**Abnormal Circadian Phenotype in Clock Mutant Mice**

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The CLOCK transcription factor is a key component of the molecular circadian clock within pacemaker neurons of the hypothalamic suprachiasmatic nucleus. We found that homozygous Clock mutant mice have a greatly attenuated diurnal feeding rhythm, are hyperphagic and obese, and develop a metabolic syndrome of hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia, and hypoinsulinemia. Expression of transcripts encoding selected hypothalamic peptides associated with energy balance was attenuated in the Clock mutant mice. These results suggest that the circadian clock gene network plays an important role in mammalian energy balance.

Major components of energy homeostasis, including the sleep-wake cycle, thermogenesis, feeding, and glucose and lipid metabolism, are subjected to circadian regulation that synchronizes energy intake and expenditure with changes in the external environment imposed by the rising and setting of the sun. The neural circadian clock located within the hypothalamic suprachiasmatic nucleus (SCN) orchestrates 24-hour cycles in these behavioral and physiological rhythms (1–3). However, the discovery that clock genes can regulate circadian rhythm in vitro in other central as well as peripheral tissues, including those involved in nutrient homeostasis (e.g., mediobasal hypothalamus, liver, muscle, and pancreas), indicates that circadian and metabolic processes are linked at multiple levels (4–9). The recent finding that changes in the ratio of oxidized to reduced nicotinamide adenine dinucleotide phosphate control the transcriptional activity of the basic helix-loop-helix (bHLH) protein NPAS2—a homolog of a primary circadian gene, Clock—suggests that cell redox may couple the expression of metabolic and circadian genes (10). Mice harboring a mutation in Clock show profound changes in circadian rhythm (11) and offer an experimental genetic model to analyze the link among circadian gene networks, behavior, and metabolism in vivo.

Positional cloning and transgenic rescue of normal circadian phenotype identified Clock as a member of the bHLH Per-Arnt-Sim (PAS) transcription factor family (12, 13). Relative to wild-type mice, the most pronounced alteration in circadian phenotype in Clock mutants is a 1-hour increase in the free-running rhythm of locomotor activity in heterozygous mice in constant darkness (DD) and a 3- to 4-hour increase (i.e., period = 27 to 28 hours in DD) in circadian period in homozygous mice, which is often followed by a total breakdown of circadian rhythmicity (i.e., arrhythmicity) after a few weeks in DD.

Although previous studies that used running wheel behavior as a marker of locomotor activity did not reveal major differences between homozygous Clock mutant and wild-type mice maintained on a light-dark (LD) cycle, use of infrared beam crossing to monitor total activity revealed a significant increase in activity during the light phase and a change in the temporal pattern of total activity during the dark phase (Fig. 1A) (14). In particular, wild-type mice showed two pronounced peaks of activity—one occurring after lights off, the other before lights on—whereas these peaks were attenuated in Clock mutant mice. Surprisingly, despite there being a clear (but dampened) diurnal rhythm in locomotor activity in Clock mutant mice (Fig. 1B), the diurnal rhythm in food intake was severely altered in these mice. The altered diurnal rhythm in food intake was even more pronounced in Clock homozygous mice maintained on a 12:12 LD cycle (Fig. 1C).

**Fig. 1.** Altered diurnal rhythms in locomotor activity, feeding, and metabolic rate in Clock mutant mice. (A) Activity counts over the 24-hour cycle during light (unshaded) and dark (shaded) periods [wild-type, n = 5; black line; Clock, n = 9, blue line]. Inset: Actograms showing locomotor activity over a 30-day period in representative adult wild-type (top) and Clock mutant (bottom) mice individually housed in 12:12 LD (at 23°C) and provided food and water ad libitum. Activity bouts were analyzed using ClockLab software in 6-min intervals across 7 days of recording (selected days are indicated by red vertical lines to the left of the actograms). (B) Diurnal rhythm of locomotor activity for mice in (A). Activity counts were accumulated over the 12-hour light and 12-hour dark periods and are expressed in each period as a percentage of total 24-hour activity (*P < 0.05). Total activity over the 24-hour period was similar between wild-type (WT) and Clock mutant (CL) genotypes. (C) Diurnal rhythm of food intake. Different groups of adult WT (N = 7) and Clock mutant (N = 5) mice were maintained on a regular diet (10% kcal/fat), and food intake (in grams) was measured during light and dark periods. Results shown are average food intake during light and dark periods as a percentage of total food intake (*P < 0.001). (D) Diurnal rhythm of metabolic rate. Metabolic rate was determined in additional groups of WT (N = 7) and Clock mutant (N = 9) mice by indirect calorimetry under 12:12 LD conditions over a 3-day continuous monitoring period (*P < 0.05). Results shown are average metabolic rates during the light and dark periods as a percentage of total metabolic rate. All results shown are expressed as group means ± SEM.
mice (Fig. 1C): Only 53% of the food intake occurred during the dark phase in Clock mutant mice, versus 75% in wild-type mice. In a preliminary analysis, we found that the diurnal rhythm of food intake was already attenuated in 3-week-old mice before an increase in weight (Fig. S1). Similarly, the rhythm in energy expenditure, as measured by respiratory gas analysis, was attenuated in the Clock mutant mice (Fig. 1D). Overall there was a net 10% decrease in energy expenditure in the mutants.

