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Obesity and Metabolic Syndrome in Circadian *Clock* Mutant Mice

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Abstract

The bHLH-PAS transcription factor, CLOCK, is a key component of the molecular circadian clock within pacemaker neurons of the hypothalamic suprachiasmatic nucleus. Here we report 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 and hyperglycemia, with insufficient compensatory insulin production, a hallmark of type 2 diabetes mellitus. In addition, the levels of expression of hypothalamic peptides associated with energy balance were greatly attenuated in the *Clock* mutant animals. Taken together, these results indicate that the circadian clock gene network plays an important role in mammalian energy balance that involves a number of central and peripheral tissues, and disruption of this network can lead to obesity and the metabolic syndrome in mice.

Major components of energy homeostasis, including the sleep-wake cycle, thermogenesis, feeding, 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 hr cycles in these behavioral and physiological rhythms [1–3]. However, the discovery that clock genes can regulate circadian rhythmicity *in vitro* in other central, as well as peripheral tissues, including those involved in nutrient homeostasis (e.g., mediobasal hypothalamus, liver, muscle, pancreas), indicates that circadian and metabolic processes are linked at the systems, cellular and molecular levels [4–8]. The recent finding that changes in the ratio of oxidized to reduced NAD(P) controls transcriptional activity of the basic helix-loop-helix (bHLH) protein, NPAS2, a homologue of a primary circadian gene, *Clock*, suggests that cell redox may couple the expression of metabolic and circadian genes [9, 10]. The identification of the *Clock* mutant mouse which shows profound changes in circadian rhythmicity [11] offers an

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experimental genetic model to analyze the link between 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]. The most pronounced alteration in circadian phenotype in *Clock* mutant animals, compared to wild-type (WT) mice, is a 1-hour increase in the free-running rhythm of locomotor activity in heterozygous animals in constant darkness (DD) and a 3–4 hour increase (i.e. period = 27–28 hours in DD) in circadian period in homozygous animals, which is often followed by a total breakdown of circadian rhythmicity (i.e. arrythmicity) after a few weeks in DD. Here we report that mice homozygous for the *Clock* mutation show an attenuated diurnal rhythm of feeding behavior, as well as profound changes in body weight regulation and fuel metabolism, which leads to obesity and markers indicative of diabetes.

While previous studies using running wheel behavior as a marker of locomotor activity did not reveal major differences in *Clock* mutant and WT mice maintained on a light-dark (LD) cycle, use of infra-red beam crossing to monitor total activity revealed a significant increase in activity in the light phase, and a change in the temporal pattern of total activity in the dark phase (Fig. 1A). In particular, while WT animals showed two pronounced peaks of activity, one occurring after lights off, the other prior to lights on, these peaks were attenuated in *Clock* mutant animals. Surprisingly, despite there being a clear (but dampened) diurnal rhythm in locomotor activity in *Clock* mutant animals (Fig. 1B), the diurnal rhythm in food intake was severely altered in the *Clock* mutant animals (Fig. 1C), such that 53% of the food intake occurred during the dark in *Clock* mutant animals, while in WT mice 75% occurred during the dark. Similarly, the rhythm in energy expenditure, as measured by respiratory gas analysis, was also attenuated in the *Clock* mutant animal (Fig 1D). Overall there was a net (10%) decrease in energy expenditure in *Clock* mutant animals.

In addition to an alteration in the diurnal pattern of food intake, *Clock* mutant animals fed either a regular or a high fat diet showed a significant increase in energy intake and body weight compared to WT controls (Fig 2A, 2B). Fig. 2C shows the time course for the weight gain in *Clock* mutant and WT adult animals fed either a control or high fat diet for a period of 10 weeks beginning at 6 weeks of age. 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 or high fat diet was specifically due to visceral adiposity (Fig. 2D, E) with about a 20–25% increase in lipid content on either diet.

Because the *Clock* mutation could affect early fetal growth and development, we analyzed body weight in *Clock* and littermate pups throughout the first eight weeks of life. Body weights were similar in *Clock* mutant and wild-type animals during the first five weeks of life, but by 6 weeks of age *Clock* mutant animals were significantly heavier (Fig 2F), suggesting that the mutation did not effect fetal growth or nutrition. In a preliminary analysis, we found that the diurnal rhythm of food intake was already attenuated in 3 week old animals prior to any evidence of an increase in weight gain (See Supplemental Fig 1).

