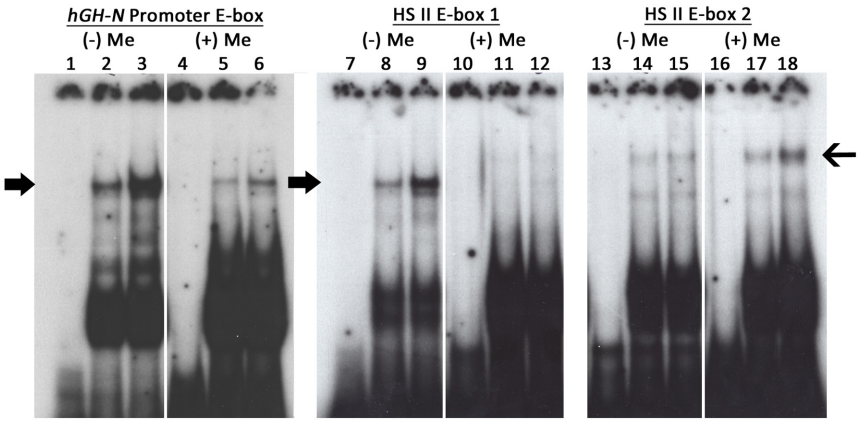

Figure 9
EMSA results using (–) Me (unmethylated) and (+) Me (methylated) oligonucleotides (probes) for putative BMAL1/CLOCK E-box DNA binding element in the *hGH-N* promoter and putative E-box 1 and E-box 2 in locus control region (LCR) hypersensitive site II upstream of *hGH-N* promoter. Lanes 1, 4, 7, 10, 13, and 16 represent free probe/radioactive oligonucleotides only, lanes 2, 5, 8, 11, 14, and 17 represent nuclear extract from HEK293 cell transfected with an empty vector and lanes 3, 6, 9, 12, 15, and 18 represent nuclear extract from HEK293 overexpressing *BMAL1/CLOCK*. Arrows point to the low mobility DNA bound by protein complexes.

-264/-259, but also related putative E-box-like elements 1 and 2 within HS II (HS II E-box 1 and E-box 2). The core E-box hexanucleotide sequence (5′-CACGTG-3′) containing a central CpG is identical in both *hGH-N* promoter E-box and HS II E-box 1, however, only *hGH-N* promoter E-box CpG was relatively hypomethylated. By contrast, the core sequence of HS II E-box 2, which shares five of six nucleotides with the core of *hGH-N* promoter E-box, was also relatively hypomethylated. However, while a decrease in the CpG methylation ratio at *hGH-N* promoter E-box was suggested during the light/inactive phase in HFD mice, the opposite effect (increased ratio) was suggested at HS II E-box 2 in the dark/active and light/inactive phases. In addition, when the data are re-plotted to assess fold-effects of acute SD in the absence of HFD at a specific CpG, an increase in CpG methylation of the HS II E-box 2 sequence is also suggested in both the dark/active and light/inactive phases among RCD. Thus, a trend toward increased CpG methylation within HS II E-box 2 sequences correlates with the common decrease in *hGH-N* RNA levels suggested with acute SD and HFD in *171hGH/CS* TG mice.

Interestingly, while an increase in CpG methylation of *hGH-N* promoter E-box was associated with decreased binding of nuclear protein from HEK293 cells overexpressing *Bmal1*/*Clock*, an increase in binding was detected with HS II E-box 2 sequences under the same conditions. HS I/II represents a pituitary enhancer region (31) and when methylated, would be expected to repress gene expression since CpG dinucleotides located in a gene body typically leads to increased gene expression while CpGs located in promoters or enhancers leads to transcriptional silencing in the case of hypermethylation (23). However, in addition to changing the chemical signature of CpGs to regulate gene expression, cytosine methylation can also affect DNA structure (23, 45). Direct contact of a protein to a methyl group could obliterate or improve binding through formation or alteration of van der Waals interactions (45). Therefore, CpG methylation could very well enhance transcription factor binding and evoke changes to chromatin to facilitate or repress gene expression. Interestingly, a recent study has shown CCAAT/enhancer-binding protein β binds the sequence 5′-TTGCGCAA-3′ with greater affinity when the central CpG dinucleotide (underlined) is methylated relative to the unmodified sequence *in vitro* (46). A role for DNA methylation also complements previous studies implicating epigenetic control through posttranslational modification of histones, including acetylation and methylation, in the regulation of *hGH-N* expression (33, 47, 48, 49, 50).

Taken together, this is the first report that hGH synthesis is negatively affected by acute SD, and that DNA methylation may play a role in regulating *hGH-N* expression through increased methylation at a central CpG in a putative E-box (5′-CACGTG-3′) within the hGH LCR at HS II *in situ*, which in turn increases protein-DNA binding *in vitro*.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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