Clock mutant mice fed either a regular or high-fat diet showed a significant increase in energy intake and body weight (Fig. 2, A and B). In Clock mutant and wild-type adult mice fed either a control or high-fat diet for a period of 10 weeks beginning at 6 weeks of age (Fig. 2C), the increase in body weight was 24% in wild-type and 29% in Clock mutant mice fed a regular diet, versus 38% in wild-type and 49% in Clock mutant mice fed a high-fat diet. Comparison of somatic growth and solid organ mass did not reveal genotype-specific differences. Instead, the marked weight gain in Clock mutants fed a regular diet was attributable to a 65% increase in lean mass and a 35% increase in fat mass, whereas in mutants fed a high-fat diet, the weight gain was due to a 25% increase in lean mass and a 75% increase in fat mass relative to wild-type control mice (fig. S2).

Because the Clock mutation could affect early fetal growth and development, we analyzed the body weights of Clock and littermate pups throughout the first 8 weeks of life. Body weights were similar in Clock mutant and wild-type mice during the first 5 weeks, but by 6 weeks of age Clock mutant mice were consistently heavier (Fig. 2D), which suggests that the mutation did not affect fetal growth or nutrition.

We next investigated whether the Clock mutation altered the adipose–central nervous system (CNS) axis that regulates feeding and energy expenditure. Histological analysis revealed adipocyte hypertrophy and lipid engorgement of hepatocytes with prominent glycogen accumulation (fig. S3A) in Clock mice fed a high-fat diet relative to wild-type controls; these are hallmarks of diet-induced obesity in wild-type mice. At 6 to 7 months of age, Clock mutant mice also had hypercholesterolemia, hypertriglyceridemia, hyperglycemia, and hypoinsulinemia (Table 1). In addition, serum leptin levels increased during the light phase in Clock mutant mice fed a regular diet; this increase was enhanced in mice fed a high-fat diet (fig. S3B). These markers of metabolic dysregulation were not due to an increase in glucocorticoid production, because levels of corticosterone were lower in the Clock mutant mice across the 24-hour LD cycle (wild-type, 5.5 ± 1.4 μg/dl; Clock mutant, 2.6 ± 0.4 μg/dl; P < 0.05). Thus, the Clock mutant mice developed a spectrum of tissue and biochemical abnormalities that are hallmarks of metabolic disease.

To explore whether the Clock mutation affects expression of neuropeptides involved in appetite regulation and energy balance, we analyzed transcripts corresponding to selected orexigenic and anorexigenic neuropeptides expressed in the mediobasal hypothalamus (MBH). We studied the orexin transcript because the orexigenic system is involved in both feeding and sleep-wake regulation (15, 16); we also studied the transcripts for ghrelin and CART (cocaine- and amphetamine-regulated transcript) because the corresponding genes contain CLOCK-responsive E-box elements (17, 18). In addition, we examined the expression of a second circadian clock gene, Per2, which has a diurnal rhythm of expression in the retrochiasmatic area. The expression levels of Per2, orexin, and ghrelin mRNA were markedly reduced in Clock mutant mice at virtually all time points of the 12L:12D cycle (Fig. 3). A small but significant decrease in the expression level of CART in Clock mutant mice occurred at the beginning and end of the 12-hour light phase (Fig. 3).

These broad effects of the Clock gene mutation on nutrient regulation reveal an unforeseen role for the circadian clock system in regulating more than just the timing of food intake and metabolic processes.

Table 1. Metabolic parameters in WT and Clock mutant mice. Serum triglyceride, cholesterol, glucose, insulin, and leptin concentrations were determined in 7- to 8-month-old WT and Clock mutant mice fed a regular diet ad libitum (n = 4 to 8 mice per group). For measurement of glucose, insulin, and leptin, blood was collected at 4-hour intervals over a 24-hour time period via an indwelling catheter (40 μl per blood sample), and the data were pooled to provide an overall mean (±SEM) value. For triglyceride and cholesterol measurement, a single blood sample (160 μl) was collected at zeitgeber time 0.