To determine if differences in weight and increases in adiposity in the *Clock* mutant mice were associated with changes in the regulation of fuel homeostasis, we sought to determine whether the *Clock* mutation altered the adipose-CNS axis that regulates feeding and energy expenditure. Histological analysis revealed adipocyte hypertrophy and lipid engorgement of hepatocytes with prominent glycogen accumulation (Fig. 3A) in *Clock* animals fed a high-fat diet; hallmarks of diet-induced obesity in WT animals. When measured at 6–7 months of age, *Clock* mutant animals also had hypercholesterolemia, hypertriglycerdemia, hyperglycemia and hypoinsulemia (Table 1). In addition, serum leptin levels increased

during the light phase in *Clock* mutant animals fed a regular diet; this increase was accentuated in animals fed a high fat diet (Fig. 3B). These markers of metabolic dysregulation were not due to an increase in glucocorticoid production because levels of corticosterone were actually lower in the *Clock* mutant animals across the 24 hr LD cycle (WT = 5.5 ± 1.4 μg/dl *Clock* mutant = 2.6 ± 0.4, $P < .05$). Moreover, changes in blood lipid levels were correlated with tissue signs of lipid overload and glycogen accumulation in the liver, as well as with marked adipose hypertrophy compared to wild-type animals (Fig. 3A). Thus, the *Clock* mutant developed a spectrum of tissue and biochemical abnormalities that are hallmarks of metabolic disease.

To test the hypothesis that obesity and metabolic dysregulation in the *Clock* mutant animals are associated with altered expression of neuropeptides involved in appetite regulation and energy balance, we analyzed transcript levels of known orexigenic and anorexigenic neuropeptides in the mediobasal hypothalamus (MBH) at 4-hr intervals across the light-dark cycle in wild-type and *Clock* homozygous mutant mice. For this analysis, we focused on the orexin transcript, because the orexinergic system is involved in both feeding and sleep-wake regulation [14, 15]. We also focused on *ghrelin* and *CART*, since these transcripts contain CLOCK responsive E-box elements [16, 17]. In addition, we examined the expression of a second circadian clock gene, *Per2*, a gene known to have a diurnal rhythm of expression in the retrochiasmatic area. The expression levels of *Per2*, *orexin* and *ghrelin* were dramatically reduced in *Clock* mutant mice at virtually all time points of the 12L:12D cycle (Figure 4). A small, but significant decrease in the expression level of *CART* in *Clock* mutant mice occurred during the beginning and end of the 12-hr light phase (Figure 4).

The present findings demonstrate that *Clock* mutant animals develop obesity, hyperphagia, reduced energy expenditure, adiposity, as well as dysregulation of glucose and lipid metabolism. The results also reveal significant changes in the expression of mRNAs encoding the major neuropeptides that regulate feeding and energy expenditure within the hypothalamus. 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. The breadth of effects on metabolism at many different levels of organization makes the *Clock* mutant animal a unique model to extend analysis of behavior and fuel homeostasis from the complex neural system level down to cell and molecular levels in both central and peripheral tissues.

It should be noted that the effect of the *Clock* mutation on body weight in animals fed a regular diet was similar in magnitude to the effect of a high fat diet in wild-type animals (Fig 2). In addition, when *Clock* mutant animals 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). Thus, a dysfunctional circadian system may be a risk factor equivalent to poor diet in causing weight gain and obesity.

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 [18, 19], in particular the feeding rhythm, that is greatly attenuated in *Clock* mutant animals. 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, since 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, 20]. The observation that mRNAs of some of the major energy regulatory peptides are altered in both diurnality and absolute expression levels in the MBH supports 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 circadian clock controlled (CCG) genes, the circadian cellular oscillator regulates approximately 3–10% of transcripts expressed in any tissue [21–24].

Clues to the effects of the *Clock* mutation on energy balance may be indicated from the emerging map of SCN projections to critical energy centers within the hypothalamus. For example, SCN projections synapse directly upon lateral hypothalamic area (LHA) neurons that express orexins [25, 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 [27–30]. Our real time 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 animals, suggesting 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 animals, and the normal diurnal variation in expression was abolished. Together, these observations raise the possibility that expression of the *Clock* gene may direct a transcriptional network involving either a direct or indirect activation of E box elements within target genes; both *CART* and *ghrelin*, which are diminished in the *Clock* mutant, contain E box elements that function in transcriptional regulation [16, 17, 31–33].

In addition to changes induced by the *Clock* mutation within the SCN and/or the MBH, cell autonomous function of the *Clock* gene in peripheral cells may indirectly cause a cascade of deficits that lead to hyperphagia and metabolic dysregulation. For example, the changes in glycogen accumulation and insulin in *Clock* mutant mice, may lead to altered glycemic control and nutrient sensing, and thereby create a perceived state of energy deficiency. This hypothesis is particularly intriguing in view of the fact that many tissues involved in fuel metabolism have recently been shown to possess a circadian clock core machinery and can produce circadian oscillations *in vitro* [4, 5, 8]. Indeed, we have recently found that the Islets of Langerhans express the *Per2* gene and sustain circadian oscillations when maintained *ex vivo* (unpublished data FWT, JST, and JB). Thus, a mutation in a circadian clock gene would not only be altering molecular rhythms in SCN cells, and associated SCN-controlled behavioral and physiological rhythms, but also in all of the cells in which clock genes are expressed including liver, pancreas, fat and muscle. It will now be of great interest to discern downstream targets of the *Clock* gene within individual cells, and the interplay between peripheral and central actions of the *Clock* gene on whole animal physiology.

It is important to note that recent genome-wide expression profiling shows that nearly 10% of the mammalian genome varies as a function of circadian time, and that the expression levels of both clock controlled genes and non-rhythmic genes are altered in *Clock* mutant animals [21–24]. Importantly, 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 [21]. Input of the *Clock* gene into metabolic pathways may occur either directly through binding to E-box motifs or through tissue-specific transcription factors. Interestingly, both the core circadian machinery, and many of the same genes that are expressed according to a circadian pattern in the liver, are similarly expressed in other peripheral tissues including heart and muscle [8, 23], as well as in adipose and pancreatic tissue (unpublished results). Thus, at the local peripheral level, a change in the circadian core molecular machinery can be expected

to alter the expression of clock controlled genes whose dysregulation at the local level could contribute to hepatic steatosis and hallmarks of the metabolic syndrome, through a bottom up cascade of events. The connection between metabolism and circadian rhythmicity is particularly intriguing in view of the recent finding that genes involved in mitochondrial redox metabolism account for a large fraction of the circadian transcriptome in the brain, liver and most tissues [9]. Thus, while these earlier results indicated that cell redox flux can alter the molecular circadian core machinery, our results in *Clock* mutant animals indicate that alterations in this molecular clock may alter cell metabolism, as well.

We previously demonstrated that *Clock* mutant mice have alterations in the amount of sleep they produce each day (1–2 hours less than wild-type controls). Recent epidemiological studies have demonstrated a close association between sleep time and obesity. Both human and animal studies have shown that experimental chronic sleep restriction or sleep deprivation lead to notable changes in energy balance and in hormone levels known to regulate adiposity and satiety [34–37]. The *Clock* mutant mouse represents an intriguing genetic animal model in which chronic sleep reduction, circadian dysregulation and obesity (and other metabolic changes) are associated with a single mutation, and represents a novel genetic model to investigate the complex neural and molecular mechanisms linking circadian rhythms, sleep and energy metabolism.