<table>
<thead>
<tr>
<th>Metabolic parameter</th>
<th>WT</th>
<th>Clock</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>136 ± 8</td>
<td>164 ± 8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>141 ± 9</td>
<td>163 ± 6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>130 ± 5</td>
<td>161 ± 7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>3.4 ± 0.4</td>
<td>4.6 ± 0.3</td>
<td>&lt;0.05</td>
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the Clock mutation on body weight in mice fed a regular diet was similar in magnitude to the effect of a high-fat diet in wild-type mice (Fig. 2). In addition, when Clock mutant mice were fed a high-fat diet, the combined effect of the diet plus mutation led to the most severe alteration in body weight and markers of metabolism (Fig. 2). Note that the weight gain in the Clock mutant mice is similar to that observed in a number of metabolic mutants (19, 20).

Alterations in fuel metabolism in animals carrying a mutant circadian Clock gene could emerge from a cascade of neural events initiated by an alteration in circadian rhythms under the direct control of the SCN (21), in particular the feeding rhythm, which is greatly attenuated in Clock mutant mice. Thus, the misalignment of food intake and/or the near-loss of feeding rhythmicity could create metabolic instabilities that lead to hyperphagia and associated obesity and lipid/glucose irregularities. On the other hand, because circadian clock genes are also expressed in nearly all CNS and peripheral tissues, alterations in metabolism could be due to cell-autonomous effects associated with altered expression of Clock in CNS feeding centers and/or peripheral tissues involved in metabolism (5, 22). The observation that mRNAs of selected energy-regulatory peptides are altered in both diurnality and absolute expression level in the MBH supports the idea of a molecular coupling between circadian and metabolic transcription networks. These results are consistent with the recent finding that in addition to regulating the timing of many genes, the circadian clock regulates the absolute expression levels of approximately 3 to 10% of transcripts (23–25).

Clues to the effects of the Clock mutation on energy balance may be derived from the emerging map of SCN projections to critical energy centers within the hypothalamus. For example, SCN projections form synapses directly on lateral hypothalamic area neurons that express orexins (26), as well as indirectly via the subparaventricular nuclei (SPV). Additional evidence suggests that connections between the SCN and neurons within the MBH may have important effects on cell and molecular functions. Specifically, recent analyses from several groups have indicated that the growth hormone agonist ghrelin, originally discovered as an incretin hormone within the stomach, may also be produced within the MBH/SPV (27, 28). Our real-time polymerase chain reaction (PCR) results provide further support for expression of ghrelin within the MBH. Remarkably, we find that ghrelin mRNA is greatly reduced in the MBH from Clock mutant mice, which suggests that signaling from SCN neurons and/or expression of the Clock gene within the MBH may play a critical role in transcriptional control of target genes within the MBH. Similarly, we found that orexin levels were lower in Clock mutant than wild-type mice, and the normal diurnal variation in expression was abolished.

Previous transcriptome analysis in the SCN and liver of Clock mutant mice has uncovered global changes in metabolic pathways, including those encoding enzymes of glycolysis, mitochondrial oxidative phosphorylation, and lipid metabolism (29). The connection between metabolism and circadian rhythmicity is particularly intriguing in view of the finding that genes involved in mitochondrial redox metabolism account for a large fraction of the circadian transcriptome in most tissues (10). Although these earlier results indicated that cell redox flux can alter the molecular circadian core machinery, our results in Clock mutant mice indicate that alterations in this molecular clock may alter cell metabolism as well.

**Fig. 3.** Clock mutant mice display altered diurnal mRNA expression from selected hypothalamic peptides involved in energy balance (A to D). Real-time PCR was used to determine transcript levels as they varied across a 12:12 LD cycle (indicated by bar at bottom). Values for WT (red line) and Clock mutant (black line) mice are displayed as relative abundance (RA; mean ± SEM) after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels in the same sample. Note that for visual clarity the RA scales vary for the different transcripts and vary between genotypes for orexin (Orx) and ghrelin (Ghr). Brains of four WT and four Clock mutant mice were collected at 4-hour intervals across the 12:12 LD cycle. At each time point, genotype comparisons were made by independent-sample t tests (*P < 0.05). CART, cocaine- and amphetamine-regulated transcript.

References and Notes
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