In just the past few years, two major developments have transformed our understanding of the circadian clock system in mammals: 1) the elucidation of the transcriptional-translational feedback loop(s) that drives cellular rhythms [2, 21], and 2) the discovery that circadian clocks are expressed in nearly all mammalian cells where they coordinate the cell cycle, growth, and metabolism [38]. These two observations have provided great insight into the challenges that organisms face to adapt their life style on the behavioral level to the 24-hr external world with its associated physical (e.g. light-dark) and biotic (e.g. predator-prey relationships) diurnal rhythms, and to coordinate events within the organism to maintain internal 24-hr temporal organization. The circadian clock is central for maintaining this temporal order, and the finding that a mutation of a canonical clock gene, *Clock*, leads to wide ranging alterations in fuel metabolism as well as metabolic characteristics associated with obesity, diabetes mellitus, and the metabolic syndrome, emphasizes how critical normal diurnal timekeeping is, from molecular to behavioral levels, for the health and well being of the organism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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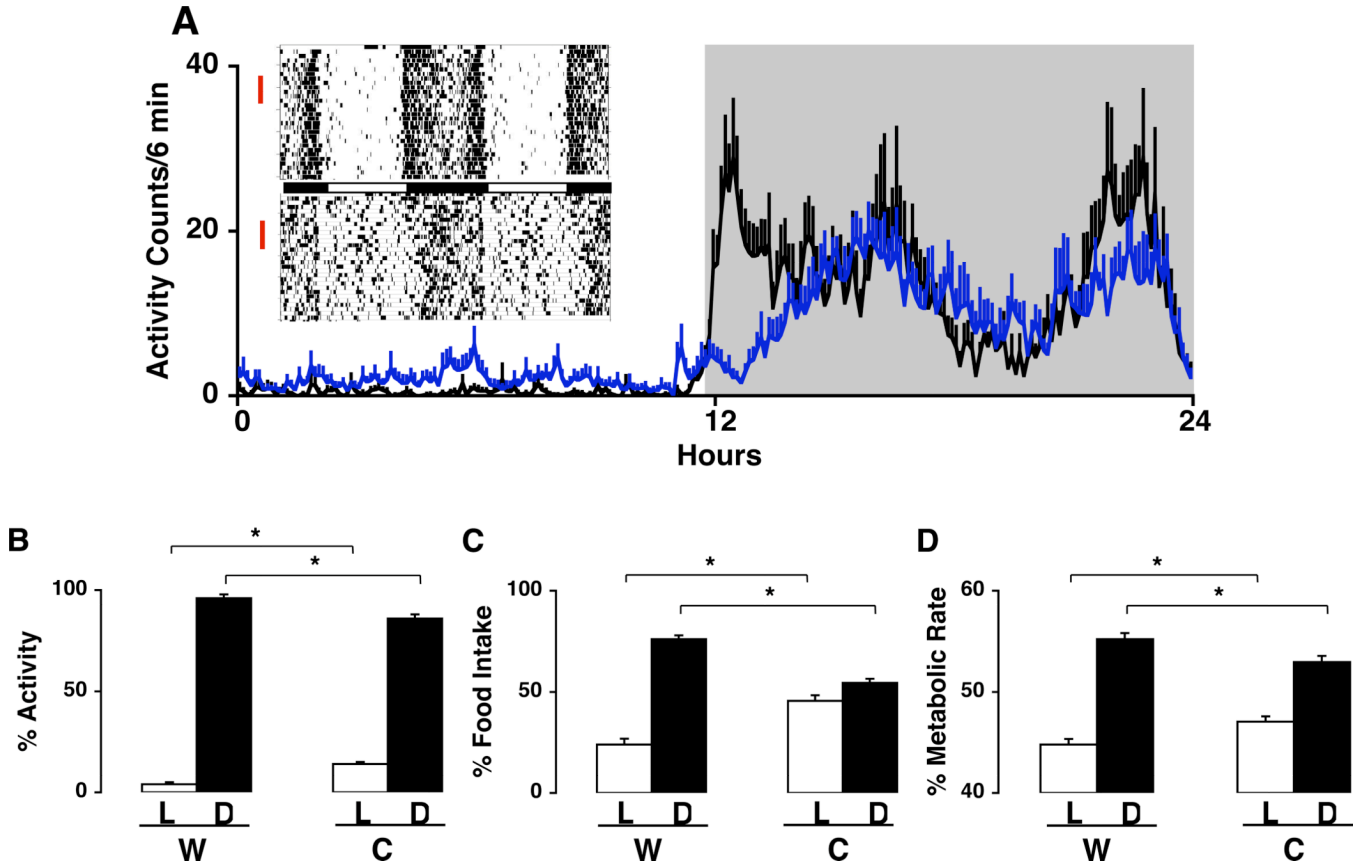

Fig. 1.

Altered diurnal rhythms in locomotor activity, feeding and metabolic rate in *Clock* mutant mice. **(A)** Insert on left: Actograms showing locomotor activity over a 30 day period in representative adult wild-type (WT) (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-minute intervals across 7 days of recording (selected days are indicated by red vertical lines to the *left* of the actograms). Shown over the 24 hr cycle are activity counts during light (unshaded) and dark (shaded) periods (WT, n=5, black line; *Clock*, n=9, blue line). **(B)** Diurnal rhythm of locomotor activity for mice shown in (A). Activity counts were accumulated over the 12-hour light and 12-hour dark periods and expressed in each period as a percent of total 24-hour activity (*, $p<0.05$). Total activity over the 24-hour period was similar between 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 (g) 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. Results shown (A–D) are expressed as group means ± SEM.

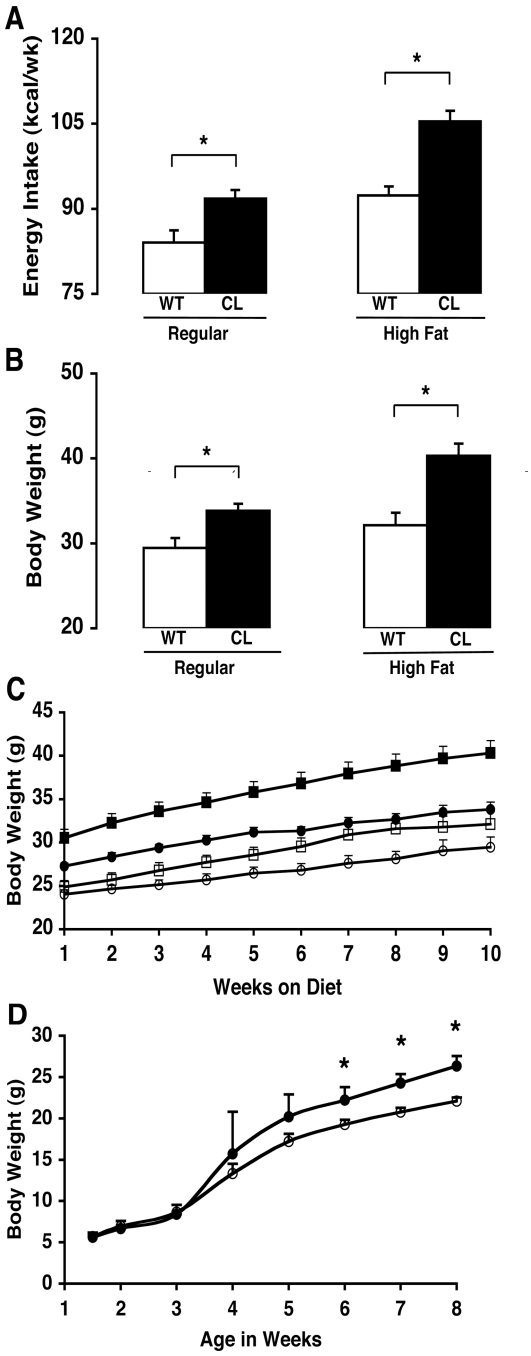

Fig. 2.

Obesity in *Clock* mutant mice. **(A)** Energy intake. Average caloric intake over a 10 week period in male WT and *Clock* mutant mice. WT and *Clock* mutant mice were provided *ad libitum* access to regular (10% kcal/fat, WT, n=8, *Clock*, n=10) or high-fat chow (45% kcal/fat, WT, n=7, *Clock*, n=11) for 10 weeks beginning at 6 weeks of age. Weekly food intake was analyzed in the two groups (*, $p<0.01$). **(B)** Body weight. Body weights for the animals depicted in (A) after the 10 week study (*, $p<0.01$). **(C)** Longitudinal weight gain. Body weights WT (open) and *Clock* mutant (closed) mice over the 10 week study for animals depicted in (A) fed either regular (circle) or high fat (square) diets. **(D)** Post-weaning body

weight of mice beginning at 10 days through 8 weeks of age. Growth curves in WT (open circle) and *Clock* mutant (closed circle) mice on regular chow were obtained by weighing animals weekly. Significant differences did not appear until 6 weeks of age (*, $p<.05$). All values (A–D) represent group means ± SEM

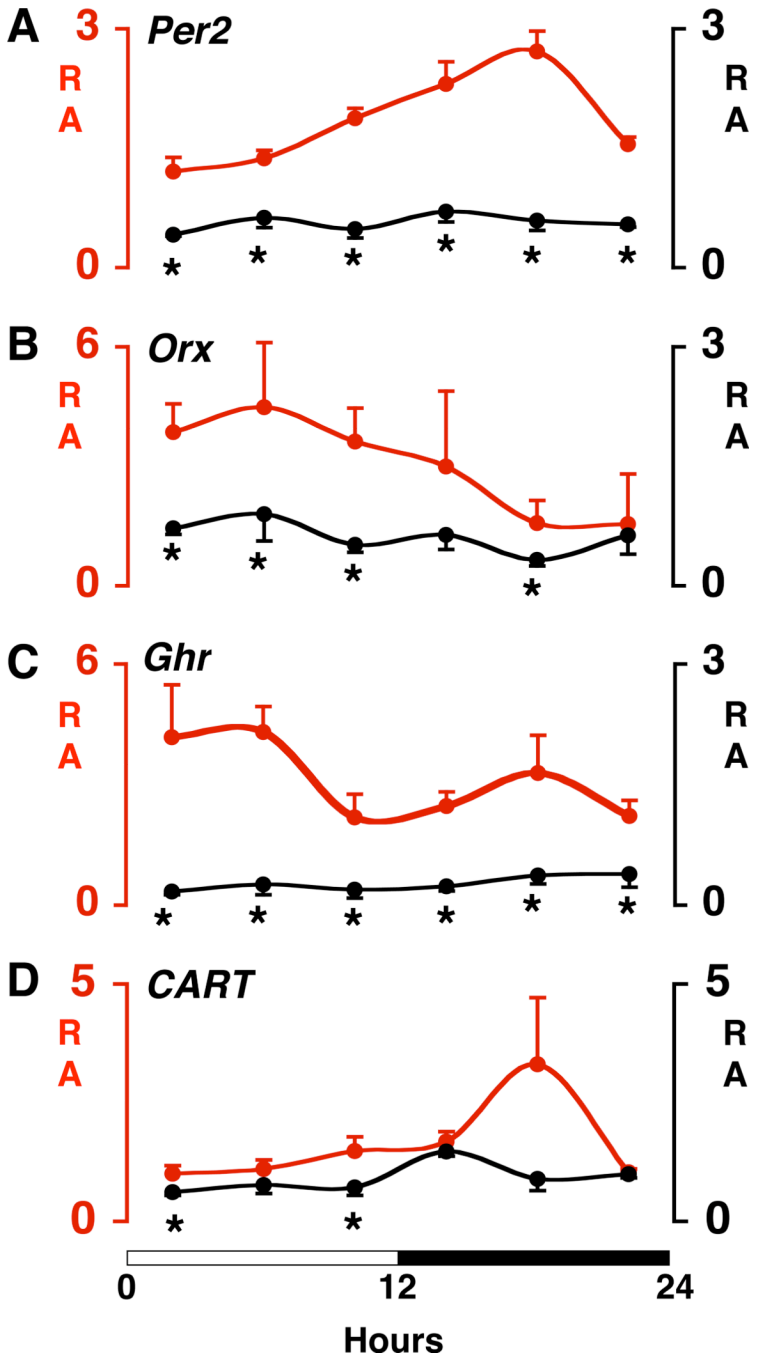

Fig. 3.

Altered diurnal rhythms and abundance in *Clock* mutant mice of *Per2* mRNA and mRNAs encoding selected hypothalamic peptides involved in energy balance. **(A–D)** mRNA relative abundance (RA) curves. Time-course variation of transcripts in the hypothalamus of WT (red line) and *Clock* mutant (black line) mice across a 12:12 LD cycle (indicated by white-black bar on bottom). Real-time PCR was used to determine transcript levels. Values are displayed as RA (mean ± SEM) after normalization to glyseraldehyde-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 and

ghrelin. Brains were collected at 4-hour intervals across the 12:12 LD cycle using four WT and four *Clock* mutant mice at each time point. Genotype comparisons were made at each 4-hour time point using independent sample t-tests with a significance level of $p<.05$ (*). *Per2* = Period-2; *Orx* = Orexin; *Ghr* = Ghrelin; *CART* = Cocaine- and amphetamine-regulated transcript.

Table 1

Metabolic parameters in WT and *Clock* mutant mice. Serum triglyceride, cholesterol, glucose, insulin and leptin concentrations were determined in 7–8 month old WT and *Clock* mutant mice fed a regular diet ad libitum (n=4–8 mice per group). For the measurement of glucose, insulin and leptin, blood was collected at 4-hour interval 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 ZT 0.

Metabolic parameters	**WT**	***Clock***	***P* value**
Triglyceride (mg/dl)	136 ± 8	164 ± 8	$P < 0.05$
Cholesterol (mg/dl)	141 ± 9	163 ± 6	$P < 0.05$
Glucose (mg/dl)	130 ± 5	161 ± 7	$P < 0.01$
Insulin (ng/ml)	1.7 ± 0.3	1.1 ± 0.1	N.S.
Leptin (ng/ml)	3.4 ± 0.4	4.6 ± 0.3	$P < 0.05$